BIOLOGICAL AND OCEANOGRAPHIC PROBLEMS REQUIRING HIGH PRESSURE AQUARIA— BIOCHEMICAL ASPECTS[1]

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Introduction

The "state of the art" in the field of high pressure research leaves much to be desired. As a result of this situation, there will naturally be many questions that arise for which there are no answers as yet. The discussion presented here will deal with general concepts of the physiological and biochemical aspects of hydrostatic pressure. For the latest, detailed literature review on the effects of hydrostatic pressure on biological systems, Zimmerman (1970) should be consulted.

All organisms in the marine biosphere live under various levels of hydrostatic pressure. The rule of thumb is to increase the pressure one atm for every 10 m of depth.The average and the deepest depths of the oceans are approximately 3,800 m, and 10,915 m, respectively, equivalent to 380 and to 1091 atm. Actually, the latter is closer to 1,100 atm due to the increased density caused by the compression of the sea water. In the deep sea, the main environmental variable is the pressure. The temperature is more or less constant, varying between approximately 3° and 5°C. Since the temperature is uniformly cold, the test organisms that we are currently employing are termed "obligate psychorophiles," (Morita, 1966) and it is only logical that such organisms be employed in studies dealing with life in the deep sea. Many of our past experiments, as well as those of others, have not been done with the obligate psychrophiles, since their existence in pure culture has only recently been scientifically established. However, many of the past experiments do shed light on the mechanisms of action of hydrostatic pressure. From the Ideal Gas Law ($PV=nRT$) we realize that there is an interaction between pressure, temperature and volume. Molecular volume changes (partial molecule volume changes) are very important in the interpretation of data. This subject will be discussed in more detail by my colleague, Dr. Robert Becker. Salinity is another important variable in the marine environment, and when one throws salt into the above Ideal Gas Law, then things can become extremely complicated. In other words, there are interrelationships between salt effects and pressure (Palmer and Albright, 1970), temperature (Stanley and Morita, 1968), and molecular volume (Kettman, *et al.*, 1965).

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Complicating Factors Involved in Pressure Research

When one applies pressure to marine organisms, the data are usually interpreted in terms of the pressure applied to the system. However, when doing so, the investigator should always bear in mind that there are many factors to be taken into consideration. Some of these complicating factors are listed in Table 1. The pH changes brought about by increased pressure depend on the buffer system employed. pH changes also take place in sea water and this subject will be discussed later by Disteche. Molecular volume changes caused by hydrophobic bonding or by electrostriction will also be

Table 1

Factors Complicating the Effects of Pressurization on Biological Systems.

pH	Buch and Gripenberg, 1932; Johnson et. al., 1954; Disteche, 1959; Pytkowicz and Conners, 1964.
Ionization of water and water structure	Owen and Brinkley, 1941; Hamann, 1963; Horne and Johnson, 1966 and 1967.
Chemical reaction rates and ionization of various substances (inorganic)	Owen and Brinkley, 1941;Ewald and Hamann, 1956; Disteche and Disteche, 1965; Hamann and Strauss, 1965; Pytkowicz and Fowler, 1967; Pytkowicz et al., 1967.
Aggregation of macromolecules and conformational changes of macromolecules	Linderstrom-Lang and Jacobsen, 1941; Gill Glogovsky, 1955; Kettman, et al, 1965; Murayama, 1966; Josephs and Harrington, 1966 and 1967; TenEyck and Kauzmann, 1967; Kegeles, et al., 1967; Murayama and Hasegawa, 1969; Morita and Becker,

Table 2

Relative turbidity caused by the multiplication of marine bacteria in nutrient broth for six days at 20 C, four days at 30 C, or one day at 40 C at different hydrostatic pressures

(All cultures listed below, except those marked with an asterisk, which failed to grow at 40 C, showed four plus (++++) growth in the controls incubated at normal pressure

CULTURE	300 ATMOSPHERES			400 ATMOSPHERES			500 ATMOSPHERES			600 ATMOSPHERES		
	20 C	30 C	40 C	20 C	30 C	40 C	20 C	30 C	40 C	20 C	30 C	40 C
Achromobacter fischeri	++++	++	•	++	—		—	—		—	—	
Achromobacter harveyi	++++	++++		+	++++	—	—	—		—	—	
Achromobacter thalassius	—	++++	++	—	—	+++	—	—	—	—	—	—
Bacillus abysseus	+	++++	++++	—	++++	++++	—	—		—	—	—
Bacillus borborokoites	++	++++	++++	—	++++	++++	—	++	++++	—	—	++++
Bacillus cirroflagellosus	++	+++	•	—	++		—	—		—	—	++++
Bacillus submarinus	++	++++	++++	+	++++	++++	—	++++	++++	—	++++	++++
Bacillus thalassokoites	+++	++++	++++	++	++++	++++	—	++++	++++	—	++++	++++
Flavobacterium okeanokoites	++++	++++	++++	++++	++++	++++	++	++++	++++	—	—	++++
Flavobacterium oliginosum	++++	++++	++++	++++	++++	++++	—	++++	+	—	—	—
Micrococcus infimus	+	++++	•	—	+		—	—		—	—	
Photobacterium splendidum	++++	++++	•	++	+++		—	+		—	—	
Pseudomonas pleomorpha	++	++++	•	++	+++		—	++		—		
Pseudomonas vadosa	++	++++	++++	++	++++	++++	—	++++	++++	—	—	++++
Pseudomonas xanthochrus	++++	++++	•	++++	+++	+	++	++	++	+	+	—
Vibrio hyphalus	—	—	•	++++	—	—	—	—		—	—	
Mixed microflora from mud	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

