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Effects of Hydrostatic Pressure and Temperature on the Uptake and Respiration of Amino Acids by a Facultatively Psychrophilic Marine Bacterium[1]

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Studies of pressure and temperature effects on glutamic acid transport and utilization indicated that hydrostatic pressure and low temperature inhibit glutamate transport more than glutamate respiration. The effects of pressure on transport were reduced at temperatures near the optimum. Similar results were obtained for glycine, phenylalanine, and proline. Pressure effects on the transport systems of all four amino acids were reversible to some degree. Both proline and glutamic acid were able to protect their transport proteins against pressure damage. The data presented indicate that the uptake of amino acids by cells under pressure is inhibited, which is the cause of their inability to grow under pressure.

It is well recognized that marine bacteria live under varying hydrostatic pressure, but many of the bacteria isolated from the surface waters of shallow sediments can be adversely affected by pressure. Oppenheimer and ZoBell (16) subjected 63 species of marine bacteria to various hydrostatic pressures and found that they all grew better at 1 atm than at 200 atm or higher. When these 63 species were subjected to 400 atm for 8 days at 27 C in nutrient medium, 28 failed to grow, and 11 were killed. At 600 atm, under the same conditions, 23 of the 56 species that failed to grow were killed. There are many reasons presented as to why hydrostatic pressure adversely affects bacteria, including the interruption of energy production due to pressure (3), molecular volume changes that affect enzyme reaction rates (2, 5, 6, 9, 12, 13, 15), and loss of the ability to synthesize macromolecules (1, 10, 11, 17). Molecular volume decreases with decreasing temperature and with increasing pressure. Both environmental stresses occur as terrestrial forms descend through the water column. The data presented here, we believe, may indicate the primary basis for the adverse affect of pressure on many bacteria.

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MATERIALS AND METHODS

A facultatively psychrophilic marine bacterium, designated MP-38, isolated from station NH-4 (Department of Oceanography, Oregon State University, R/V Acona, Cruise 6406) by R. D. Haight, was used for experiments on pressure and temperature effects on transport. This organism is a *Vibrio* species, and its taxonomic characteristics have been determined by R. R. Colwell (*personal communication*). MP-38 exhibits a wide temperature tolerance range; it is able to grow below 0 C and above 30 C. The optimum growth temperature [temperature at which the greatest optical density at 600 nm (OD_{600}) is reached in 8.5 hr] is about 22 C. Stock cultures of this organism were kept on agar slants at 5 C and transferred bimonthly.

Cells were grown in a dilute nutrient saline medium (NSM) containing (in g/liter): Rila Marine Salts (Rila Products Co., Teaneck, N.J.), 35; succinic acid, 0.2; polypeptone (BBL), 0.5; yeast extract (Difco), 0.3. The nutrients were kept in a sterile concentrated solution, *p*H 7.4, and diluted 1:10 with artificial seawater (ASW) before being dispensed. ASW was adjusted to *p*H 7.4 with NaOH. The dispensed medium was autoclaved at 120 C for 10 to 20 min, depending on volume, and cooled to 15 C before inoculation. ASW, in all cases, refers to a solution of Rila salts of 35 g/liter, *p*H 7.4. When a solid medium was needed, NSM was solidified with 1.5% agar (Difco).

Cells were grown for 12 hr in NSM using a 1% (v/v) inoculum from a 12-hr culture. The cells were harvested by centrifugation for 5 min at 10,400 $\times g$ in a Sorvall RC2-B refrigerated centrifuge, washed twice with membrane filtered (MF) ASW, and suspended to an OD_{600} of 0.25, corresponding to approximately 6.0 $\times 10^8$ to 8.0 $\times 10^8$ cells/ml. Viable count versus OD was established by plate count versus OD_{600} on a spectrophotometer (Bausch & Lomb Spectronic 20). The

