THE EFFECTS OF HYDROSTATIC PRESSURE AND SALT ON CELL

GROWTH AND ON PHENYLALANYL tRNA SYNTHETASE ACTIVITY

OF <u>VIBRIO</u> <u>MARINUS</u> AND <u>ESCHERICHIA</u> <u>COLI</u>

by

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ABSTRACT

In nondefined 35‰ synthetic seawater salts medium, Vibrio marinus MP-1, assayed by viable cell counts, grew to 425 atm at 9 C and 320 atm at 4 C respectively. At 35‰ NaCl concentration in a defined medium, the growth of this bacterium was completely inhibited at hydrostatic pressures of 422 atm at 9 C and 327 atm at 4 C respectively. At the lowest NaCl concentration (8‰) which permitted cell growth in a defined medium, cell division was inhibited at 88 and 61 atm at 9 and 4 C respectively. The ability of several salts to replace the NaCl requirement of the marine vibrio was dependent on ionic composition of the replacing salt as well as on the concentration of available NaCl. Escherichia coli B/r, assayed turbidimetrically, grew to the same maximum pressure (P_{max}) of 500 atm at 37 C independent of the salt concentration in a nondefined medium.

Salts and pressure were demonstrated to affect the aminoacylation of phenylalanyl tRNA during protein synthesis in both V. marinus and E. coli. At 33 C, phenylalanyl tRNA synthetase (PAS) activity from E. coli showed stimulation in low levels of NaCl and at all levels of KCl greater than 0 M, except near 1 M. NH_4Cl caused inhibition of activity at concentrations less than 0.5 M or greater than 0.6 M. At 15 C,

synthetase from $\underline{V}$. $\underline{marinus}$ showed a response to NaCl
similar to that of $\underline{E}$. $\underline{coli}$. All concentrations of KCL
and NH_4Cl greater than O M caused a decrease in PAS
activity from the marine organism however. The effects
of combinations of these salts on PAS activity from
each organism could be predicted from the data for each
salt. In media lacking all three of these salts,
synthetase from $\underline{V}$. $\underline{marinus}$ was stimulated to a peak of
activity by 270-300 atm, whereas $\underline{E}$. $\underline{coli}$ PAS was un-
affected in the 1-300 atm range of pressure. The effect
of pressure on PAS activity in both organisms was modi-
fied by the ionic environment. The synthetase from
$\underline{V}$. $\underline{marinus}$ was not only more sensitive to salts but was
also more adversely affected by pressures greater than
300 atm than was $\underline{E}$. $\underline{coli}$ PAS.

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INTRODUCTION

Hydrostatic pressure in the sea increases at
approximately 0.1 atm per meter of depth, and the
pressure range in this environment varies from 1 atm
at the surface to 1150 atm in the Challenger Deep (12).
Marine bacteria, that is, bacteria which require salt
in the growth medium on their initial isolation from the
sea (29), differ in their ability to grow under pressure.
They have therefore been classed as barophiles, baro-
phobes, and barodures (53). Barophiles are those
bacteria which grown preferentially or exclusively at
high pressures; such organisms are found in the deep
sea and have also been isolated from deep oil wells.
Barophobes grown only at relatively low pressures, such
as 200 atm whereas barodures can tolerate high pressures
but grow best at 1 atm pressure. Escherichia coli, a
terrestrial bacterium, and Vibrio marinus, from the
ocean, are both baroduric bacteria. Numerous studies
of hydrostatic pressure effects on microbial growth,
morphology, and metabolism have been reported. For
example, ZoBell and Cobet (51) observed that at
increased pressures, cells of three different strains of
E. coli produced long filaments. Similarly, pressure

induced filament formation in many marine bacteria (53).
Other morphological variations induced by pressure in-
clude a general increase in cell mass, marked pleomor-
phism, and loss of motility (36). In addition, ZoBell
and Oppenheimer (53) obtained some evidence that
pressure induces genetic changes in bacteria. McElroy
and de la Haba (32) found that pressures from 150 to
600 atm increased the number of biochemical mutants in
cultures of Neurospora crassa treated with nitrogen
mustard. Several studies (36, 52, 53) revealed that the
rates of growth of many terrestrial bacteria were
retarded by a pressure of 400 atm. In some cases, a
pressure of 300 atm was sufficient to cause some cell
death. Of the various marine bacteria examined, some
grew readily at 600 atm whereas others resembled ter-
restrial bacteria in their response to pressure. Enzy-
matic reactions which involved an increase in volume are
inhibited by increased pressures. Morita (33) observed
that at 1000 atm, formic, malic, and succinic dehydrogen-
ase activity at 30 C was almost completely inhibited in
E. coli. Borrowman has also reported that pressures
above 700 atm inhibited formic dehydrogenase activity
at 30 C in E. coli (6). Macromolecular synthesis in
whole cells is adversely affected by increased hydro-
static pressures. Landau (19) demonstrated that at 408

atm and 37 C, ^{14}C-glycine was incorporated into protein at the same rate as at 1 atm in E. coli K-12. At this temperature, higher pressures inhibited and lower pressures stimulated this incorporation. Pressure which completely prevent protein synthesis were insufficient to stop nucleic acid synthesis however. Landau (20) also found that, at 37 C, a pressure of 265 atm prevented induction of the enzyme β-galactosidase (β-gal). At 670 atm, a pressure which was sufficient to prevent translation of messenger RNA for β-gal synthesis, transcription of messenger still occurred. Pollard and Weller (39), using E. coli 15 T$^-$L$^-$, found that 900 atm prevented the incorporation of ^{14}C-valine into protein whereas uptake of ^{14}C-uracil was less affected. At pressures insufficient to prevent cell division of E. coli, the order of sensitivity to pressure was protein > DNA and RNA synthesis. Albright (2) and Albright and Morita (3) further demonstrated that protein synthesis was more adversely affected than either DNA or RNA synthesis by increased hydrostatic pressures in V. marinus MP-1 and MP-4 and in E. coli B/r. Albright (2) showed that pressure inhibition of these processes was completely reversible. The response of DNA and RNA synthesis to pressure may be the direct result of inhibition of protein synthesis since both DNA and chromosome repli-

cation require protein synthesis for initiation (21, 28).

The specific site of action of pressure on protein

synthesis may be due to the complete or partial inhibi-

tion of the transport of any or all 20 amino acids (3),

inhibition of transcription (20, 39) and aminoacyl tRNA

synthetase activity (3, 19) or inhibition of formation

of the aminoacyl tRNA, ribosome mRNA complex (2, 3, 39).

The salinity of the ocean varies from the polar

seas to the tropics and in estuarine environments.

Numerous reports of salt effects on cell growth, morph-

ology, and metabolism are in the literature. Abram and

Gibbons (1) demonstrated that the halophilic bacterium

Halobacterium cutirubrum requires NaCl at concentrations

of 3.5 M or greater to maintain its rod shape. Cessation

of cell wall synthesis occurs in the halophile Micro-

coccus halodenitrificans at low NaCl levels (48).

Various salts are known to prevent cell lysis of several

marine bacteria (1, 4, 7, 18, 31, 40, 48) and to stimu-

late the activities of enzymes involved in substrate

utilization (30, 37, 49). The terrestrial bacterium

Pseudomonas aeruginosa requires the divalent cations

Mg^{++}, Mn^{++}, and Zn^{++} to preserve the organization,

structure, and assembly of the cell wall (4). In ter-

restrial bacteria, specific salts are known to affect

macromolecular synthesis. Lubin (26) found that stimu-

lation of the synthesis of ^{14}C-phenylalanyl-tRNA

occurred in medium enriched with KCl. This salt also

stimulated uptake of ^{14}C-guanine into RNA and ^{14}C-leucine

into protein. KCl affects cell mass by controlling the

uptake of assimilable carbon, nitrogen, and phosphorus

(13). Lubin and Ennis (27) demonstrated that a high

level of KCl was required for the transfer of an amino

acid from aminoacyl-tRNA to a polypeptide in E. coli

B. Various salts are also known to affect aminoacyl

tRNA synthetase activity (27, 38, 43), amino acid poly-

merization (10), and the formation of the aminoacyl tRNA,

ribosome mRNA complex (44). The salinity of the growth

medium has been demonstrated to cause changes in the

response of marine bacteria to hydrostatic pressure (53).

The response of terrestrial bacteria to pressure when

grown in media of differing salinity has not been

reported.

The effects of hydrostatic pressure on bacterial

cells can be altered by changing the growth temperature.

The temperature of the oceans varies from -1.9 C in the

polar regions or in deep water, to 30 C in the tropics

(50). By volume, more than 90% of the ocean is colder

than 5 C (50). An increase in incubation temperature

permits both terrestrial and marine bacteria to tolerate

higher pressures (52). Within the growth temperature

range of bacteria, lower temperatures greatly accentuate the growth retarding effects of hydrostatic pressure above 1 atm (52). Thermal denaturation can frequently be reversed or inhibited if the hydrostatic pressure is increased (52). At pressure up to 700 atm for example, malic dehydrogenase exhibits no activity at 101 C. However, pressures above 700 atm permit this enzyme to remain active at this temperature. Under these conditions, optimal activity occurs at 1300 atm (34). Further evidence on reversal of thermal denaturation by increased pressure has been reported by Brown et al. (8) for luciferase in luminescent bacteria, by Johnson and Campbell (15) for globulins and egg albumin, and by Johnson et al. (17) for tobacco mosaic virus. During protein synthesis, a pressure of 408 atm at 30 C permits the incorporation of ^{14}C-glycine into protein at the 1 atm rate (20). This pressure inhibits uptake at 27 C however. Even at 272 atm, temperatures below 27 C inhibit this incorporation (20). Temperature optima and maxima for growth of several marine bacteria are known to be affected by salinity of the growth medium. (45). Hence both temperature and pressure tolerance of bacteria could be altered by changes in salinity of the environment.