Reprinted by permission. After ZoBell and Johnson, 1949.

Pressure Effects on Enzyme Reaction Rates

The majority of enzymes studied under pressure are affected adversely. All of the dehydrogenases thus far studied, whether in cell-free form or in intact bacterial cells, display decreased activity with increased pressure (Morita, 1967; ZoBell, 1964). An example of this is shown in Fig. 23. Much of this can be attributed to the molecular volume change of the enzyme. At constant temperature, pressure decreases the molecular volume of the enzyme so that it will not accept the substrate. As a general rule, the enzyme must undergo an increase in molecular volume to be able to accept the substrate if the enzymic reaction is to occur (Laidler, 1951). With this in mind, one begins to wonder how the organisms in the deep sea carry on their metabolic activities, and the questions that naturally come up are the following:

1. Are the enzymes different in deep sea forms compared to those at the surface?
2. If so, how are they different?

The answer to these questions will have to await further studies when instrumentation becomes available.

However, if surface bacterial forms (ZoBell and Johnson, 1949; Oppenheimer and ZoBell, 1952) were investigated as to their pressure tolerance in terms of their enzymes, this investigator would venture to say that they would be very similar in terms of the amino acid composition, temperature characterisites, behavior to pressure, etc. The difference between organisms may lie in the particulate enzymes such as those attached to membranes or other cellular structure. Being associated with cellular structures, the enzymes could then undergo concomitant conformational changes, since the associated structure would undergo a conformational discussed by Becker. Nevertheless, all the complicating factors, as listed in Table 1, should be taken into consideration.

Life in the Deep Sea

During the Galathea Expedition, life was demonstrated in the various hadal portions of the oceans. This was visually verified by the descents of the bathyscaph *Trieste*. It appears that the number of species existing in the deeper portions of the ocean decreases with increasing pressure. The inquiring mind must ask how these forms exist under the conditions that prevail in the depths of the ocean—especially when it appears that pressure adveresely affects enzyme reaction rates and macromolecular synthesis.

Instrumentation

Although bacteria have been isolated faom the various deeps and trenches (ZoBell and Morita, 1957), these types of organisms still remain an academic curiosity because of the difficulties encountered in isolating them in pure culture, and in obtaining sufficient numbers for biochemical studies. In other words, our instrumentation is not adequate to grow the cells with a constant supply of air and to bleed off the carbon dioxide resulting from respiration. Even after we have grown the cells, we would encounter difficulties because the material would need to be transferred to the pressure reaction vessel for biochemical studies. In other words, a single depressurization step in the entire procedure may allow the biochemical system to change its conformation so that it will no longer resemble the original form under pressure. It is well known that the conformation of macromolecules will change depending on the perturbing forces applied.

As a result of our present types of instrumentation, most of our pressure research is done with forms that can be grown at 1 atm. Such studies will give us some insight as to how pressure can act on metabolic systems.

The various methods presently employed to investigate the effects on microbial systems under pressure are described by Morita (1970).

Species Difference

ZoBell and Johnson (1949), and Oppenheimer and ZoBell (1952), subjected various marine bacteria to various pressures (1,200, 400 and 600 atm) in media. Depending upon the pressure employed, some of the bacteria were killed, some did not multiply, while others were not affected (Table 2). In other studies, ZoBell and Oppenheimer (1950) noted that *Serratia marinorubra* formed long filaments (growth but not reproduction) and this has been noted with other forms subsequent to their studies of 1950 (ZoBell and Cobet, 1964). Why various species within a single genus are different is still not known.

change. For instance, it is known that the mitochondria possess many hydrophobic groups and are responsible for the binding of phosholipids to the protein of the mitrochondrial membranes (Lenaz, *et al.*, 1970), and therefore they would naturally be influenced by both temperature and