suspension was diluted 1:10^3 with MF ASW, and ^{14}C-amino acids were added to a concentration of 200 μg/liter and 0.02 μCi/ml. The radioactive amino acids, L-glycine-*UL*-^{14}C (Amersham/Searle), L-phenylalanine-*UL*-^{14}C, L-methionine-*UL*-^{14}C, L-proline-*UL*-^{14}C, and L-glutamic acid-*UL*-^{14}C (International Chemical and Nuclear Corp.), were made up to a concentration of 2 μg/ml (0.2 μCi/ml) with cold DL-amino acid (Nutritional Biochemicals Corp.) and stored frozen at −10 C. The specific activity of each solution was 100 μCi/mg. After addition of the substrate, 10-ml portions of the iced cell suspension were drawn into plastic syringes, tightly capped, and kept on ice until pressurized. The syringes were placed in pressure cylinders (19) and pumped up to the desired pressure. The blank was treated as a 500-atm sample, only up to temperature equilibration time, after which the cylinder was depressurized. The syringe contents were then emptied into 50-ml serum bottles containing 0.6 ml of 0.1 N H_2SO_4 to fix the cells and release CO_2. The bottles were quickly stoppered with rubber serum caps equipped with a plastic bucket containing a piece of fluted Whatman no. 1 filter paper (25 by 57 mm). At least 10 min after fixing, 0.2 ml of phenylethylamine was introduced with a syringe onto the filter paper through the serum cap for $^{14}CO_2$ adsorption. All serum bottles were left sealed at room temperature at least 1 hr before uncapping. (4). The fluted filter papers were placed in scintillation vials and counted in 5 ml of scintillation fluor containing 5 g of 2,5-diphenyloxazole (PPO) per liter and 0.3 g of triphenyldioxazole (POPOP) in toluene. Twelve hours of incubation of the filter papers in fluor at 15 C was necessary to permit clearing of the fluor and stabilization of counts. Cells were collected by filtration through membrane filters (0.45 μm, 25 mm diameter), air dried at 60 C for 2 hr, and counted in 2 ml of fluor in a liquid scintillation counter (Nuclear Chicago Mark I). The counting efficiency was determined from channels ratio by using toluene-*UL*-^{14}C (International Chemical and Nuclear Corp.) quenched with chloroform.

The viability of MP-38 under pressure was checked by plate count to make certain that no substantial decrease in viable cell number occurred during the course of the experiments. Experiments on the effect of substrate concentration or exposure time were set up in the same manner described above, with varied parameters. Experiments were performed to determine whether preincubation with substrate could protect the transport system from pressure inhibition. Samples were prepared as above, incubated with ^{14}C-glutamate or ^{14}C-proline for 3 hr at 5 C, 1 atm, and pressurized to 500 atm. Cells were depressurized and fixed at 3 and 7 hr after pressurization. To determine whether pressure damage was reversible or irreversible, samples in syringes were incubated at 5 C under 500-atm pressure for 5 hr. The pressure was then released, and the samples were allowed to incubate further at 1 atm for 6 hr. Sampling was done on release of pressure and during atmospheric incubation.

In these experiments, uptake of ^{14}C material into the cell was measured in disintegrations per minute. The rate of uptake is measured in disintegrations per minute of ^{14}C material taken up per hour. Relative uptake refers to uptake under pressure, taken as a percentage of the uptake of an identical sample at atmospheric pressure.

RESULTS AND DISCUSSION

Figure 1 presents the uptake of a variety of amino acids by MP-38, indicating both the specificity of transport proteins and their differing substrate affinities. These data include a CO_2 correction for uptake. MP-38 shows a marked preference for amino acids over the other organic substances presented.

Most pressure studies with MP-38 were done with glutamic acid because of the key role this amino acid plays in cellular metabolism. Pressure-viability studies over the temperature range of 5 to 25 C showed that MP-38 remained viable for at least 5 hr at 5 C up to 400 atm. At 20 C, the organism grew within 3 hr at pressures below 200 atm but started to expire at 400 atm after 2 hr. At 25 C, MP-38 grew at atmospheric pressure but died at 200 and 400 atm after 3 hr. The following experiments were run for a period of time at each temperature such that there was no change of cell numbers during the time of exposure.

Glutamate uptake by MP-38 was linear with concentration up to 200 μg/liter, the highest concentration used (Fig. 2). Uptake was also linear with time for the time interval and over the temperature and pressure range used in these experiments (Fig. 3 and 4). This information, taken with the viability studies, indicates that there were no substantial numerical alterations in the bacterial population during the course of the experiment and that decreases in uptake resulted from the effects of temperature and pressure. The linearity of uptake with time also indicates that the event or events which accounted for decreased uptake with application of pressure happened at the outset of the timed period. There was no progressive pressure effect indicated for the time period observed; the uptake rate is constant for each time interval.