This study was initiated to determine the tolerance of a terrestrial and a marine bacterium to increased

pressures. An analysis of the effects of salinity on pressure tolerance was examined to discover whether salt-dependent pressure tolerance was found in one or both organisms. In addition, a detailed analysis of the effects of temperature and ionic environment on the pressure tolerance of $\underline{V}$. $\underline{marinus}$ was performed.

In order to answer the question whether there was one step of protein synthesis which is primarily affected at pressures and salinities which prevent cell growth and division, the aminoacylation reaction involving phenylalanyl-tRNA in $\underline{E}$. $\underline{coli}$ B/r and $\underline{V}$. $\underline{marinus}$ MP-1 was examined. The effects of various concentrations of NaCl, KCl, and NH_4Cl on phenylalanyl tRNA synthetase (PAS) activity at pressures from 1 to 1088 atm were also determined. A comparison of the effects of salt effects on cell growth and PAS activity may reveal whether requirements by intact cells for specific salts are reflected in the response of PAS to different ionic environments.

MATERIALS AND METHODS

Cultures and Media

The obligately psychrophilic marine bacterium
Vibrio marinus MP-1 and the mesophilic terrestrial
bacterium Escherichia coli B/r were used throughout this
investigation. Stock cultures for the marine vibrio were
maintained in 16 x 150 mm culture tubes at 15 C in a
nondefined marine broth containing peptone (Difco), 10 g;
Rila salts (Rila Products Co., Teaneck, N. J.), 35 g;
succinic acid, 10 g; yeast extract (Fisher), 3 g; and
distilled water, 1000 ml. E. coli was maintained, in
similar tubes in nutrient broth (Difco). Both cultures
were transferred daily. Nondefined medium for hydro-
static pressure-salinity studies of both organisms con-
sisted of the marine broth described above to which Rila
salts were added in quantities of 2.5, 5, 10, 15, 20 or
35 g/l respectively. This broth was adjusted to pH 9.2
with NaOH, boiled, cooled and filtered, and adjusted
back to pH 7.3 with HCl. This medium was used in the
preparation of marine agar by the addition of 1.5% agar.
For the detailed pressure-salt study on V. marinus, a
defined basal medium (pH 7.3) consisting of $NH_4H_2PO_4$,
2.3 g; $(NH_4)_2SO_4$, 1.32 g; $CaCl_2$, 5 mg; $FeCl_3 \cdot 6HOH$,
0.05 mg; $MgCl_6 \cdot 6HOH$, 200 mg; $K_2HPO_4 \cdot 3HOH$, 4.57 g;

and 1000 ml distilled water, was employed. Additional salts were added to this basal medium as required and the volume increased two-fold. One-half ml of 20% (w/v) glucose solution was added to 100 ml of this medium immediately before inoculation. For the Mg^{++} and Mn^{++} studies, the level of PO_4^{3-} salt was reduced to 4.7 g with its replacement to an equivalent ionic strength with 5.8 g KCl and 0.3 g NH_4Cl. This was necessary to prevent salt precipitation. Dilution blanks for viable count analysis of V. marinus contained 3.5% NaCl (w/v) at a pH 7.3. All media were dispensed into appropriate vessels and autoclaved. Salinity-temperature effects were measured by the method of Stanley and Morita (45) in the above defined medium.

For PAS studies, E. coli was maintained in nutrient broth (Difco), and grown and harvested as previously described (22). V. marinus was maintained, grown and harvested from the nondefined medium first described. Both media (pH 7.3) were autoclaved at 121 C for 20 min. Incubation temperature for the maintenance and harvesting of cells was 33 C for E. coli and 15 C for V. marinus.

Growth Under Pressure

An inoculum of 0.5 ml from a 12-18 hr culture of either organism was mixed with 100 ml of each salt

medium to be tested. Then, 3.5 ml portions of this suspension were pipetted into sterile 10 x 75 mm culture tubes. The tubes were sealed with sterile No. 000 neoprene stoppers and the samples were pressurized according to the method of ZoBell and Oppenheimer (53) in pressure apparatus like that described by these authors. Cell growth was measured by one of the following methods. Initial cell titre was measured either turbidimetrically at 550 mu (Spectronic 20) or by viable count analysis. Both measures were taken 40 min after media was inoculated. Cells required this amount of time to reach stable equilibrium in the different salt media. Initial absorbance readings of both bacteria were compared with readings obtained after cells had been subjected to 72 hr under pressure. Growth in these samples was recorded as (+) if the absorbance had increased 0.01 to 0.1 units and as (++) if the readings were greater than 0.1 units. If the absorbance had increased less than 0.01 units, growth was recorded as (-) (Table I). The second method involved dilution-plate counts for V. marinus only for the detailed study of temperature and ionic environment effects on P_{max} (maximum growth pressure). This method provided a more accurate quantitative measure of differences of hydrostatic pressure tolerance of these cells. Using this procedure, the pressure which

prevented cell division (i. e. the maximum growth pressure) was determined by comparing initial cell titres to titres from depressurized samples. P_{max} was calculated as the average of the pressure interval within which final and initial cell titres were equivalent. All experiments involving cell viability were performed at 14 or 27 atm intervals. The kinetics of cell growth near P_{max} revealed that data variance was never more than ± 7 atm regardless of the interval used. The effects of pressure and salinity were tested at 9 or 4 C for V. marinus or at 37 C for E. coli. A temperature no greater than 9 C was employed for the growth studies of V. marinus because media of low salinity prevent cell growth at higher temperatures (22). Initial cell titres for all experiments involving cell viability were 10^3 - 10^5 cells/ml. Initial absorbance readings were approximately 0.05 at 550 mμ. Pipettes, culture media, and all equipment were kept at 0 C during inoculation of media before pressurization and after pressure was released. This ensured that negligible cell growth occurred before either absorbance measurements or viable counts could be taken.

Harvesting of Cells and Preparation of Extract for PAS
Assay

E. coli and V. marinus were grown with vigorous
aeration in 12 l of medium, equilibrated at either 33 C
or 15 C respectively, in a 14 l MicroFerm Fermenter (New
Brunswick Co., Inc., New Brunswick, N. J.). The cells
were harvested in late log phase in a Sorvall continuous
flow centrifuge rotor at 35,000 x g. Harvesting of cells
and preparation of crude extract were both done at 0-5 C.
The resulting cell pellets, washed once in 200 ml of
buffer containing 0.01 M tris (hydroxymethyl) amino-
methane, 0.01 M $MgCl_2$, and 0.006 M mercaptoethanol (pH
7.6), were quick frozen in liquid nitrogen and stored at
-15 C. Freshly thawed cells were ruptured in a Sorvall
Omni-Mixer (Sorvall Inc., Norwalk, Conn., U. S. A.) with
three times their wet weight of glass beads (0.17-0.18
mm dia) in three extractions with the above buffer at
pH 7.3. Rupture of cells occurred effectively after
three exposures of 2 min each, at top speed, with 1 min
cooling intervals. The total volume of buffer used for
extractions was 2 ½ times the wet weight of the cells;
each addition of buffer was followed with a brief ex-
posure at top speed in the Omni-Mixer. The resulting
brei was then centrifuged by the method of Nirenberg and
Matthaei (35). The S-100 fluid (crude extract) was

aspirated, quick-frozen under liquid nitrogen, and stored at -15 C with no noticeable loss of activity after 2 months of storage.

Purification of Crude Extract

Freshly thawed crude extract was passed through a 1.5 x 20 cm column of Sephadex G-25 (coarse) which was equilibrated with buffer (pH 7.3) at 2-5 C. Twenty-five drop fractions were collected using a refrigerated LKB Ultrorac fraction collector, (Model 7000, Stockholm, Sweden) at a flow rate of 25 drops per min. A column load of 1 ml of E. coli extract or 2 ml of V. marinus extract provided sufficient activity and protein con-centration in the 7th and combined 7th and 8th fractions of each organism. Protein concentration in these frac-tions was determined using the method of Lowry et al. (25), modified for small amounts of protein. Bovine serum albumin was used as a standard.

Assay for Phenylalanine Activation

PAS activity was determined using a modification of the method of Fangman and Neidhart (11). The standard reaction mixture contained in a volume of 1 ml, 100 μm $MgCl_2$, 2 μm glutathione (GSH, pH 7.0, Sigma Chem. Co.), 2 μm diK ATP (pH 6.8, Sigma Chem. Co.), 1.32 mμm (10 ul) of ^{14}C-phenylalanine (uniformly labelled, 475 mC/mM; Amersham-Searle), 1 mg E. coli W tRNA (Schwartz Bio-research Inc.), and 0.05 Or 0.5 mg protein from the

Sephadex fractions of E. coli and V. marinus respectively. GSH and ATP were adjusted to the required pH with additional Tris. The incubation temperature of 33 C for E. coli and 15 C for V. marinus, at the above protein concentrations, maintained a linear rate of acylation up to 15 min (Table IV). The reactions were initiated by the addition of enzyme, isomolar with respect to salt concentration, to the remaining reaction mixture. They were terminated by the addition of an equal volume of cold, 10% trichloroacetic acid (TCA). After 10 min on ice, the TCA samples were passed through a 25 mm dia, 0.45 μ pore size, millipore filter under mild vacuum (5 psi). The filters were washed three times with 2 vol each of cold 10% TCA, heat-dried and then dropped into scintillation vials which contained 15 ml of cocktail. The cocktail consisted of 42 ml liquifluor in 1000 ml toluene to which ethylene glycol monomethyl ether (6 parts per 10 parts toluene liquifluor). All activities were determined in a Beckman Scintillation Counter (Model 250) operating at 95% efficiency for ^{14}C. Quench effects, which were constant irrespective of the amount of precipitate collected on the filter, reduced efficiency to 70%.