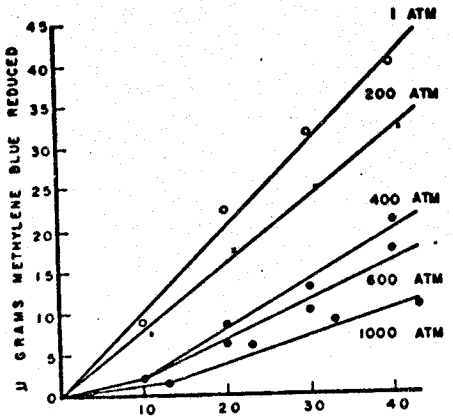

Figure 23. Effect of hydrostatic pressure on oxalosuccinic dehydrogenase activity by mitochondria of *Allomycetes macrogynus*. The reaction was run at room temperature (ca. 26.5°C). All values are corrected for controls. Time in minutes. (After Hill and Morita, 1964).

pressure (see discussion by Becker in this book). If the lipids of the membrane undergo hydrophobic changes due to temperature and/or pressure, it can readily be visualized that the enzymes associated with the mitochondira would undergo certain changes also, or have their active sites hidden. In our laboratory, we have been doing some studies which appear to bear this concept out a little more. When certain bacterial cells (we have not tried all of them) are subject to cold temperature (below its minimum temperature for growth) the cells cease to be permeable to certain of the amino acids. The same phenomenon happens when the cells are subjected to elevated pressures. My guess is that the differences between species with reference to pressure and temperature tolerance lies mainly in the membrane. In other words, membranes possess lipoproteins and the lipid portions are hydrophobic.

Influence of Temperature on Pressure Reactions

There is a temperature-hydrostatic pressure relationship in terms of the maximum temperature for growth under pressure (Table 2) and enzyme reactions under pressure. In the study of pressure effects on luminescence (bacterial bioluminescence), a good correlation between temperature and pressure is noted. An increase in luminescence is noted when the temperature is 35°C (Brown, *et al.*, 1942). At 15°C there is a progressive decrease in luminescence with increased pressure, while at 0°C the decrease in luminescence with increased pressure is very great. At the lower temperature, two forces (pressure and low temperature) combine to bring about a decrease in the molecular volume, hence a rapid decrease in the reaction rate.

This temperature-pressure relationship is further illustrated in the data of Haight and Morita (1962). In Fig. 24, it can readily be seen that between the temperatures of 37° and 45°C, the Q_{10} rule is affecting the reaction at all pressures employed. These data were obtained with washed cells of *Escherichia coli*. Between 45° and 50°C, there is a drop in the reaction rate for the pressures of 100, 200, 300 and 400 atm. However, the reaction rate still increases when the pressure is 500, 600, 700, 800, 900 and 1,000 atm. Between the temperatures of 50° and 53°C, all the reaction rates are decreased with the exception of the 900 and 1,000 atm rates. At 56°C, the highest rate of reaction is shown under a pressure of 1,000 atm and the lowest at 1 atm, indicating the reversal of everything that happens at 37°C. The 1 atm curve in Fig. 24 illustrates the effect of temperature on the system. An increase of temperature from 37° to 45°C results in an increase of activity as one would expect according to Vant Hoft's Law. However, above 45°C, the 1 atm curve shows a decrease in activity with temperature, thereby demonstrating that thermal inactivation has taken place. When the data of Fig. 24 are replotted as shown in Fig. 25 using pressure as the abscissa instead of temperature, one can see the effect of pressure upon the enzyme reaction rates for any given temperature. The 37°, 45° and 50°C curves illustrate the effects of pressure on the reaction rates: the pressure decreases the reaction rate at temperatures near the optimum for the organism, or even at the enzyme's optimum temperature for reaction. However, if the temperature is above the maximum for the reaction mixture at 1 atm, a different picture unfolds. The 53°C curve demonstrates that the effects of temperature are counteracted by pressure equally, and as a result there is no decrease or increase in reaction rates. When the temperature is raised to 56°C, the reaction rate increases with incresed pressure. In other words, we are beginning to see a pressure-temperature interrelationship where the pressure counteracts the effects of temperature so that the enzyme does not undergo inactivation, and therefore can carry out its catalysis at temperatures above its maximum at 1 atm.