Figure 5 presents a comprehensive picture of glutamic acid transport over the temperature range 5 to 25 C and the pressure range of 1 to 500 atm. It can be seen readily that increased pressure decreased the total amount of glutamate taken up by the cells in a given amount of time. The continued production of CO_2 at a constant or increasing proportion of the total indicates that the cells respired under pressure. It also indicates that glutamate respiration and incorporation into cellular material are affected differently by pressure. The proportion of CO_2 increased as pressure increased at all temperatures up to 400 atm.

At temperatures below 25 C, the percentage of

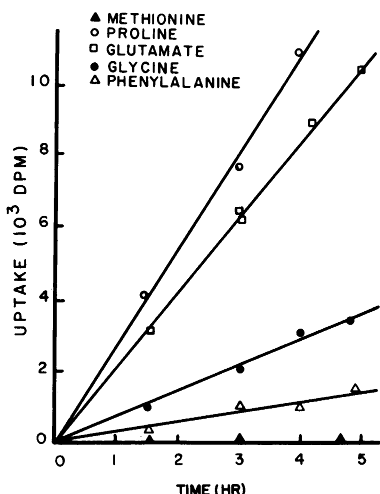

FIG. 1. *Time course of total uptake of ^{14}C-methionine, ^{14}C-glycine, ^{14}C-phenylalanine, ^{14}C-glutamate, and ^{14}C-proline by marine bacterium MP-38 at 5 C, 1 atm. All amino acid concentrations were 200 μg/liter with an activity of 0.02 μCi/ml in 10 ml of artificial seawater; the cell concentration was 6.0 × 10^5/ml.*

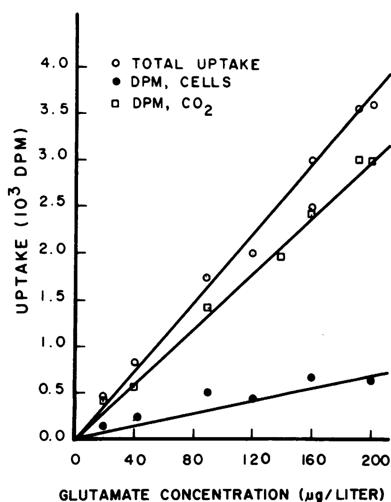

FIG. 2. *Uptake of ^{14}C-glutamic acid by marine bacterium MP-38 versus concentration at 15 C, 1 atm. Cell concentration was approximately 6.0 × 10^5/ml in artificial seawater.*

CO_2 dropped or remained constant between 400 and 500 atm; but at 25 C, the proportion of CO_2 increased at 500 atm. The overall increase in CO_2 production may be due to the failure of the cell to maintain a balance in the proportion of substrate which goes into any of the metabolic or synthetic pathways. The factors which alter the distribution of radioactivity may reflect the different effects of temperature and pressure on the enzymes of those pathways. Any set of conditions which favors the activity of the enzymes of the respiratory pathway or inhibits those of the synthetic pathways, or both, would result in an increased proportion of CO_2. If pressure depressed incorporation more than respiration, the availability of substrate for the respiratory enzymes would be effectively increased; that is, the competition for glutamate would be decreased. It is possible that the effects of pressure on enzyme structure, solvent structure, substrate ionization, etc., might result in increased enzyme-substrate

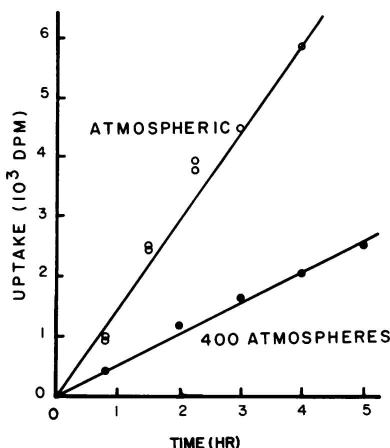

FIG. 3. *^{14}C-glutamic acid uptake in artificial seawater by marine bacterium MP-38 at 5 C, 1 atm and 400 atm. Cell concentration was approximately 6.0 × 10^5/ml; glutamic acid concentration was 200 μg/liter with an activity of 0.02 μCi/ml in 10 ml.*