Assay for Activity Under Pressure

The amount of coupling of phenylalanine to tRNA prior to pressurization and after depressurization, before the addition of TCA, was determined for all samples because the reaction could neither be initiated nor terminated under pressure. Linearity of the rate of acylation was maintained under pressure and even after exposure to 1088 atm, the rate of acylation proceeded at the 1 atm rate immediately after pressure was released. The amount of acylation which occurred when samples were not pressurized was subtracted from the total observed acylation so that only coupling which occurred under pressure is shown in the PAS hydrostatic pressure data. Specifically, 0.18 ml of enzyme, at the appropriate concentration, was added to 0.72 ml of the reaction mixture in a 6 x 15 mm culture tube. The tube was sealed with a neoprene stopper (#124, S-1) and was then inverted several times to permit a small air bubble to mix the tubes contents. Pressurization, with apparatus previously described (53), occurred 108 sec after the addition of enzyme. Depressurization 10 min later was followed by removal of either a 0.2 or 0.25 ml sample of the reaction mixture. This sample was injected into an equal volume of cold, 10% TCA within 80 sec. These samples were then treated as described previously.

All P_{max} and PAS activity data are the results of duplicate assays. P_{max} data is subject to a variance no larger than ± 7 atm. Data on salt and pressure effects on PAS activity is subject to variance from 2 to 10%.

RESULTS AND DISCUSSION

Salinity and NaCl concentration of the growth medium has previously been reported to affect the maximum growth temperature (T_{max}) of the marine bacterium Vibrio marinus (45). Reexamination of the effect of NaCl concentration on the T_{max} of this organism (Fig. 1) gave results very similar to those obtained previously (45). It is doubtful that the T_{max} for E. coli was affected by media of different salinity since equivalent growth response was observed (Table I). The P_{max} for V. marinus was dependent on the salinity of the medium, whereas that for E. coli displayed no such response (Table I). Since pressures in the range of 300-500 atm are known to cause filament formation in E. coli (51), turbidimetric measurements for this organism reflect both an increase in cell size and cell titre. No such alterations of cell morphology were observed for V. marinus however. Turbidimetric measurements for this bacterium therefore indicate an increase in cell titre only. The ability of V. marinus to grow at elevated pressures was found to be related to the growth temperature as well as to salinity (Fig. 1). This dependence of the P_{max} upon the temperature of incubation has been noted by several authors for both marine and terrestrial bacteria (16, 52).

E. coli (16, 52) and V. marinus (Fig. 2 and 3) show an
increased P_{max} with an increase in incubation tempera-
ture. The effects of pressure on cell growth at tem-
peratures above T_{max} for any salinity were not examined.
Observations by other authors (16, 52) indicate that,
within limits, increasing pressure reverses the thermal
inactivation of cell growth. The response of the marine
bacterium to different NaCl concentrations and salinities
was very similar. At 35‰ NaCl concentration, the P_{max}
at 9 and 4 C was 422 and 327 atm respectively. At a
salinity of 35‰, the P_{max} at these temperatures was 425
and 320 atm (Fig. 2 and 3). This suggests that it is
NaCl which permits V. marinus to grow at elevated
pressures. The fact that the marine vibrio would not
grow at NaCl concentrations less that 8‰ illustrates the
need for NaCl by this bacterium (Fig. 3). The presence
of other salts in the artificial seawater mix and salt
impurities in both peptone and yeast extract are prob-
ably responsible for permitting cell growth at only
2.5‰ salinity in the nondefined medium (Fig. 2). The
effectiveness of various salts in replacing the NaCl
requirement of V. marinus was dependent not only on the
ionic composition of the replacing salt but also upon
the concentration of the available NaCl (Table II). The
concentration of the replacing salt may also be important.

For example, at 8‰ NaCl an increase from 0.19 M to 0.2 M Na_2SO_4 raised the P_{max} from 298 to 326 atm. Table III, a rearrangement of the data from Table II, lists the relative order of effectiveness of various ions in replacing either Na or Cl ion. The ion which allows growth to occur to the highest P_{max} is listed first (underlined) and all other ions are listed as either equivalent or less effective in replacing this ion. The data for this table was tested at 95% level of significance. Cell death at low salinities or NaCl concentrations and at elevated pressures may be due to a loss of membrance integrity (1, 4, 18) as well as to inhibition or denaturation of enzymes involved in general cell metabolism (5, 30, 37, 49). Inhibition of macromolecular synthesis at low salt levels may also be involved (10, 13, 26, 27, 38, 43, 44). Instability of cellular proteins of V. marinus at low NaCl concentrations may be the primary cause of salt-dependent pressure and temperature tolerance of this organism (23).

Salts are known to affect the aminoacylation of various tRNA's (24, 38, 43). In addition hydrostatic pressure and salts have both been shown to affect total protein synthesis (2, 3, 13, 26, 27). The stimulation of PAS activity by low NaCl levels (Fig. 4 and 5) has been noted before (43). The response of E. coli and

$\underline{V}$. $\underline{marinus}$ PAS to NH_4Cl and KCl were quite different
however and it was not only ionic strength, as suggested
by Loftfield and Eigner (24), which affected synthetase
activity. Peterkofsky $\underline{et}$ $\underline{al}$. (38) also observed that
NaCl was a more potent inhibitor of $\underline{E}$. $\underline{coli}$ PAS activity,
at concentrations greater than 0.3 M (Fig. 4) than was
either NH_4Cl or KCl. These salts proved more inhibitory
than NaCl to PAS from $\underline{V}$. $\underline{marinus}$ however. At NaCl con-
centrations from 0 to 0.3 M, greater stimulation of $\underline{V}$.
$\underline{marinus}$ PAS than $\underline{E}$. $\underline{coli}$ PAS occurred (Fig. 4 and 5).
At concentrations of NaCl greater than 0.7 M, severe in-
hibition of PAS from the marine vibrio occurred, unlike
$\underline{E}$. $\underline{coli}$ PAS (Fig. 4 and 5). Unlike the halophilic system
examined by Griffiths and Bayley (14), no irreversible
inactivation of the enzyme occurred for the moderate
halophile $\underline{V}$. $\underline{marinus}$ in negligible KCl levels. In general,
the response of PAS from both $\underline{E}$. $\underline{coli}$ and $\underline{V}$. $\underline{marinus}$ to
combinations of NaCl, KCl, and NH_4Cl, near seawater con-
centration, could be predicted from the effects of in-
dividual salts (Table V). It appears that the greatest
activity of the synthetase from these two bacteria occurs
at NaCl levels lower than is found in the marine environ-
ment, except perhaps in estuarine environments. $\underline{E}$. $\underline{coli}$
PAS appears to be more tolerant of high NaCl concentra-
tions and of concentrations of KCl and NH_4Cl from 0 to

1.0 M than is the enzyme from $\underline{V}$. $\underline{marinus}$. Salts may be acting either on the enzyme only, on the tRNA only, or on both (24, 38, 43). If $\underline{E}$. $\underline{coli}$ W tRNA is affected by salts and pressure in a manner significantly different than is $\underline{V}$. $\underline{marinus}$ tRNA, it is conceivable that the data obtained for the marine organism does not present a true picture of the response of phenylalanine activation. The response of whole cells to salts (Fig. 2 and 3) is in direct contrast with the PAS data obtained for $\underline{V}$. $\underline{marinus}$. The enzyme clearly functions most efficiently at 0 to 0.3 M NaCl (Fig. 5). This suggests that the NaCl requirement of this organism does not involve amino-acylation. $\underline{E}$. $\underline{coli}$ grows equally well under pressure regardless of the salinity of the medium (Table I). However, as for $\underline{V}$. $\underline{marinus}$, the greatest activity of PAS occurs at relatively low NaCl concentrations (Fig. 4). This suggests that NaCl may be effectively compartmenta-lized or removed from the intracellular environment. A sodium pump has been demonstrated for $\underline{E}$. $\underline{coli}$ (41, 42). Concentrations of NH_4Cl up to 1.0 M do not adversely affect $\underline{E}$. $\underline{coli}$ PAS, however this salt very effectively inhibits synthetase activity in $\underline{V}$. $\underline{marinus}$. $\underline{E}$. $\underline{coli}$ is known to accumulate K ion (41). This ion not only stimulates PAS activity in this organism (26, Fig. 4) but also known to stimulate overall protein synthesis (13,

26, 27). KCl and NH_4Cl both aid formation of the amino-
acyl tRNA, ribosome mRNA complex (44). In $\underline{V}$. $\underline{marinus}$,
KCl inhibits synthetase activity (Fig. 5). Investigation
on intracellular Na and K ion levels in a moderately
halophilic marine bacterium indicated that intracellular
and extracellular Na ion concentrations are equivalent
at all Na^+ levels (47). K ion was accumulated to two-
fold the extracellular concentration at all levels
tested (47). A study by Christian and Waltho (9)
revealed that cellular Na ion concentration was always
below that of the medium for two moderate halophiles.
Cellular K ion levels were higher in the cell however and
were also greater than intracellular Na^+ concentration.
If the cytoplasm of $\underline{V}$. $\underline{marinus}$ was equimolar to extra-
cellular Na^+ and had 2 x the concentration of extracellular
K^+, as suggested by the work of Takacs $\underline{et}$ $\underline{al}$. (47), then
it appears that the PAS in this organism would be func-
tioning at much less than optimal activity (Table V).