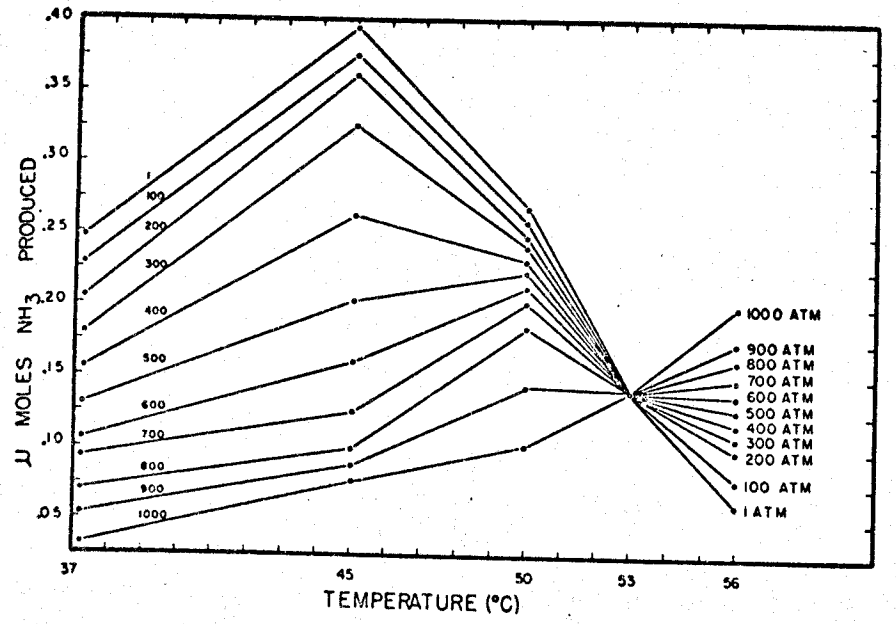

Figure 24. Effect of temperature and pressure on the deamination of L-aspartic acid by washed cells of *Escherichia coli*. (After Haight and Morita, 1962).

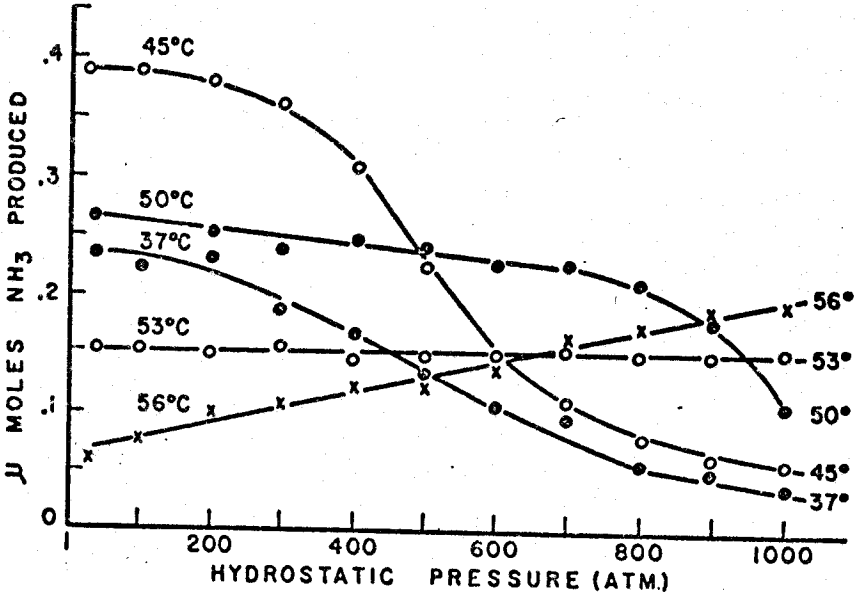

Figure 25. Temperature—pressure effects on the deamination of L-aspartic acid by washed cells of *Escherichia coli*. Replot of Fig. 2, using pressure as the abscissa instead of temperature. (After Haight and Morita, 1962).

Haight and Morita (1962) demonstrated that there is a stimulation of activity above 45°C for the cell-free system, and 53°C for the washed cell preparation. There appears to be a difference between cell-free systems and whole-cell systems which might be explained partially by the fact that in the cell-free system the enzymes are not attached to any particulate substance of the cell. If an examination of the aspartase activity in Fig. 26 illustrates the