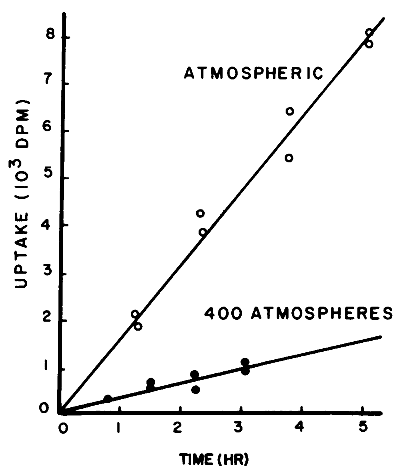

FIG. 4. *^{14}C-glutamic acid uptake in artificial seawater by marine bacterium MP-38 at 20 C, 1 atm and 400 atm. Cell concentration was approximately 6.0 × 10^5/ml; glutamic acid concentration was 200 μg/liter with an activity of 0.02 μCi/ml in 10 ml.*

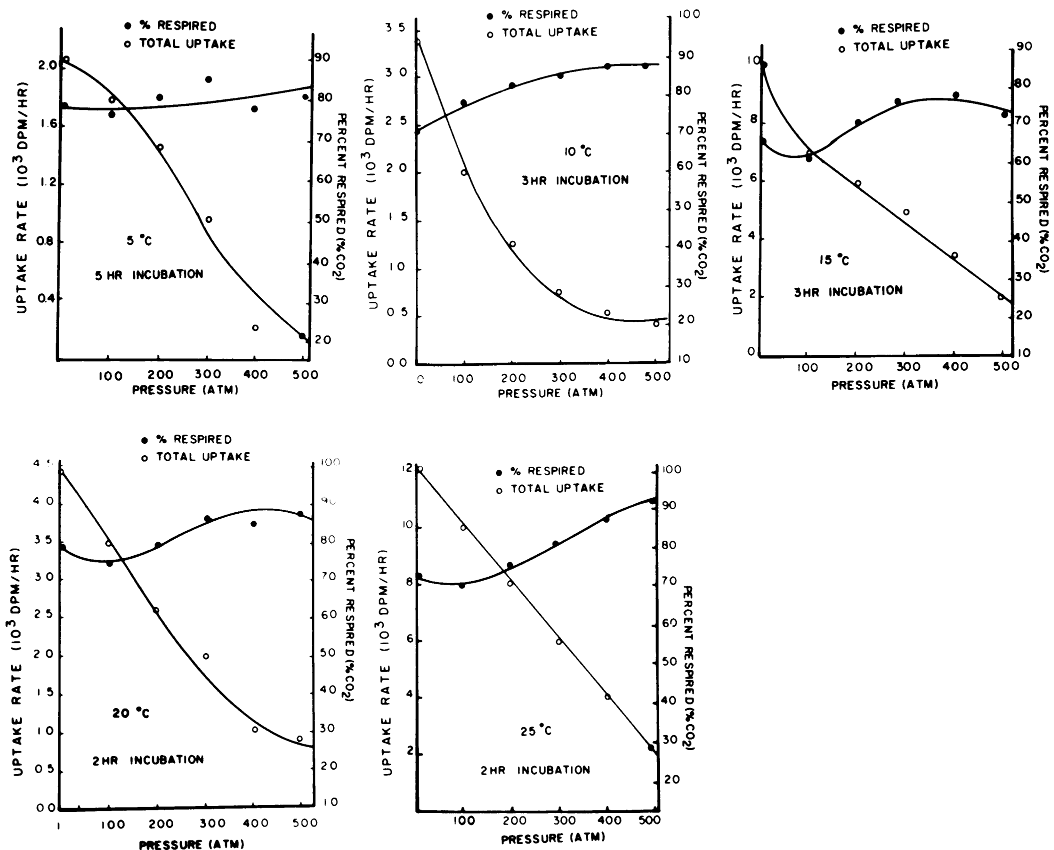

FIG. 5. *Uptake and respiration of* [14]*C-glutamic acid in artificial seawater at 5, 10, 15, 20, and 25 C. Cell concentration was approximately 6.0* $\times$ *10*[5]*/ml; glutamic acid concentration was 200 µg/liter with an activity of 0.02 µCi/ml in 10 ml.*

affinity which would permit more effective competition for substrate on the part of the respiratory enzymes.