Cell-free $\underline{E}$. $\underline{coli}$ PAS was unaffected from 1-300
atm pressure in basal salts solution. The synthetase
from $\underline{V}$. $\underline{marinus}$ reached optimal activity at 270-300 atm
however (Fig. 6 and 7). In general, salts did not
reverse the effects of hydrostatic pressure on PAS
activity (Fig. 6, 7, 8, 9). Unlike the effects of in-
creasing NaCl concentration on whole cells, the PAS from

V. marinus did not become more resistant to pressure.
In basal salts, synthetase from the marine bacterium was
more pressure sensitive than that from E. coli. The
interplay of salts on this response makes direct com-
parison between the two systems difficult however. In
basal salts solution, V. marinus PAS was 70% as active at
425 atm as at 1 atm, whereas E. coli PAS was of com-
parable activity at 544 atm (Fig. 6 and 7). Thus
sufficient synthetase activity remains, at pressures
sufficient to prevent cell division, to sustain protein
synthesis. At a concentration of NaCl near seawater
levels, 27% of V. marinus activity remains compared to
basal salts activity (Fig. 5). E. coli prevents entry
of NaCl into the intracellular environment (41), hence
all PAS activity should be preserved even in the marine
environment. The effects of salts and pressure on amino-
acylations involving amino acids other than phenylalanine
may be more adverse. General conclusions on the effects
of these parameters on inhibition of aminoacylation and
protein synthesis are therefore difficult to make. If
the results obtained here are representative of the
responses of all other amino acids to pressure, then the
aminoacylation reaction in protein synthesis does not ap-
pear sufficiently inhibited to prevent protein synthesis
and cell division in either E. coli or V. marinus. Though

NaCl, KCl, and NH_4Cl affect PAS activity of E. coli appreciably, it is doubtful that these salts are present in the intracellular environment at levels which would limit aminoacyl tRNA esterification. This would be true even if this bacterium were exposed to the marine environment. These salts would severely depress V. marinus PAS activity however. Even under these conditions though, sufficient activity remains so that protein synthesis could occur even at 425 atm, the P_{max} for this organism. The marine organism prefers salt levels near 35‰ for optimal growth. The fact that various salts found in the marine environment are detrimental to PAS activity suggests that the NaCl requirement may involve the integrity of the cell membrane rather than intracellular metabolism. The interplay of salts on V. marinus PAS activity may also account for this bacterium being less tolerant of pressure than is E. coli which has no specific NaCl requirement.

Table I. The effect of salinity on cell densities of
Escherichia coli and Vibrio marinus grown
under hydrostatic pressure in nondefined
medium. Growth was determined as described
in Materials and Methods.

Salinity (‰)	Organism	Hydrostatic pressure (atm)						
		1	100	200	300	400	500	600
5	E. coli	++	++	++	++	++	+	−
5	V. marinus	++	+	−	−	−	−	−
10	E. coli	++	++	++	++	++	+	−
10	V. marinus	++	++	++	−	−	−	−
15	E. coli	++	++	++	++	++	+	−
15	V. marinus	++	++	++	+	−	−	−
35	E. coli	++	++	++	++	++	+	−
35	V. marinus	++	++	++	++	+	−	−

Table II. The maximum hydrostatic pressure (atm) at

which cell division by <u>Vibrio marinus</u> occurs

in a defined medium enriched with 0.16 M

and 0.2 M concentrations of various salts.

P_{max} was determined as described in Materials

and Methods. * denotes no growth at any

pressure; ** denotes 0.1 M each of sodium

mono- and dihydrogen phosphate salt. Incu-

bation temperature was 9 C.

Addition to defined medium:	P_{max} (atm) at an additional NaCl concentration of:			
	35‰	18‰	8‰	1.8‰
0.16 M NaCl	313	400	299	197
0.16 M KCl	327	367	218	NG
0.16 M NH$_4$Cl	299	340	177	NG
0.16 M MgCl$_2$	NG*	NG	NG	NG
0.16 M MnCl$_2$	NG*	NG	NG	NG
0.2 M NaCl	--	415	326	238
0.2 M NaBr	--	313	286	197
0.2 NaI	--	NG*	NG	NG
0.2 M NaNO$_3$	--	299	204	177
0.2 M Na$_2$SO$_4$	--	313	326	259
0.2 M Na$_x$H$_x$PO$_4$**	--	367	326	204
None	422	306	88	NG

Table III. The relative order of cation and anion

replacement of Na and Cl ions for <u>Vibrio</u>

<u>marinus</u> grown at elevated pressures in a

defined medium. The data is presented

with the ion which permitted growth to the

highest pressure appearing first (*).

NaCl added to defined medium:	Order of cation replacement
35‰	<u>K</u>*, Na, NH_4 > Mg, Mn
18‰	<u>Na</u> > K > NH_4 > Mg, Mn
8‰	<u>Na</u> > K > NH_4 > Mg, Mn
1.8‰	<u>Na</u> > K, NH_4, Mg, Mn

	Order of anion replacement
18‰	<u>Cl</u> > PO_4 > Br, SO_4 > NO_3 > I
8‰	<u>Cl</u>, PO_4, SO_4 > Br > NO_3 > I
1.8‰	<u>SO_4</u>, Cl > PO_4 > Br > NO_3 > I

Table IV. The activities of <u>Escherichia</u> <u>coli</u> and

Vibrio marinus PAS at 33 C and 15 C res-

pectively. Activities in the standard

reaction mixture were determined as described

in Materials and Methods. Specific activity

is expressed as mμm phenylalanyl tRNA formed

per 15 min at the indicated protein concen-

tration and temperature.

Synthetase	Temperature (c)	Specific Activity
<u>E</u>. <u>coli</u>	33	0.3054
<u>V</u>. <u>marinus</u>	15	0.0883

Table V. The effects of combinations of various salts

on PAS activity in Escherichia coli and

Vibrio marinus. Activities were determined as

described in Materials and Methods. Incubation

temperature was 33 C and 15 C for Escherichia

coli and Vibrio marinus PAS respectively.

* denotes that this point was not tested.

Salts added to standard reaction mixture:	% Activity compared with activity in 0 M salts.	
	E. coli	V. marinus
0.41 M NaCl	63.9	73.4
0.09 M KCl	115.3	92.2
0.01 M NH_4Cl	98.8	87.8
0.41 M NaCl + 0.09 M KCl	83.2	90.0
0.41 M NaCl + 0.01 M NH_4Cl	*--	66.3
0.41 M NaCl + 0.09 M KCl + 0.01 M NH_4Cl	104.4	69.3

Figure 1.   The effect of NaCl on the maximum growth

temperature of <u>Vibrio</u> <u>marinus</u> in a defined

medium.   The method of Stanley and Morita

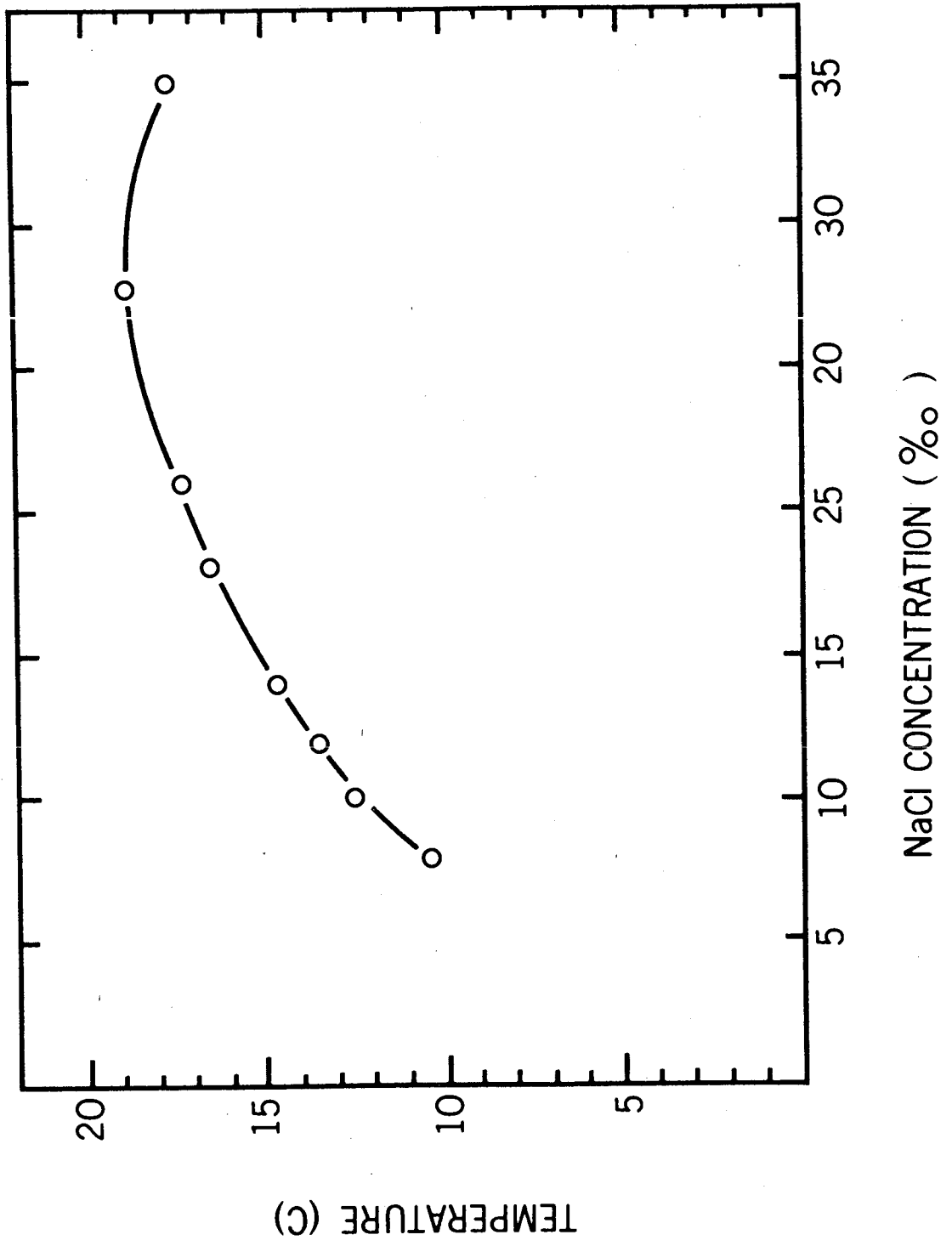(45) was employed for T_{max} determinations.