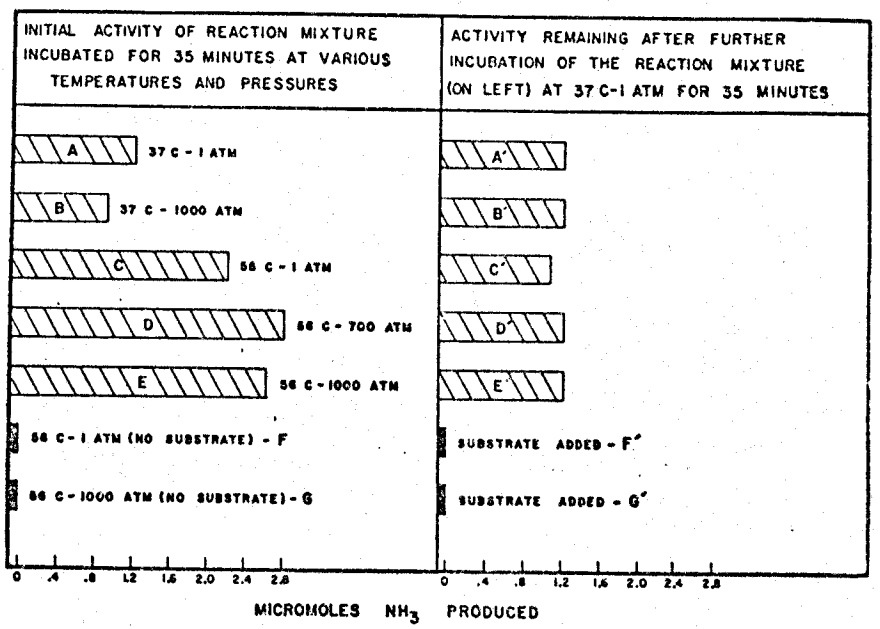

Figure 26. Activity of aspartase remaining after treatment at various hydrostatic pressures and temperatures in the presence and absence of L-aspartic acid. (After Haight and Morita, 1962).

effect of temperature on the inactivation of the enzyme, pressure then can actually counteract this thermal inactivation. There is no substrate limitation in the experiment, and as a result, the amount of ammonia produced at 37°C at one atm and 35 min of incubation is represented by Bar A of Fig. 26. Further incubation of the same reaction mixture under identical conditions for an additional 35 min produces approximately the same level of ammonium as indicated by Bar A'. Bar A' indicates that there is no inactivation of the enzyme during the total 70-min incubation period. However, at 37°C and 1,000 atm, the reaction does not take place so readily as indicated in Bar A of Fig. 26. However, Bar B' is equal to Bar A', indicating that the enzyme has not undergone any inactivation due to the pressure to which it was subjected. From Fig. 24 and Fig. 25, we recognize that temperature of 56°C does inactivate the enzyme. Bar C represents a temperature of 56°C and one atm and indicates the amount of aspartase

activity is quite great in the first 35 min. Further incubation of the same reaction mixture indicates that the enzyme does undergo thermal inactivation when subjected to 56°C at one atm for a total of 70 min., which is represented in Bar C'. Bar D, which is a reaction mixture at 56°C and 700 atm, does illustrate that it is much better than Bar C, indicating that within the first 35 min the pressure has prevented the enzyme from undergoing inactivation. And as a result, when the pressure is released Bar D has the same amount of activity as Bar A and B. The same can be said for Bar E and Bar F. In other words, in Bar C, for the first 35 min of incubation there is some inactivation of the enzyme taking place, which is further illustrated by Bar C'. This thermal protection by pressure is not noted unless the substrate is present in the reaction mixture (Fig. 26 F and F' and G and G').

This concept can be carried further, and from an academic viewpoint we have checked to see whether or not we could make an enzyme reaction take place above 100°C. Fig. 27 illustrates the effect of pressure on the malic dehydrogenase activity (Morita ahd Haight, 1962). The 56°C, one-atm curve in Fig. 27 illustrates an optimal condition for the malic dehydrogenase taken from a thermophile. However, reaction does take place at 101°C at 1300 atm. No activity can be seen at 101°C at one atm. Again, the data illustrate that pressure can counteract the effects of temperature on an enzyme. A pressure of 1300 atm does not allow the enzyme to undergo complete thermal inactivation. Since complete thermal inactivation is not brought about, there is reaction at 101°C; whereas, at 101°C at one atm, no reaction can be detected. Further evidence of this temperature-pressure relationship is illustrated by Morita and Mathemeier (1964). In this case, inorganic pyrophosphotase activity was shown to take place at 105°C. However, in this study, it appears that the metal cofactor is more important than the substrate in protecting the enzyme against heat inactivation when pressure is applied to the system. Morita and Haight (1962) found that substrate was essential in the protection of the enzyme against thermal inactivation at elevated pressure. However, another important fact should be recognized: When enzymes are subjected to elevated temperatures the cofactor can change. With inorganic pyrophosphotase, cobalt replaces manganese as the cofactor when temperatures above 80C are used (Mathemeier and Morita, 1964). Whether or not there is an inner change of cofactors when pressure is applied to the system is still not known. Harold Evans, on our campus, is investigating whether or not sodium or potassium can be interchanged in sodium and potassium activated ATP-ase under pressure.

The temperature at which bacterial cells are grown does influence the action of pressure in intact cells. For his master's thesis, Albright (M.S. Thesis, Oregon State University, Corvallis, Oregon; Albright and Morita 1965), studied the effect of pressure on *V. marinus* MP-1 cells grown at two different temperatures, harvested and then tested at two different tempera-

