THE INFLUENCE OF HYDROSTATIC PRESSURE ON THE GROWTH AND VIABILITY OF TERRESTRIAL AND MARINE BACTERIA[1]

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Although there is abundant evidence that a rich microflora exists at the bottom of the ocean, in deep oil well brines, and in other habitats characterized by high pressure (cf. ZoBell, 1946), no clear understanding has been reached concerning the influence of pressure on the growth, viability, and metabolism of the organisms in such environments. The results of older (Certes, 1884*a*,*b*,*c*; Regnard, 1884; Certes and Cochin, 1884; Roger, 1895; Krause, 1902; Chlopin and Tammann, 1903; Hite, Giddings, and Weakley, 1914; Larson, Hartzell, and Diehl, 1918), as well as more recent, studies (cf. reviews by Macheboeuf and Basset, 1934; Cattell, 1936; Bridgman, 1946) with respect to the effects of presure on microbial processes in general are not only inadequate but in some cases apparently inconsistent. Furthermore, before the advent of the theory of absolute reaction rates in 1935 (Eyring, 1935; Glasstone, Laidler, and Eyring, 1941) no rational basis was available for the interpretation of the action of pressure on chemical reaction rates, and it has been only within the past several years that this theory has been applied to biological reactions in living cells (cf. reviews by Johnson, 1947; Johnson and Eyring, 1948.) The latter studies have shown that, in all cases, the observed effects of pressure may be profoundly modified by temperature; a fundamental relationship that had previously remained almost unnoticed.

In view of these circumstances, we have undertaken to reinvestigate the problem, with special reference to the relation between temperature and the effects of hydrostatic pressure, as well as the relation of the natural habitat of the organism to the effects observed.

The present report is in the nature of a survey of the influence of pressures up to 9,000 pounds per square inch (about 600 atmospheres), at different constant temperatures, on the growth and viability of representative species of both marine and terrestrial bacteria in pure culture, initially in the logarithmic growth phase. Extensive quantitative data are necessary for kinetic analyses of the pressure effects and will be made the subject of later investigations, as will, likewise, the significance of certain biological and other factors, such as the specific physiology of the organism, growth phase, composition of the medium, and so forth.

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METHODS

Flasks of glucose nutrient broth for aerobes and facultative anaerobes, or of brain-heart infusion containing 0.3 per cent agar for obligate anaerobes, were inoculated from cultures in the late logarithmic growth phase. For marine bacteria, the culture medium was made in natural sea water, filtered through filter paper. The flasks were incubated for 2 or 3 hours to ensure a logarithmic rate of reproduction at the start of the experiment. Sterile test tubes, size 10 by 50 mm, were then filled with aliquot portions of these cultures and were closed by sterile vaccine bottle rubber stoppers, of the type whose cap could be folded over the neck of the tube and held firmly in place by tightly wound rubber bands. Contamination was thus effectively prevented, while hydrostatic pressure was readily transmitted through the rubber stopper. The inoculated tubes were placed in a steel pressure chamber (Johnson and Lewin, 1946*a*) filled with water at the desired temperature. Pressure was then applied to the entire system by means of a connecting hydraulic pump, and the chamber immersed in a constant temperature water bath. Control cultures, corresponding in every way to those under increased pressure, were immersed in the same water bath for incubation at normal pressure. The influence of different pressures or temperatures was generally studied simultaneously by employing several pressure chambers. In all cases the experiments were repeated sufficiently to make certain of the reproducibility of the results.

RESULTS

At 30 C, a temperature generally favorable for the development of diverse terrestrial species whose "optimum" temperatures are not all the same, visible turbidity failed to develop in broth cultures of any of over 30 species incubated under a pressure of 600 atmospheres, and growth was visibly less at 400 atmospheres than at normal pressure. Moreover, cultures of most of the species were sterilized within 48 hours under 600 atmospheres at 30 C, and some by only 400 atmospheres, as shown by their failure to develop following decompression. The data for representative species of bacteria and yeasts are summarized in table 1. Yeasts appear to be more senstive, in general, than bacteria to pressure, although the opposite has been previously reported (Chlopin and Tammann, 1903).