Carbon dioxide production may not be as affected by pressure as incorporation is, because of a possible intrinsic ability of respiratory enzymes to endure the molecular volume decrease effected by high pressure. If these enzymes, or a rate-limiting enzyme, undergo only a small molecular volume increase on forming the enzyme-substrate complex, the effects of pressure may be minimized. As the temperature is increased, the effect of pressure should be further minimized and finally offset. This may have been the case for glutamate uptake between 400 and 500 atm. At the lower temperatures, 500 atm may have been sufficient to inhibit respiration by preventing molecular volume increase. At 25 C, the effects of temperature may have compensated for the effects of pressure, permitting continued or increased activity of respiration enzymes.

Although the proportion of CO_2 increases, the actual CO_2 production decreases, as does the actual amount of glutamate incorporated. The actual amount of glutamate respired or incorporated decreases with pressure, because the total amount of glutamate taken up decreases with increasing pressure.

It is unlikely that glutamate uptake was influenced by amino acid pool levels. The high proportion of respired substrate indicates that the glutamate was rapidly utilized and spent little time in the pool. Using similar amino acid concentrations, Kay and Gronlund (7) found that pool levels did not influence amino acid uptake for *Pseudomonas aeruginosa*, also because of rapid utilization.

Figure 6 shows the uptake of glutamate as a function of temperature at 1 and 400 atm pressure. The optimal temperature of growth is lower than the optimum and maximum of the transport system, neither of which has been reached at 25 C. The temperature-pressure optimum effect discussed by Johnson (5) cannot be completely demonstrated with the data, since the optimal temperature at atmospheric pressure was not

attained. However, it can be seen that, as the optimal temperature is approached by increasing the incubation temperature, the effect of pressure is decreased. The values for uptake at low temperatures are more depressed as compared with atmospheric values than are those at temperatures closer to the optimum for the glutamate transport mechanism. The inhibiting effect of pressure should be minimized at the optimal temperature; at higher temperatures, pressure should accelerate transport and protect transport proteins from heat damage (5, 15).

Studies on the transport of glycine, phenylalanine, and proline were also done at 5 C (Fig. 7). As with glutamate, increased pressure reduced the total amount of amino acid taken up by the organism. However, the respiration patterns at 5 C are different for all four amino acids. Where glutamate respiration increased up to 400 atm and then decreased slightly, glycine respiration was depressed strongly at higher pressure, proline respiration increased, and phenylalanine-CO_2 production was relatively unaffected. Since pressure affected amino acid incorporation the same amount for each amino acid, the differences in respiration patterns are probably due to intrinsic differences in the respiration enzymes for each amino acid.

The percentage of total glycine that was respired increased little with increasing pressure and was depressed strongly at higher pressures, proamounts for incorporation and respiration may not be altered, because both systems are equally pressure-sensitive up to 400 atm.

Phenylalanine respiration also occurred at a fairly constant proportion of the total take-up. This may indicate that the relative sensitivities of the metabolic pathways using phenylalanine were maintained under pressure. It is possible that the respiratory enzymes were functioning at their maximum capacity at each pressure level with respect to the total substrate concentration, a pseudosaturation condition.

Explanations for the increase in proline respiration are the same as those for glutamate, with some exceptions. If pressure inhibited the enzymes of proline respiration less than those of other pathways, available proline may have been metabolized at all pressures, indicating a higher degree of pressure resistance at this temperature than found for the glutamate enzymes. A similar curve would be expected if pressure stimulated proline respiration, either by increasing enzyme-substrate affinity or enzyme activity. Figure 8 indicates the differences in the response of MP-38 to these four substrates. It is possible that the four amino acid transport and metabolic systems have different temperature, pressure, and concentration optima, varying pool levels, and that any

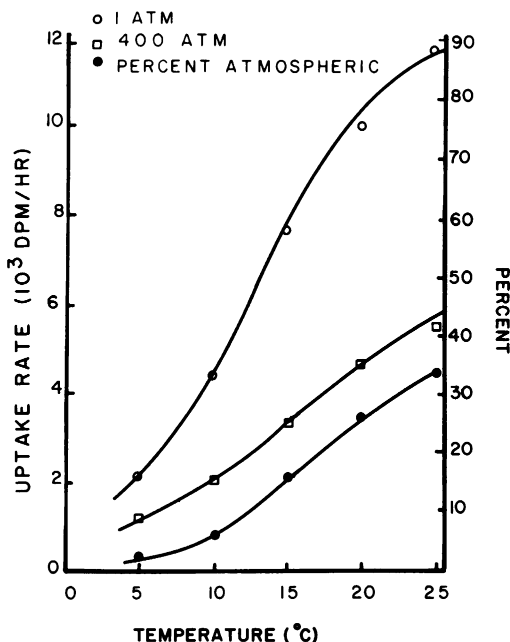

FIG. 6. *Total uptake of ^{14}C-glutamic acid by marine bacterium MP-38 at 1 atm and 400 atm over the temperature range of 5 to 25 C. Uptake at 400 atm is plotted as a percentage of the uptake at 1 atm at the same temperature.*

given amino acid may have more than one transport mechanism.