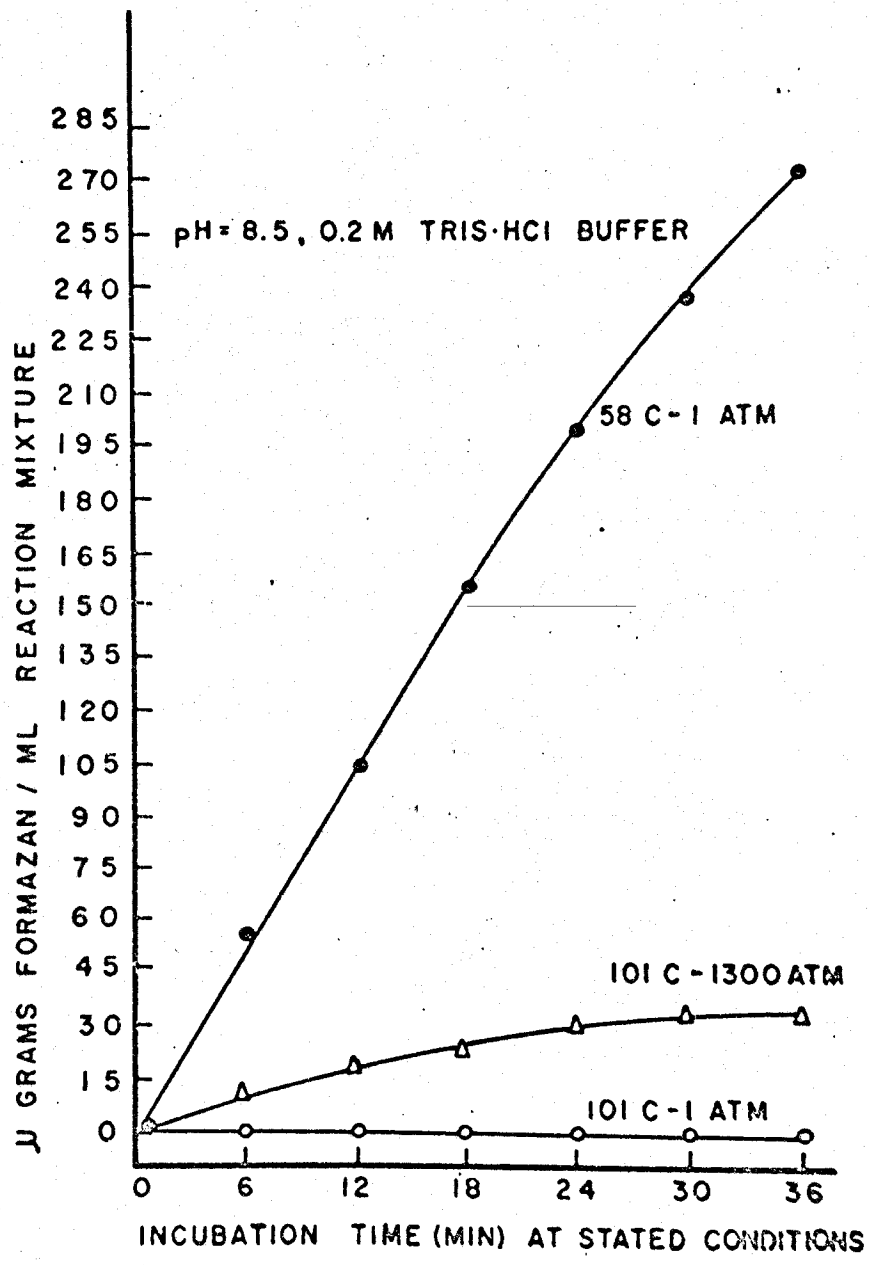

Figure 27. Rate of malic dehydrogenase activity. The curve of 101°C and 1,300 atm is corrected for the 6 min. period required for the initial pressure to reach the final pressure. Values are corrected for controls. (After Morita and Haight, 1962).

tures. In Fig. 28 the effect of hydrostatic pressure on the deamination of L-serine by cells of *V. marinus* raised at 15°C is shown. When the reaction mixture is tested at 15°C, there is an optimal activity at approximately 300 atm and a decrease in activity with increased pressure. On the other hand, when the 15°C-grown cells are tested at a temperature of 4°C, no optimum is observed when pressure is added to the system. As a result, there is a linear