Plate counts (table 2) showed that viability was, in some cases, much more profoundly affected than was evident from the degree of visible turbidity (table 1). Thus, after 48 hours under 300 atmospheres, cultures of *Alkaligenes viscosus* and *Proteus vulgaris*, which had grown to a marked turbidity, contained less than 100 viable cells per ml. The data show clearly that, in addition to retarding the rate of reproduction, pressure under these conditions accelerates the rate of death.

These results are evidently attributable to a direct effect of pressure during incubation, rather than to factors associated with the experimental procedure. Thus, although the accumulation of carbon dioxide during growth in the closed tubes might be expected to influence the results to some extent, more carbon dioxide was undoubtedly formed and remained in solution in cultures that de-

veloped abundantly under 300 atmospheres than in those that failed to grow at higher hydrostatic pressures. Oxygen may be ruled out as a critical factor for the aerobes, since all tubes contained the same amount of oxygen at the start.

TABLE 1

Multiplication or destruction of terrestrial microorganisms after 48 hours' incubation at 30 C at different hydrostatic pressures

(Plus signs show the degree of turbidity relative to the control culture at one atmosphere and a minus sign denotes no apparent multiplication. A "d" indicates that the culture had lost its ability to multiply following decompression.)

CULTURE	HYDROSTATIC PRESSURE IN ATMOSPHERES				
	1	300	400	500	600
Alkaligenes viscosus	++++	+++	++	−d	−d
Bacillus alvei	++++	+++	+	−	−
Bacillus brevis	++++	+++	+	−	−d
Bacillus cereus	++++	++++	−	−	−
Bacillus circulans	++++	++++	−	−	−
Bacillus megatherium	++++	++++	+	−	−d
Bacillus mesentericus	++++	++++	+++	++	−d
Bacillus mycoides	++++	++++	+	−	−d
Bacillus subtilis	++++	++	+	−	−
Clostridium chauvei	++++	++++	++	−	−
Clostridium histolyticum	++++	++++	+++	−	−d
Clostridium putreficum	++++	++++	++	−	−
Clostridium septicum	++++	++	+	−	−
Clostridium sporogenes	++++	+++	++	−	−
Clostridium welchii	++++	++++	+	−	−
Escherichia coli	++++	++++	+++	++	−
Micrococcus lysodeikticus	++++	++	−	−d	−d
Mycobacterium phlei	++++	+++	++	−d	−d
Mycobacterium smegmatis	++++	++	+	−	−d
Proteus vulgaris	++++	++	−	−	−d
Pseudomonas fluorescens	++++	++++	+++	−	−
Sarcina lutea	++++	+++	+	−	−d
Serratia marcescens	++++	−	−	−	−
Staphylococcus albus	++++	+++	+++	−	−
Staphylococcus aureus	++++	++	−	−d	−d
Streptococcus lactis	++++	++++	++++	++	−
Hansenula anomala	++++	++	−d	−d	−d
Saccharomyces cerevisiae	++++	++++	−d	−d	−d
Saccharomyces ellipsoideus	++++	+	−d	−d	−d
Schizosaccharomyces octosporus	++++	−	−d	−d	−d
Sporobolomyces salmonicolor	++++	+	−d	−d	−d
Torula cremoris	++++	++++	++	−d	−d

With regard to anaerobes, no significant amount of oxygen diffused through the rubber stopper closure to retard growth, as was shown by the absence of appreciable recolorization of methylene blue solutions that had been partially reduced at the start by hydrosulfite, then incubated under conditions similar to

those of the cultures. The suddenness with which cultures were compressed or decompressed, during short exposures to high pressures, was shown by plate counts to have no appreciable effect on viability.