These figures, taken together, indicate that the essential effect of high pressure is to reduce the organism's supply of nutrients at low temperature by inhibiting its transport system. Such an effect would have tremendous ecological ramifications for nutrient material in the deep sea, where the combined effects of cold and pressure could essentially prevent bacterial activity.

Figure 9 gives the reversibility patterns for each amino acid. All four transport systems exhibit reversible pressure inhibition with differing degrees of pressure damage. The proline transport system appear to be completely reversible in that the post-pressurization uptake rate is approximately the same as that at 1 atm without pressure.

However, there is a substantial decrease in the proportion of CO_2 respired compared to atmospheric conditions. The percentage respired is also lower at 1 atm than at 500, indicating the relative increase in CO_2 production with pressure for proline. The decrease of CO_2 production relative to a nonpressurized sample may indicate that some irreversible damage was done to the respiration pathway.

Glutamate, glycine, and phenylalanine transport systems all show impairment to the uptake

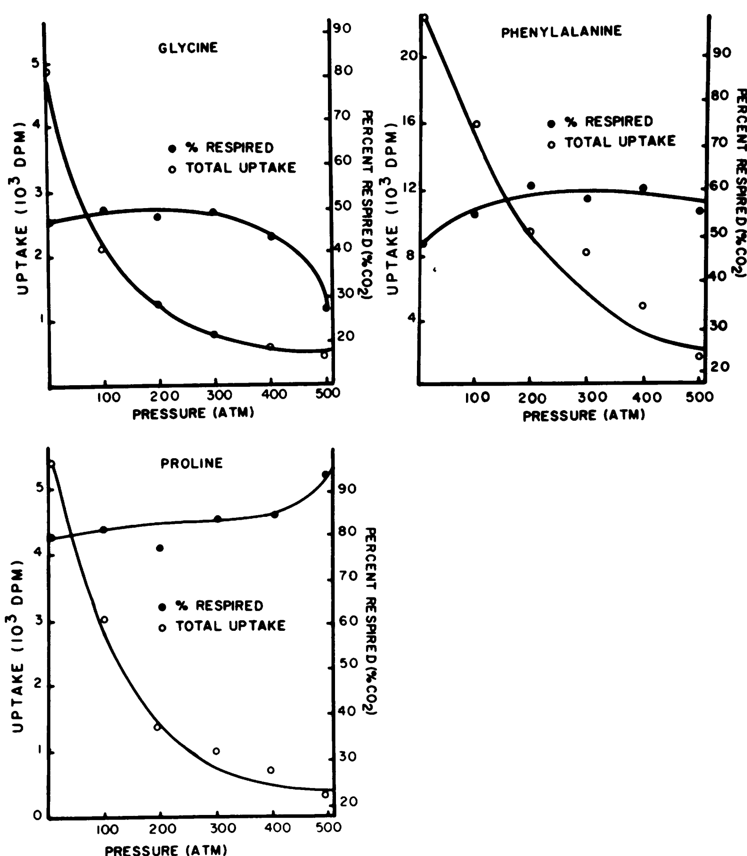

FIG. 7. *Uptake and respiration of ^{14}C-glycine, ^{14}C-phenylalanine, and ^{14}C-proline by marine bacterium MP-38 at 5 C, 5 hr of exposure. Cell concentration was approximately 6.0 × 10^5/ml in 10 ml of artificial seawater. Concentration of substrate was 200 μg/liter with an activity of 0.02 μCi/ml.*

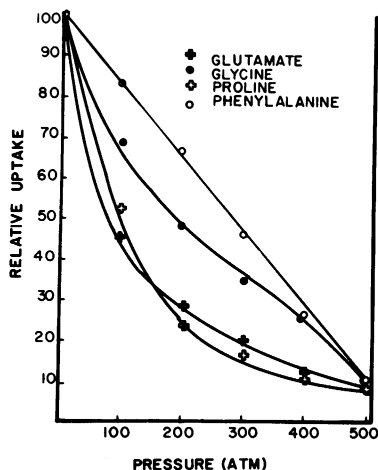

FIG. 8. *Relative uptake of four amino acids by marine bacterium MP-38, expressed as percentage of uptake at atmospheric pressure, at 5 C, 5 hr of exposure.*

mechanism, as evidenced by lowered uptake rates. The increased CO_2, relative to nonpressurized samples (percentage respired), indicates that the respiratory mechanisms are still functional and relatively undamaged after pressure release. Only glutamate transport exhibited a lag or recovery time after release of pressure.