Figure 2. The effects of salinity and temperature on

the maximum hydrostatic pressure of growth

of <u>Vibrio</u> <u>marinus</u> in a nondefined medium.

P_{max} (± 7 atm) was determined as described

in Materials and Methods. Symbols: ● ,

incubation temperature 9 C; ○ , incubation
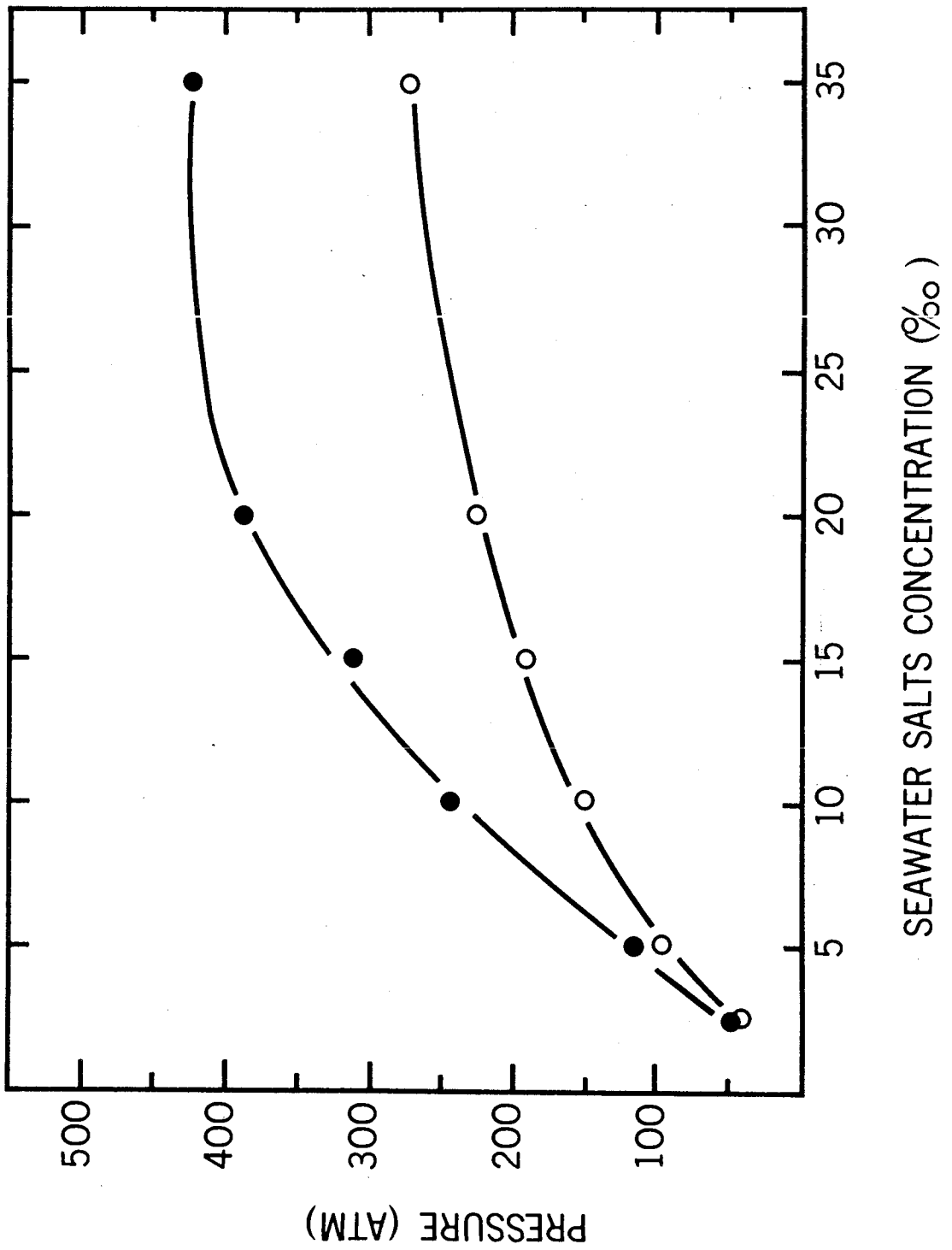
temperature 4 C.

Figure 3.   The effects of NaCl and temperature on the

maximum hydrostatic pressure of growth of

<u>Vibrio</u> <u>marinus</u> in a defined medium. P_{max}

(± 7 atm) was determined as described in

Materials and Methods.   Symbols: ● , in-

cubation temperature 9 C; ○ , incubation

temperature 4 C.

Figure 4. The effects of NaCl ($\bigcirc$), KCl ($\blacksquare$), and

NH_4Cl ($\square$) on <u>Escherichia</u> <u>coli</u> PAS activity

at 33 C and 1 atm pressure. Synthetase

activity was determined as described in

Materials and Methods. Activity is expressed

as a % of the mμm of phenylalanyl tRNA formed

in the standard reaction mixture (Table IV).

Samples were counted to ± 1% preset error

with background counted to ± 10% preset error.

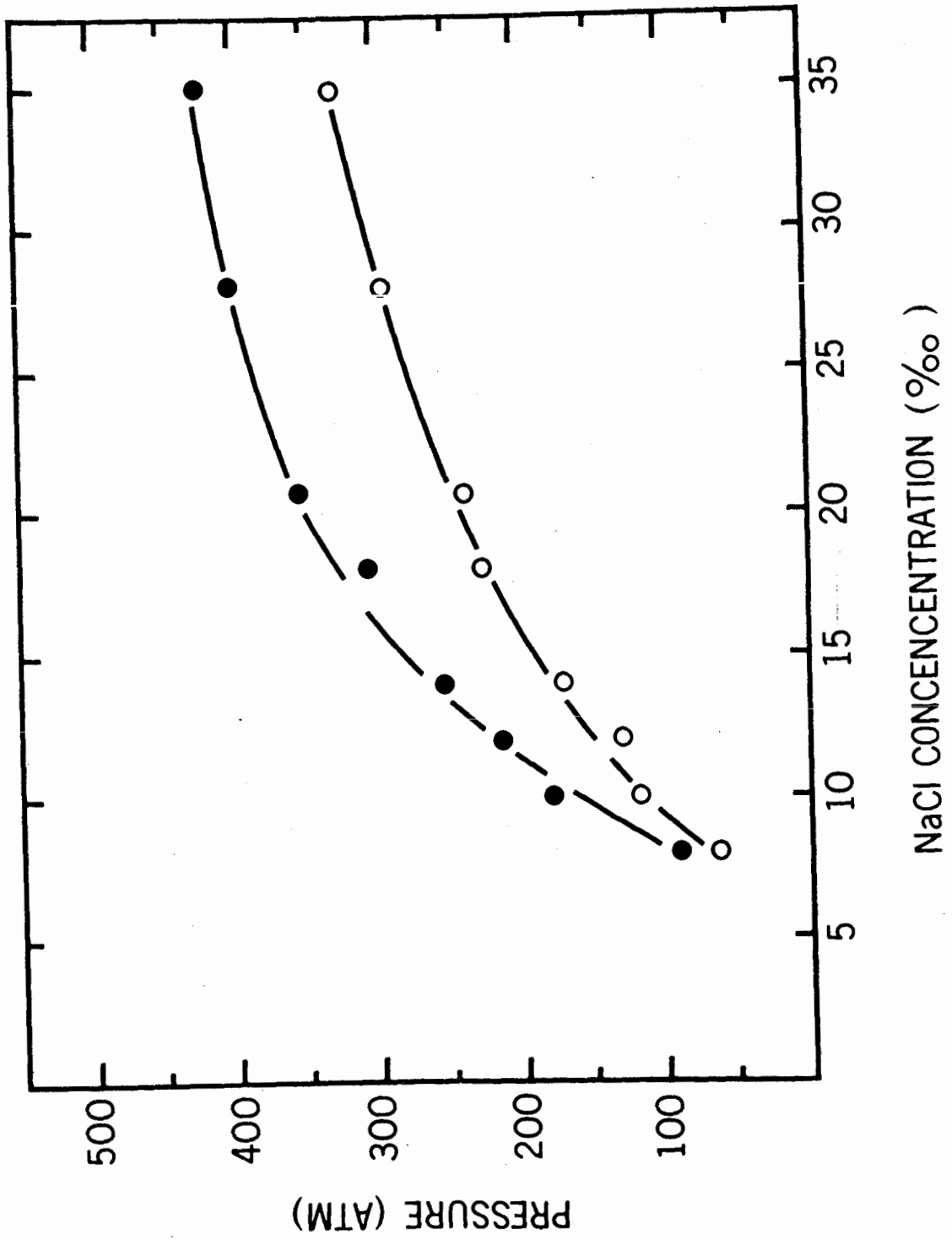% PHENYLALANYL — tRNA

SALT CONCENTRATION (M)

Figure 5. The effects of NaCl (○), KCl (■), and

NH₄Cl (☐) on <u>Vibrio</u> <u>marinus</u> PAS activity

at 15 C and 1 atm pressure. Synthetase

activity was determined as described in

Materials and Methods. Activity is expressed

as a % of the mμm of phenylalanyl tRNA formed

in the standard reaction mixture (Table IV).