In general, tables 1 and 2 would indicate that the various species are more sensitive to pressure than would be expected according to previous reports to the effect that, under some conditions, terrestrial bacteria can withstand pressures of 10 to 50 times those that we find retard multiplication or actually kill (Chlopin and Tammann, 1903; Hite, Giddings, and Weakley, 1914; Larson, Hartzell, and Diehl, 1918; Basset and Macheboeuf, 1932). A number of causes may be responsible for such apparent discrepancies. In any event, the influence of temperature as a factor in the net effect of pressure is of special significance.

TABLE 2

Plate count of nutrient broth shortly after inoculation and after 48 hours' incubation at 30 C at various hydrostatic pressures

CULTURE	INITIAL COUNT PER ML	PLATE COUNT PER ML AFTER 48 HOURS AT				
		1 atm	300 atm	400 atm	500 atm	600 atm
Alkaligenes viscosus	700	160,000,000	<100	<100	<10	0
Bacillus cereus	1,600	40,000,000	650,000	700	<100	0
Bacillus circulans	20,000	11,000,000	880,000	265,000	200	20
Bacillus mesentericus	1,900	21,000,000	230,000	60,000	10	0
Bacillus mycoides	2,250	2,000,000	160,000	114,000	700	0
Bacillus subtilis	1,600	14,000,000	600,000	42,000	30	8
Proteus vulgaris	2,600	142,000,000	<100	<100	<10	0
Pseudomonas fluorescens	10,000	95,000,000	21,000,000	5,000,000	810	80
Sarcina lutea	4,700	36,000,000	67,000	22,000	400	12
Serratia marcescens	300	64,000,000	<100	<100	<10	0
Staphylococcus albus	4,400	77,000,000	80,000,000	57,000	700	350
Staphylococcus aureus	800	156,000,000	174,000,000	9,000	0	0
Streptococcus lactis	4,000	273,000,000	149,000,000	70,000,000	160,000	18,000

In studying the relation of the pressure effect to temperature, experiments with a majority of the species listed in table 1 were repeated at 20 C, 30 C, and 40 C, for periods of 4, 2, and 1 days' incubation, respectively. All cultures developed well at normal pressure, but the influence of increased pressure was strongly dependent upon the temperature of incubation, as shown by the results summarized in table 3. Most of the species failed to develop visible turbidity under 300 atmospheres at 20 C, yet the same organisms grew abundantly under the same pressure at 40 C. Furthermore, although none of the cultures developed visibly at either 20 C or 30 C under 600 atmospheres, at least four species (*Bacillus mesentericus*, *Bacillus subtilis*, *Escherichia coli*, and *Streptococcus lactis*) grew fairly well under this pressure at 40 C. Thus, within the range of temperatures that permit cultures to develop at normal pressure, the growth-retarding effects of increased pressure are diminished at the higher temperatures. The same relation has been reported in connection with the rate of growth of *E. coli* in synthetic medium during short periods of the early logarithmic phase (Johnson and Lewin, 1946*b*).

Marine bacteria indicate the same general relation between temperature and the effects of pressure, with a somewhat greater resistance to pressure on the whole (table 4). In addition, there is a correlation between the sources from which these species were isolated and their ability to grow under pressure. Thus, the luminous bacteria (*Achromobacter fischeri*, *Achromobacter harveyi*, and *Photobacterium splendidum*), as well as other species (*Achromobacter thalassius*, *Bacillus cirroflagellosus*, *Micrococcus infimus*, *Pseudomonas pleomorpha* and *Vibrio hyphalus*) obtained at or near the surface of the ocean, tend to resemble the terrestial organisms in their response to pressure. On the other hand, some species, *Bacillus submarinus* and *Bacillus thalassokoites*, in particular, isolated from

TABLE 3

Relative turbidity of cultures in nutrient medium after four days' incubation at 20 C, two days at 30, or one day at 40 C at different hydrostatic pressures