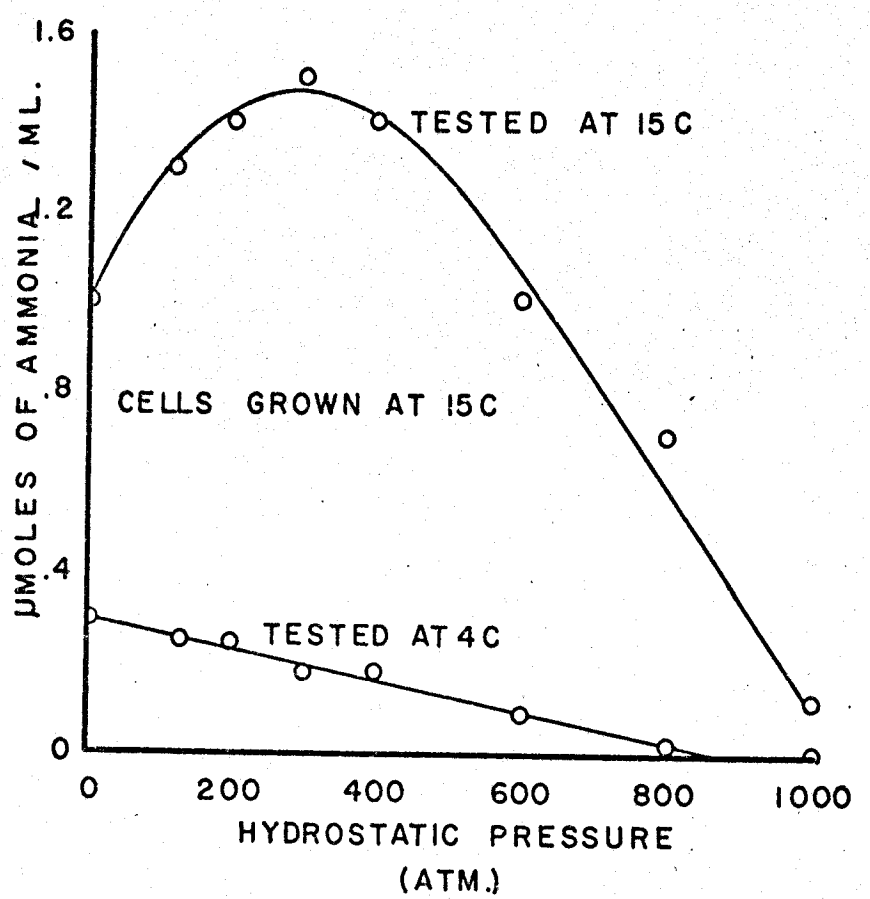

Figure 28. The effect of hydrostatic pressure on deamination of L-serine by 15°C-grown washed cells of *V. marinus*.

drop of L-serine deaminase activity with increased pressure. In Fig. 28 and 29, the effect of pressure is shown on cells grown at 4°C. When these cells are tested at 15°C at various hydrostatic pressures, again it is noted that there is an optimum activity at approximately 300 atm. When 4°C-grown cells are placed in a reaction mixture which is subjected to 4°C-grown, we also see an optimum peak of activity. However, this optimum activity occurs at approximately 150 atm