Samples were counted to ± 1% preset error

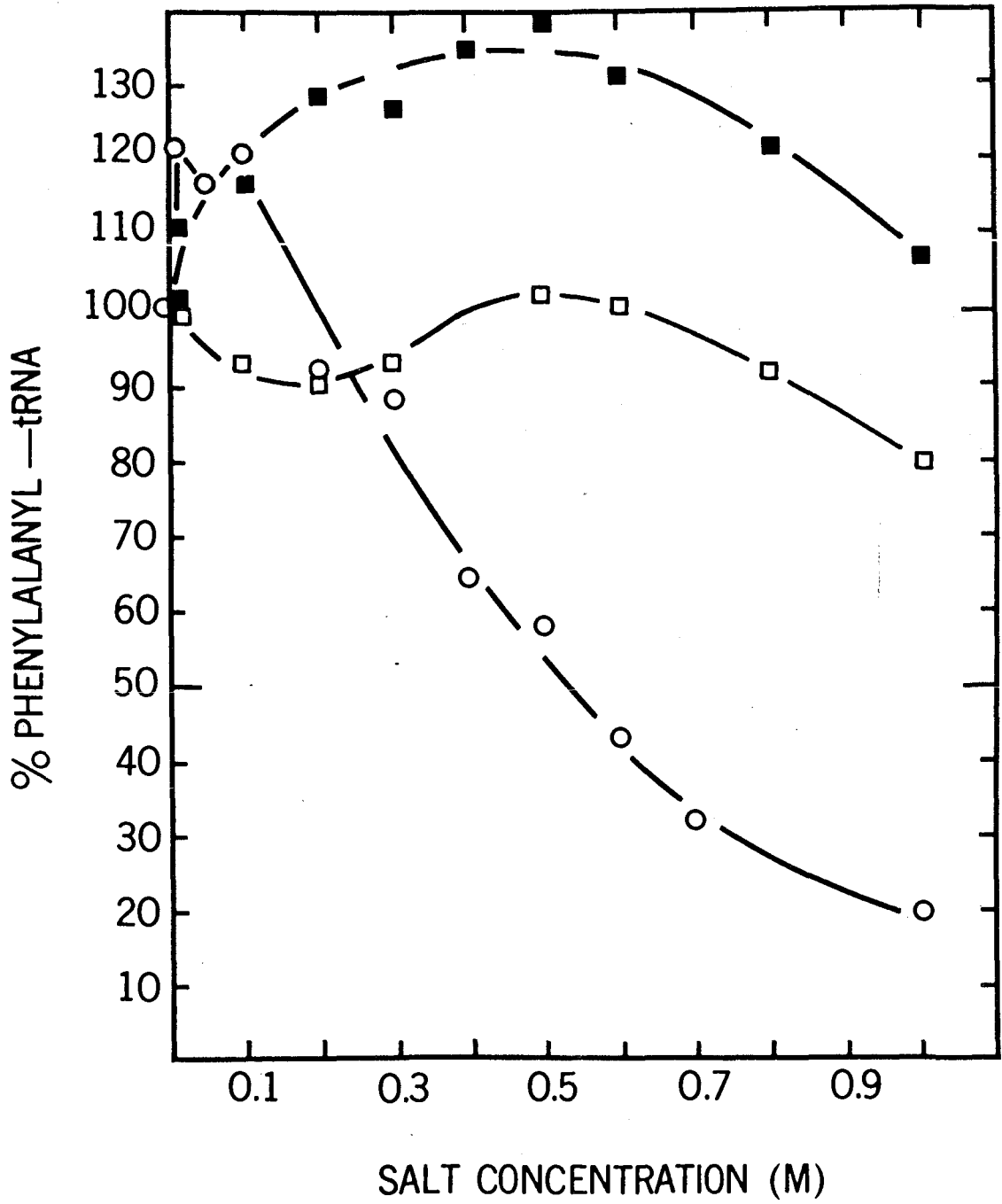with background counted to ± 10% preset

error.

Figure 6. The effect of hydrostatic pressure on <u>Escherichia</u>

<u>coli</u> PAS activity at 33 C with no added salts

(●) and 0.5 M levels of NaCl (○), KCl

(■), and NH_4Cl (□). Synthetase activity

was determined as described in Materials and

Methods. Activity is expressed as a % of the

mμm of phenylalanyl tRNA formed in the stan-

dard-reaction mixture at 1 atm pressure

(Table IV). Samples were counted to ± 1%

preset error with background counted to ±

10% preset error.

Figure 7. The effect of hydrostatic pressure on <u>Vibrio</u>

<u>marinus</u> PAS activity at 15 C with no added

salts (●) and 0.5 M levels of NaCl (○),

KCl (■), and NH_4Cl (□). Synthetase ac-

tivity was determined as described in Materials

and Methods. Activity is expressed as a % of

the mμm phenylalanyl tRNA formed in the stan-

dard reaction mixture at 1 atm (Table IV).