All cultures listed below 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
Alkaligenes viscosus	++	+++	++++	++	++	++	−	−	−	−	−	−
Bacillus brevis	−	+++	++	−	+	+	−	−	−	−	−	−
Bacillus megatherium	−	++++	+++	−	+	++	−	−	−	−	−	−
Bacillus mesentericus	−	++++	++++	−	+++	++++	−	++	++++	−	−	++++
Bacillus subtilis	−	+++	++++	−	++	++++	−	−	+++	−	−	++
Clostridium bifermentans	++	++++	++++	−	−	+++	−	−	−	−	−	−
Clostridium chauvei	−	++++	++++	−	++	+++	−	−	−	−	−	−
Clostridium histolyticum	−	++++	++++	−	−	+++	−	−	−	−	−	−
Clostridium putreficum	−	++++	++++	−	++	++	−	−	−	−	−	−
Clostridium septicum	−	+	++	−	−	+	−	−	−	−	−	−
Clostridium sporogenes	−	++++	++++	−	++	+++	−	−	−	−	−	−
Clostridium welchii	−	++++	++++	−	+	++	−	−	−	−	−	−
Escherichia coli	++	++++	++++	−	+++	++++	−	++	++++	−	−	++++
Mycobacterium phlei	−	+++	++++	−	++	+	−	−	−	−	−	−
Mycobacterium smegmatis	−	++	++	−	+	+	−	−	−	−	−	−
Pseudomonas fluorescens	++	+++	++++	−	++	++++	−	−	+++	−	−	−
Sarcina lutea	++	++	++++	−	+	++	−	−	−	−	−	−
Staphylococcus albus	++	++	++++	−	+	++	−	−	−	−	−	−
Staphylococcus aureus	−	+++	++++	−	++	+++	−	−	−	−	−	−
Streptococcus lactis	+++	++++	++++	+	++++	++++	−	++	++++	−	−	++++

depths where the pressure approximates 500 atmospheres, grew prolifically under 600 atmospheres at 30 C as well as at 40 C and were obviously more resistant to the effects of 400 and 500 atmospheres than the terrestrial bacteria. Mixed microflora, from mud freshly collected from the sea floor at depths up to 12,000 feet, multiplied abundantly at all pressures and temperatures that were studied. Growth in some cases was more rapid under 400 to 600 atmospheres than at normal pressure. To these facts we can add the observation that certain sulfate-reducing bacteria, isolated from oil well brines several thousand feet below the earth's surface, are metabolically more active when compressed at 400 to 600 atmospheres than at 1 atmosphere. The ability to grow and carry on metabolism as well or better under increased pressure would seem to justify coining the word *barophilic* to characterize such organisms.

TABLE 4

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 uliginosum	++++	++++	++++	++++	++++	++++	−	++++	+	−	−	−
Micrococcus infimus	+	++++	*	−	+		−	−		−	−	
Photobacterium splendidum	++++	++++	*	++	+++		−	+		−	−	
Pseudomonas pleomorpha	++	++++	*	++	+++		−	++		−	−	
Pseudomonas vadosa	++	++++	++++	++	++++	++++	−	++++	++++	−	−	++++
Pseudomonas xanthochrus	++++	++++	*	++++	+++	+	++	++	++	+	+	−
Vibrio hyphalus	−	−	*	−	−	−	−	−		−	−	
Mixed microflora from mud	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