Figures 10 and 11 show the result of exposing cells to substrate at 1 atm for 3 hr before pressurization. Counts from samples taken after exposure to 500 atm for given amounts of time indicate that both proline and glutamate were able to protect their transport proteins from pressure inhibition. The uptake rate at 500 atm without preincubation was 200 dpm/hr for glutamate and 85 dpm/hr for proline. With preincubation, the rates at 500 atm were 980 dpm/hr for glutamate

Cell concentration was approximately 6.0 × 10^5/ml. Substrate concentration was 200 μg/liter with an activity of 0.02 μCi/ml in 10 ml of artificial seawater.

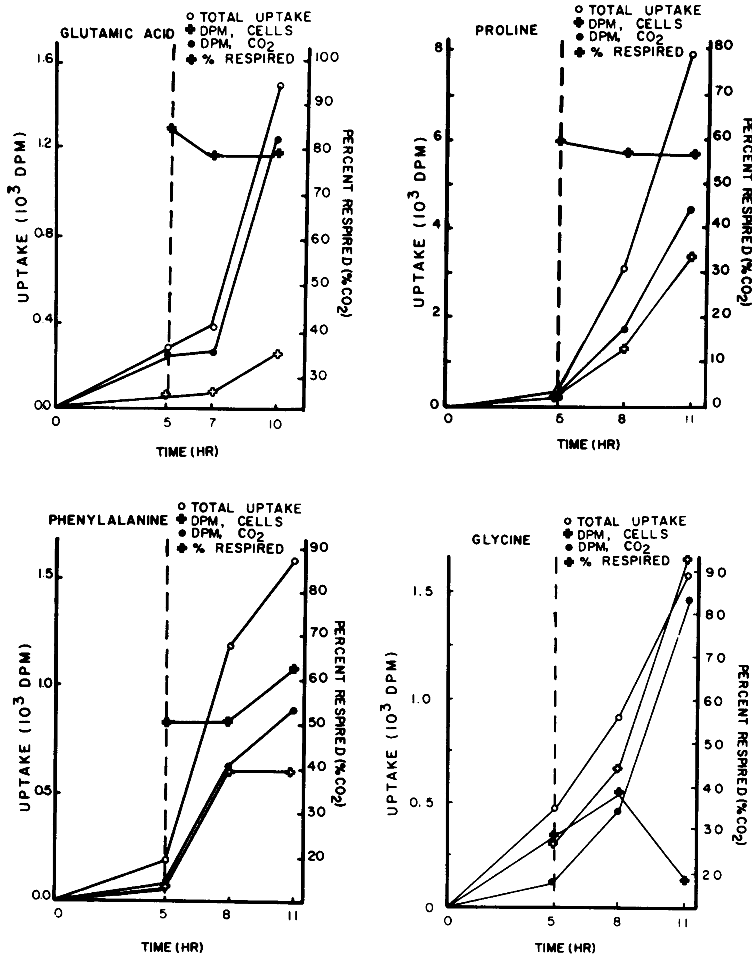

FIG. 9. *Reversibility of pressure inhibition of amino acid uptake after 5 hr of exposure to 500 atm pressure at 5 C. Cells were incubated for 6 hr after release of pressure. Cell concentration was approximately 6.0 × 10^5/ml; amino acid concentration was 200 μg/liter with an activity of 0.02 μCi/ml in 10 ml of artificial seawater.*

and 280 dpm/hr for proline. It appears that, with a large amount of available glutamate or proline, a site on the transport protein may remain charged with substrate and protected from pressure inhibition. The presence of cofactors combined with the protein in its active configuration may also help protect the transport mechanism (13).