Samples were counted to ± 1% preset error
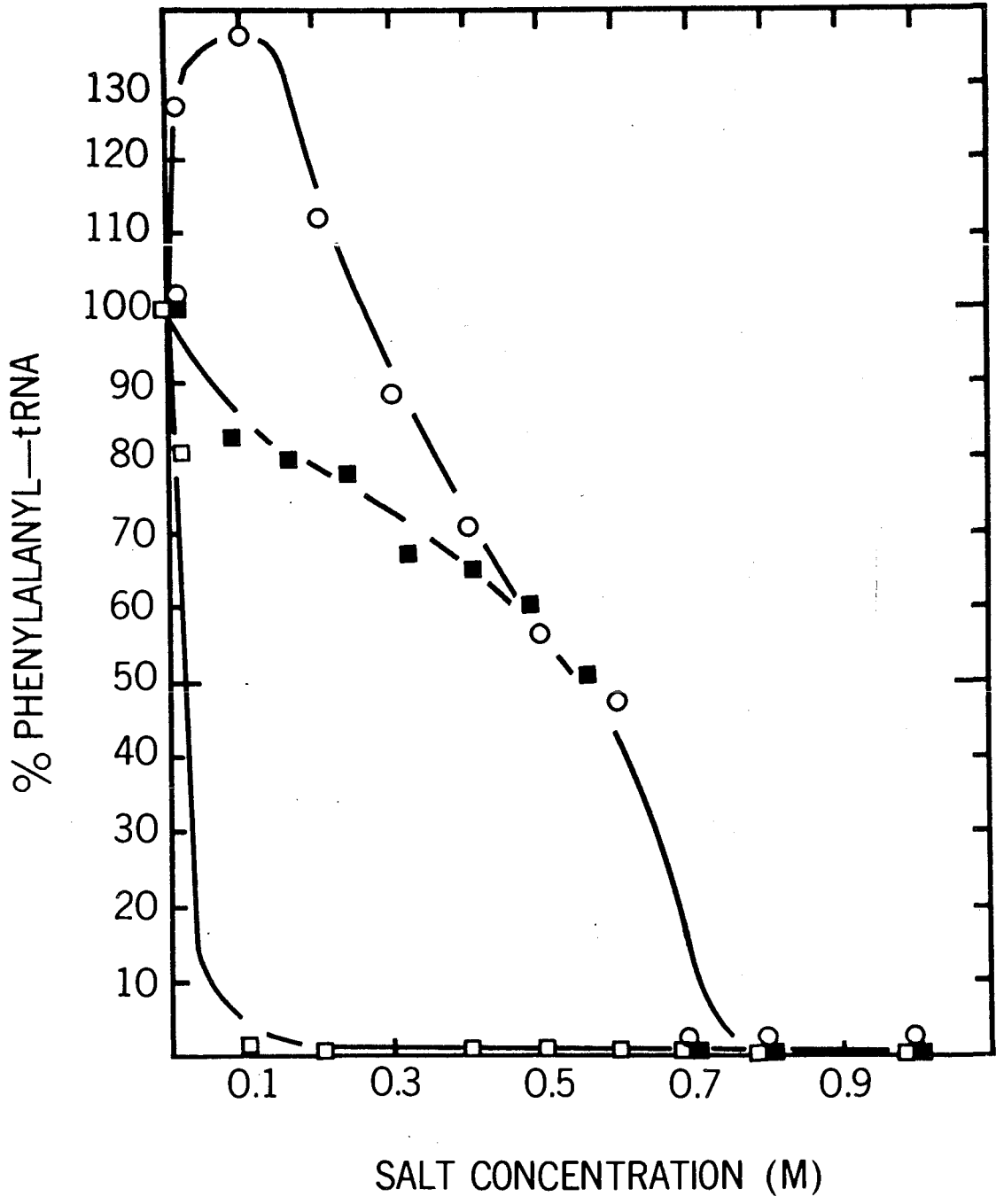
with background counted to ± 10% preset error.
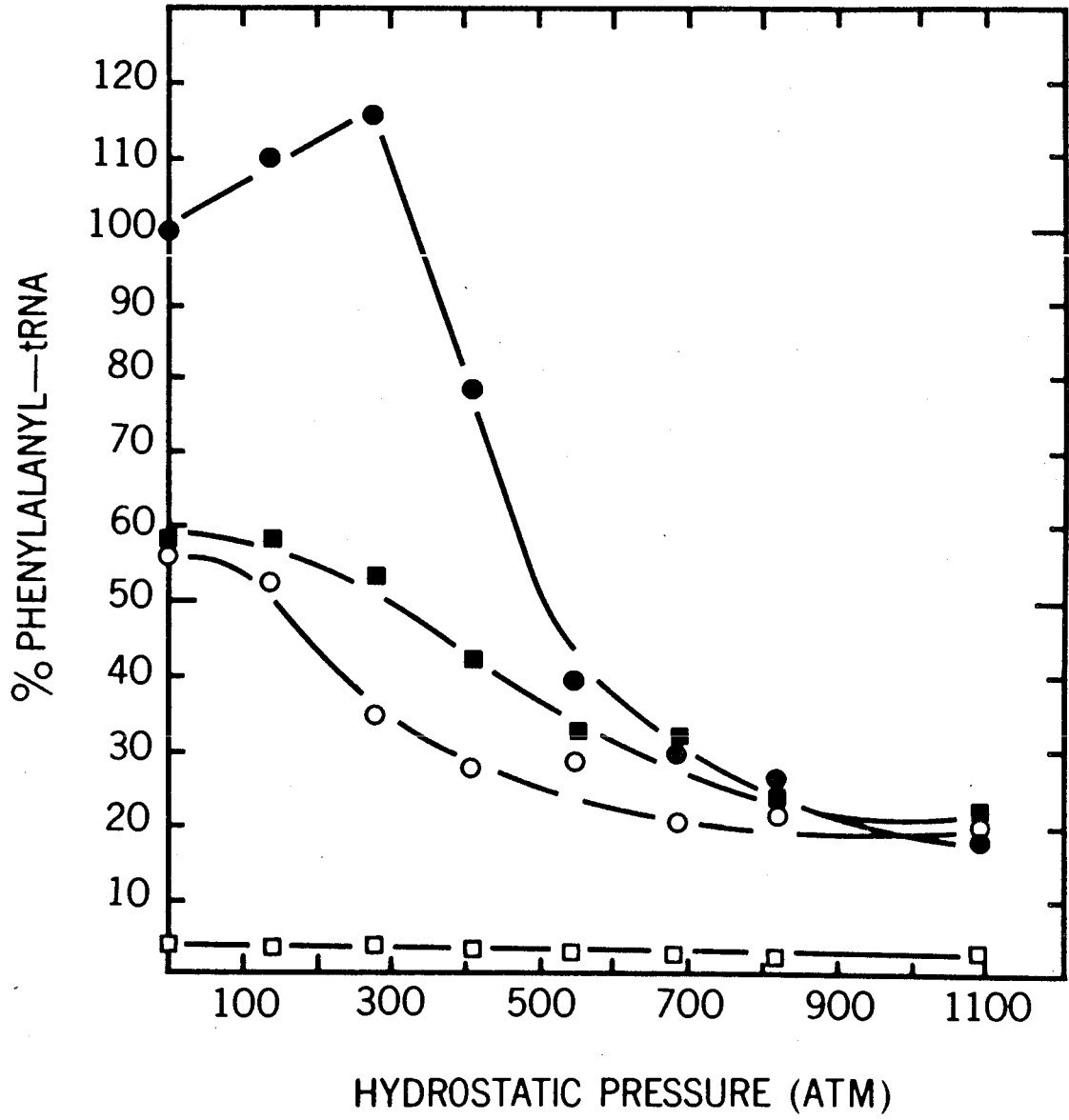
Figure 8. The effect of pressure on <u>Escherichia</u> <u>coli</u> PAS

activity at 33 C with no added salts (●)

and 1.0 M levels of NaCl (○), KCl (■),

NH_4Cl (□). Synthetase activity was deter-

mined as described in Materials and Methods.

Activity is expressed as a % of the mμm

phenylalanyl tRNA formed in the standard

reaction mixture at 1 atm pressure (Table IV).

Samples were counted to ± 1% preset error with

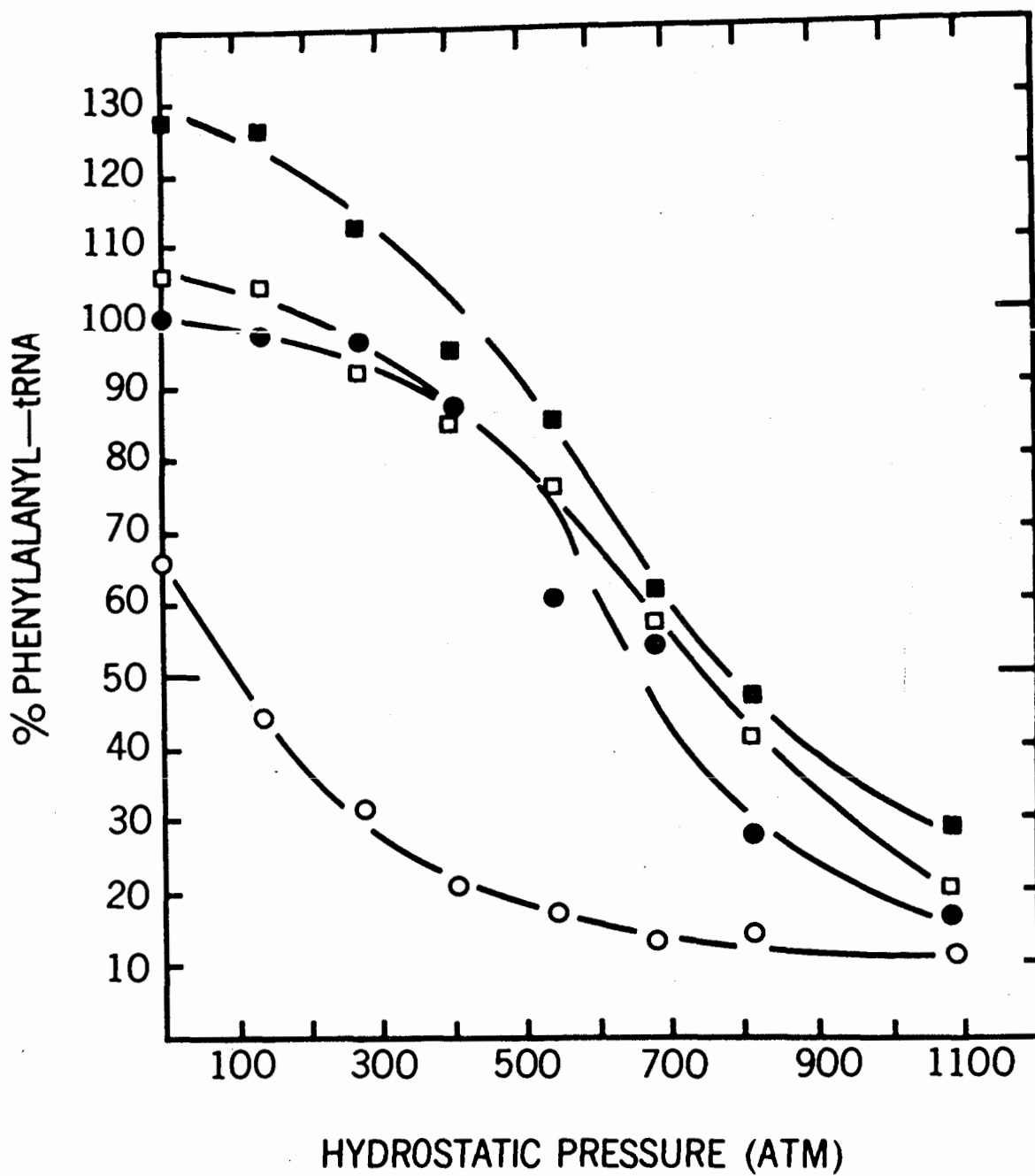background counted to ± 10% preset error.