All the pure cultures listed in table 4 had been maintained at atmospheric pressure for several years, most of the time at around 4 C, prior to the experiments described, yet the barophilic properties of the deep-sea organisms were not lost. This provides evidence of a genetic character, in adaptation to the high pressure of the habitat, as one might expect from general considerations. At the same time, however, it must be emphasized that the total effects of pressure are likely in all cases to depend upon the temperature, and a complete picture is obtainable only after exhaustive studies of the reciprocal relationships of both factors. Data concerning *Pseudomonas xanthochrus* (table 4) provide a striking illustration. At 40 C no growth was apparent at normal pressure, but cultures grew at 400 and 500 atmospheres. Under these conditions, *Pseudomonas xanthochrus* might be regarded as an obligate barophile. At 20 C, however, although it grew under as much as 600 atmospheres, growth was more abundant under lower pressures, including atmospheric. Lower temperatures remain to be studied, and it is difficult to predict whether evidence of barophilism will then be found, but it is reasonable to expect that the sensitivity of this, as well as other species, to pressure will be more pronounced as the temperature is decreased. Likewise, it remains to be seen whether there exist in nature any bacterial species that normally require high pressures for growth.

DISCUSSION

Although the results of this study confirm those of previous investigations in showing that, under given conditions, various species of terrestrial bacteria exhibit a considerable range in sensitivity to the effects of pressure, they indicate that broth cultures in the logarithmic phase are generally more susceptible to the growth-retarding and disinfecting action of pressure than was formerly appreciated. The influence of temperature as a factor modifying the effects of pressure has been shown by present data to be of considerable importance, and may account in part for apparent discrepancies between these and previously reported results. Moreover, on the basis of the more recent studies of the pressure-temperature relationships of microbial enzyme activity (Johnson, Brown, and Marsland, 1942; Brown, Johnson, and Marsland, 1942; Johnson, Eyring, and Williams, 1942; Johnson, Eyring, Steblay, Chaplin, Huber, and Gherardi, 1945; Johnson, Kauzmann, and Gensler, 1948), denaturation of proteins (Johnson and Campbell, 1946; Johnson, Baylor, and Fraser, 1948), and related phenomena some specific mechanisms, among the numerous and incompletely known complex of reactions concerned in growth and viability, may be postulated as controlling factors in the influence of pressure. Briefly, they are as follows:

First, it is evident that the action of pressure, under the conditions of the present experiments, is directly on chemical reactions: rate processes and equilibria. No significant change in concentrations of reactants resulted from the application of pressure, per se, to the purely liquid systems, i.e., systems without any considerable gas phase. The effects of pressure that are observed through changes in sol-gel systems, oxidation-reduction potentials, solubility, dissociation, enzyme reactions, etc., at given concentrations of reactants

depend upon the molecular volume change accompanying the limiting reaction. Where large molecules are concerned, as in biological systems, the volume change is frequently large, of the order of 50 to 100 cm^3 per mol, and the influence of pressures amounting to only 500 or 600 atmospheres is therefore pronounced.

In growing cells over considerable periods of time, both the internal and extracellular chemical environments undergo many changes, and it is not possible in the present stage of knowledge to single out any one or two limiting reactions whose modification by pressure would account for the total result. It is possible, however, to point to certain fundamental actions of pressure, particularly on enzyme systems and protoplasmic gels, that parallel the results obtained with growth and viability. Thus, it has been shown that oxidative enzyme reactions, such as luminescence, proceed with a large volume increase of activation. At temperatures below the normal optimum, a pressure of 500 atmospheres greatly retards the reaction by opposing the molecular volume increase. At higher temperatures, and most noticeably above the optimum, the critical enzyme undergoes a reversible denaturation that proceeds with an even larger volume increase of reaction. At these temperatures, the net effect of pressure is to increase the rate of the reaction by reversing the denaturation of the enzyme to a greater extent than opposing the catalytic reaction. At intermediate temperatures, the net effect is also intermediate, for pressure acts on two simultaneous reactions with opposite results on the observed phenomena. In luminescence, the effects of pressure are in the same direction as cooling, and the same trend is apparent in the present data on growth. Thus, pressure is more effective at the low temperatures, as further cooling would be at normal pressure. Also, at temperatures too high for growth (*Pseudomonas xanthochrus*) at normal pressure, the application of pressure permits growth to take place. Pressure has also been shown to retard the irreversible thermal denaturation of proteins, and this, too, might be expected to contribute an aid to viability under pressure at relatively high temperatures.