Comparison of proline uptake and incorporation for MP-38 at 5 C, 500 atm, with and without preincubation, indicates the effect the transport system can have on data from incorporation studies. At atmospheric pressure at 5 C, MP-38 took up and incorporated into cellular material approximately 2 × 10^{-13} mg of proline per cell per hr. At 500 atm, this figure was reduced to 2 × 10^{-15} mg per cell per hr. If cells were preincubated with proline, the incorporation at 500 atm as a trichloroacetic acid precipitate was approximately 1.6 × 10^{-13} mg per cell per hr, equivalent to the amount found in whole cells (Fig. 4). The protection of the transport system by preincubation substantially changed the picture of incorporation under the same conditions. One would have to allow substrate to enter the cell before pressurization to see what effect pressure has on its use, not its uptake. Temperature variation, which also affected amino acid transport (Fig. 6) would be reflected in incorporation data. Cellular uptake of glutamate was approximately 2 × 10^{-12} mg per cell per hr at 20 C, 1 atm. At 5 C, the value was only 3 × 10^{-13} mg per cell per hr. Comparison of data for whole cells and that from trichloroacetic acid precipitates might help indicate whether transport or incorporation was the more sensitive process.

Studies of pressure effects on cellular systems using whole cells must take into consideration

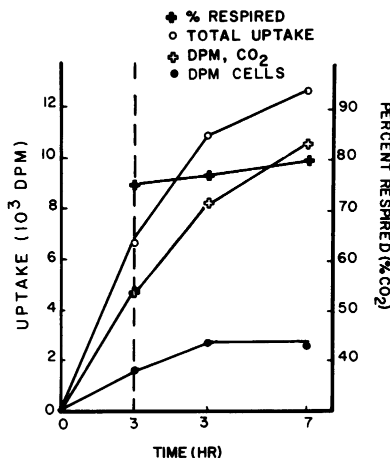

FIG. 10. *Uptake and utilization of ^{14}C-glutamic acid under pressure by marine bacterium MP-38 at 5 C. Cells were pressurized to 500 atm after being exposed to substrate for 3 hr at 1 atm. Cell concentration was approximately 6.0 × 10^5/ml. Glutamic acid concentration was 200 μg/liter with an activity of 0.02 μCi/ml in 10 ml of artificial seawater.*

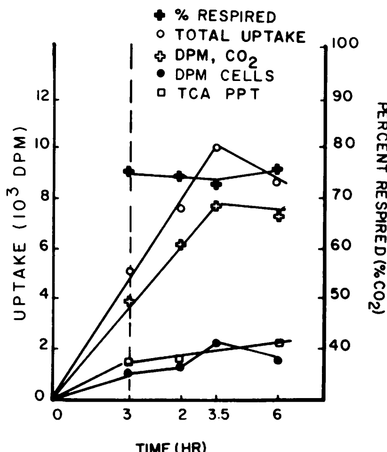

FIG. 11. *Uptake and utilization for ^{14}C-proline under pressure by marine bacterium MP-38 at 5 C. Cells were pressurized at 500 atm after being exposed to substrate for 3 hr at 1 atm. Cell concentration was approximately 6.0 × 10^5/ml; proline concentration was 200 μg/liter with an activity of 0.02 μCi/ml in 10 ml of artificial seawater.*

the effect of pressure on the transport system of compounds used for measuring incorporation and intracellular enzyme activity. Protective effects of substrates and cofactors should also be taken into account in intracellular and cell-free enzyme studies.

The data presented here suggest that the uptake of amino acids is inhibited by high pressure and low temperature; but the respiratory enzymes, necessary for subsequent amino acid metabolism, are not affected to any great degree. This naturally implies that we are dealing with a membrane phenomenon. Pressure and temperature have been shown to influence hydrophobic bonding (8). Hydrophobic groups of membrane proteins should also be influenced by both pressure and temperature changes. This should result in a conformational change of membrane proteins. This change is reflected in our studies by the lack of the ability of cells to take up amino acids.

The average pressure of the ocean has been calculated to be about 380 atm (18). This pressure has been shown to be strongly inhibitory to transport in many cases. It is interesting to speculate that, before the organism suffers a breakdown of its intracellular machinery due to pressure, it is already suffering from starvation because of an inability to obtain raw materials from outside the cell. If this is, in fact, the case, then a primary reason for the limitation of growth of organisms under high hydrostatic pressure in the ocean is the breakdown in the systems for transporting specific substrates to the metabolic machinery inside the cell.

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