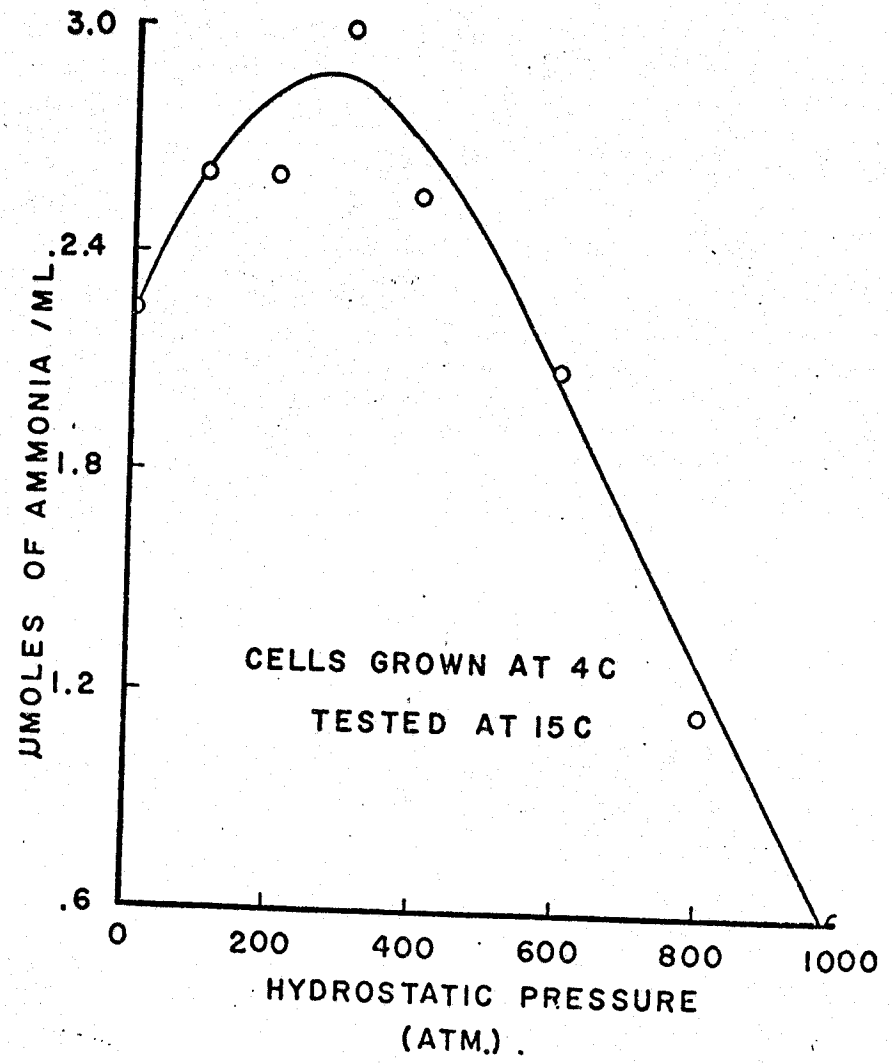

Figure 29. The effect of hydrostatic pressure on the deamination of L-serine by 4°C-grown washed cells of *V. marinus*. Incubation period was 1 hour at 15°C at various hydrostatic pressures.

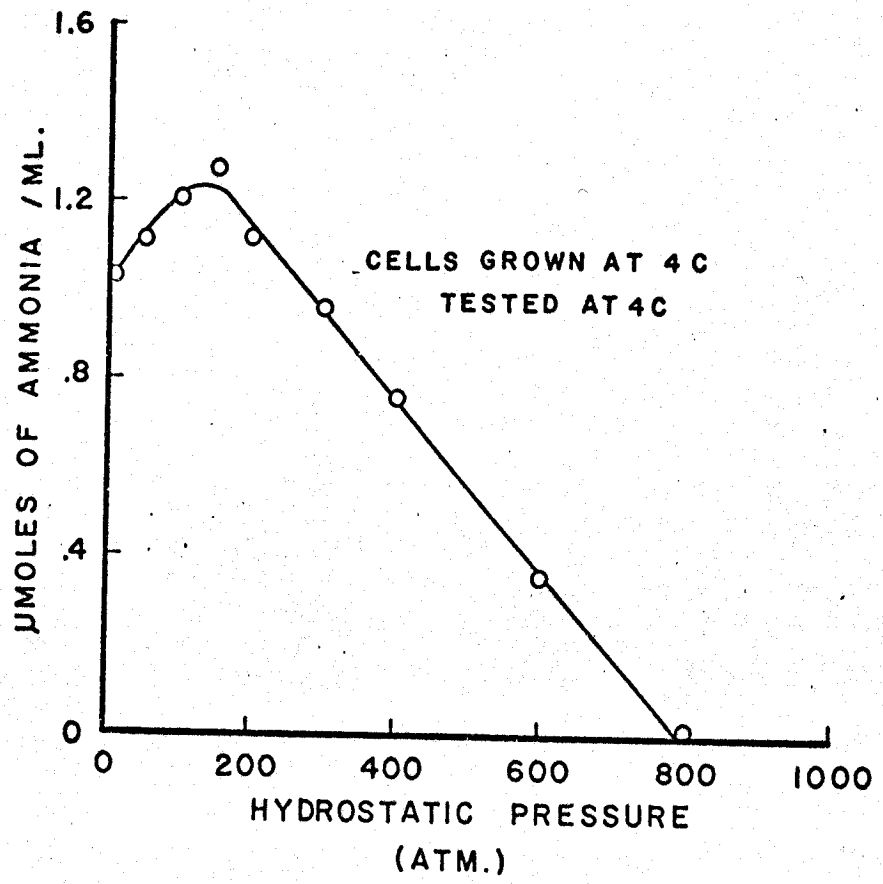

Figure 30. The effect of hydrostatic pressure on the deamination of L-serine by 4°C-grown washed cells of *V. marinus*. Incubation period was 1 hour at 4°C at various hydrostatic pressures.

The above concept applies not only to bacterial cells but to other organisms. Brown (1934) noted this phenomenon when working with muscle contraction on the pectoral fin of the red grouper *(Epinephelus mario)*. This concept should not be overlooked when animals are observed in high pressure aquaria. In other words, the temperature from which the animal is taken will govern the pressure response of the organism. In Albright's studies with L-serine deaminase, whole cells were employed. If the L-serine deaminases isolated from cells grown at 15 C and at 4 C were compared, in all probability the enzymes would react similarly to temperature and pressure. This investigator would again venture to state that one of the main reasons why intact cells function differently when grown at two different temperatures and subjected to various temperatures of reaction is that membrane permeability probably played a great role. In this case, it may be that the ability of the serine to be transported into the cells through the membrane of 4°C-grown cells is different than that of 15°C-grown cells.

Synthesis of Macromolecules

The synthesis of protein RNA and DNA was commenced in our laboratory by L.J. Albright (Albright and Morita, 1968). It is well recognized that pressure does inhibit the synthesis of these macromolecules and, generally, the greater the pressure applied, the greater the decrease in synthesis of these molecules. Since both Landau and Albright are here, I think it is best to allow them to present their investigations on this subject matter.

ACKNOWLEDGMENTS

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