The disinfecting action of pressure is less readily explained. Cells might be expected to lose their viability, however, when their energy-yielding reactions are sufficiently retarded, and by opposing such reactions, pressure might be expected to accelerate death. Irreversible changes under pressure have also been noted in protoplasmic gels (Marsland, 1942), another possible factor involved in the killing action of pressure.

In undertaking to account for species differences in susceptibility to pressure, two important relationships must be taken into account.

In the first place, the reciprocal relationship between temperature and the effects of pressure is conditioned by the specific temperature characteristics of the system of organism concerned. For psychrophilic organisms, the beneficial effects of pressure might be expected to occur at lower temperatures than with mesophilic species, and the retarding effects at much lower temperatures, even though the volume changes were the same in the reactions affected. For thermophilic organisms, on the other hand, it is reasonable to expect that pressure will act strongly against growth and viability at ordinary temperatures, but at

very high temperatures, above their normally high optimum, pressure will enable growth to occur as well as help to preserve viability. Thus, deep oil well brines at high temperatures are not unlikely to yield "obligate barophiles."

In the second place, there is a possibility that the organisms living in deep-sea muds and other habitats under high pressures have become genetically so adapted to their environments that metabolic reactions occur through mechanisms involving no appreciable volume increase, or even involving a significant volume decrease of activation. In this event, pressure would either have little effect or actually favor their activities. Although experimental methods and the theoretical basis are available, the whole field remains very largely to be explored. Practical as well as fundamentally significant consequences can be expected to result from future investigations on the action of hydrostatic pressure.

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SUMMARY

Representative species of mesophilic terrestrial bacteria and yeasts of the genera *Alkaligenes*, *Bacillus*, *Clostridium*, *Escherichia*, *Micrococcus*, *Mycobacterium*, *Proteus*, *Pseudomonas*, *Sarcina*, *Serratia*, *Staphylococcus*, *Streptococcus*, *Hansenula*, *Saccharomyces*, *Schizosaccharomyces*, *Sporobolomyces*, and *Torula* were studied in regard to the action of hydrostatic pressure on the development of pure cultures, initially in the logarithmic growth phase in broth. Species of the genera *Achromobacter*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Photobacterium*, *Pseudomonas*, and *Vibrio*, isolated from the sea at various depths, were similarly studied. In both cases, the influence of temperature (by incubating at 20, 30, and 40 C) on the effects of pressure was investigated.

At 30 C, the terrestrial organisms developed abundantly within 48 hours at normal pressure, but none multiplied perceptibly under 600 atmospheres, and failure to grow after decompression showed that some had been sterilized by this pressure during the period of incubation. Growth of most terrestrial organisms was visibly retarded by a hydrostatic pressure of 400 atmospheres and plate counts indicated that growth was slower and death faster at only 300 atmospheres than at normal pressure.

The marine species, particularly *Bacillus submarinus* and *Bacillus thalassokoites*, which were isolated from depths where the pressure approximates 500 atmospheres, grew readily under 600 atmospheres at both 30 and 40 C in the laboratory. Mixed microflora from muds of the same depths apparently grew faster under pressure. The term "barophilic" is introduced to characterize species whose growth or metabolism is favored by pressure. Other species from near the surface of the sea were intermediate, or more nearly resembled terrestrial bacteria in their sensitivity to pressure. In one case (*Pseudomonas xanthrochrus*) cultures developed under 400 to 600 atmospheres at 40 C, a temperature too high for growth of the culture at normal pressure. The same pressures retarded growth at lower temperatures.

The influence of pressure on pure cultures in all cases depended upon the temperature. In general, lower temperatures markedly accentuated the growth-retarding and disinfecting effects of pressure. Conversely, at higher temperatures, the net effect of pressure was less pronounced, or in some cases acted in the direction of opposing the unfavorable effects on growth and viability caused by the high temperature.

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