Y-axis: % PHENYLALANYL—tRNA

X-axis: HYDROSTATIC PRESSURE (ATM)

Figure 9. The effect of hydrostatic pressure on <u>Vibrio</u>

<u>marinus</u> PAS activity at 15 C with no added

salts (●) and 1.0 M levels of NaCl (○),

KCl (■), and NH_4Cl (□). Synthetase ac-

tivity under pressure was determined as

described in Materials and Methods. Activity

is expressed as a % of the mµm phenylalanyl

tRNA formed in the standard reaction mixture

at 1 atm pressure (Table IV). Samples were

counted to ± 1% preset error with background
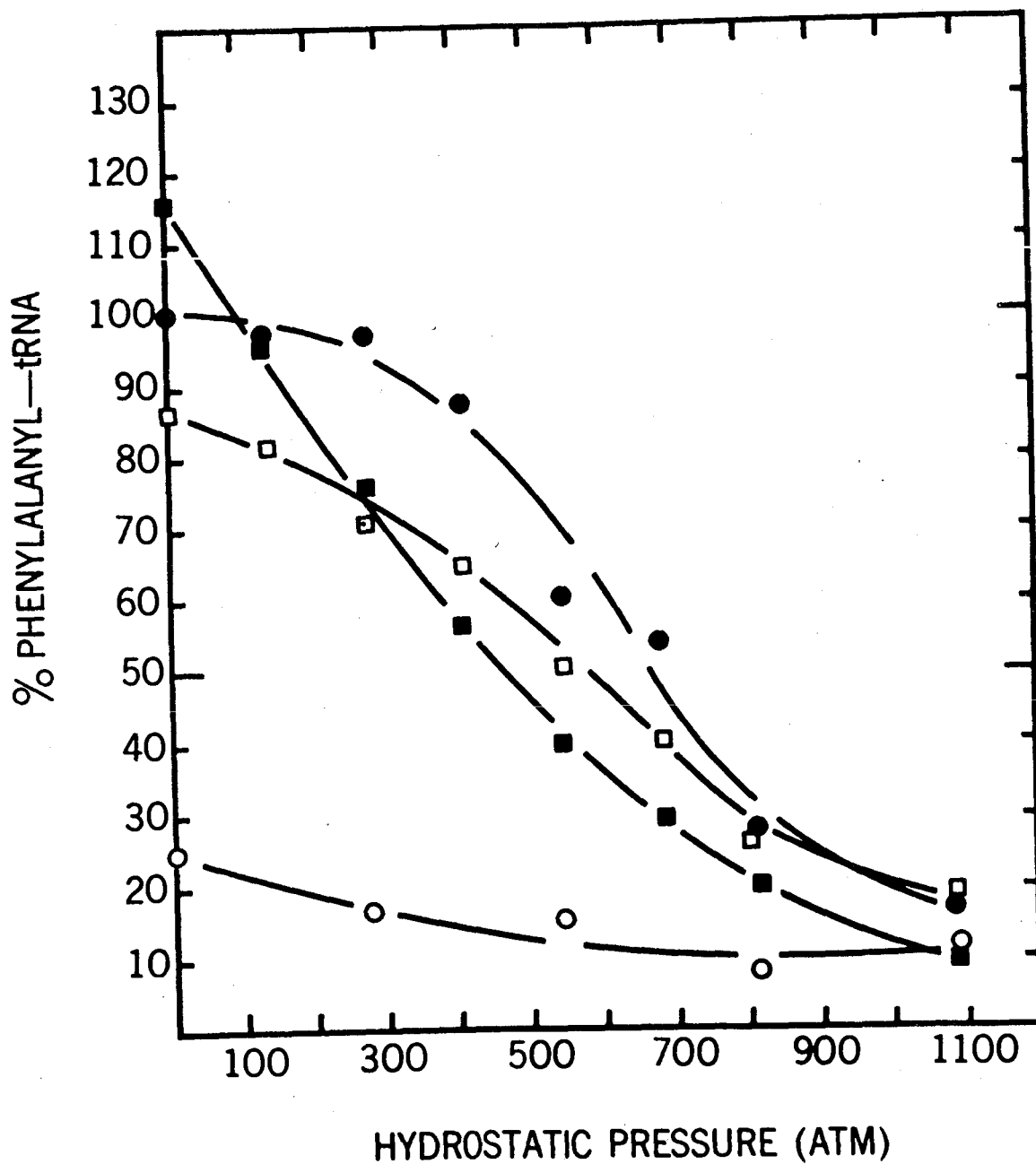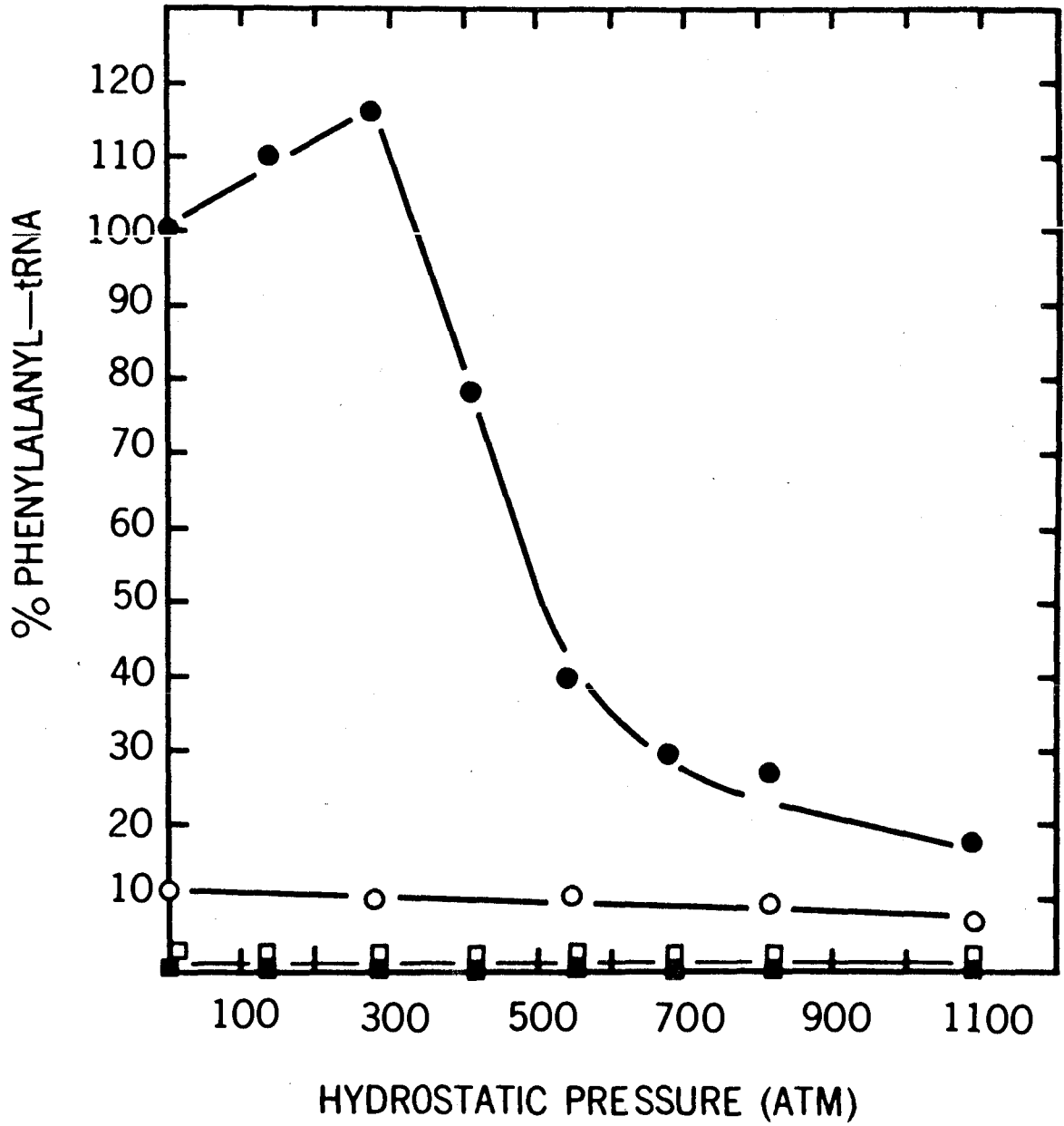
counted to ± 10% preset error.

SUMMARY

The maximum hydrostatic pressure of growth of the marine bacterium $\underline{V}$. $\underline{marinus}$ grown in a nondefined medium with 35‰ synthetic seawater salts was 425 and 320 atm at 9 and 4 C respectively. At an optimal NaCl concentration of 35‰ in a defined medium, the growth of this bacterium was completely inhibited at 422 and 326 atm at 9 and 4 C respectively. At the lowest NaCl concentration which permitted growth at 1 atm pressure (8‰), cell division was inhibited at 88 and 61 atm at these growth temperatures. The ability of other salts to replace the NaCl requirement of this bacterium was dependent on the ionic composition of the replacing salt and on the concentration of NaCl employed. $\underline{E}$. $\underline{coli}$ was unaffected by changes in the NaCl concentration of a nondefined medium and grew to a maximum pressure of 500 atm at 37 C. The results would suggest that the hydrostatic pressure tolerance of the marine organism is affected both by temperature and ionic environment. Temperature is known to affect the pressure tolerance of $\underline{E}$. $\underline{coli}$, however unlike $\underline{V}$. $\underline{marinus}$ salinity does not affect the P_{max} of this organism. Salts and pressure were demonstrated to affect the aminoacylation of phenylalanyl tRNA in cell-free extracts of $\underline{E}$. $\underline{coli}$ and $\underline{V}$. $\underline{marinus}$

at 33 C and 15 C respectively. Pressure effects on the
PAS activity of both organisms was modified by the ionic
environment. The PAS from _V. marinus_ was not only more
sensitive to salts but was also more adversely affected
by hydrostatic pressures greater than 300 atm than was
E. coli PAS. The data suggests that, in both cases,
aminoacylation is not so adversely affected by salts
and pressure so that protein synthesis and cell division
would cease. In addition both organisms probably com-
partmentalize or effectively remove PAS inhibitory salts
from the intracellular environment.

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