High-Pressure Microbiology

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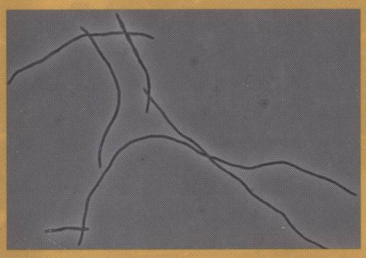
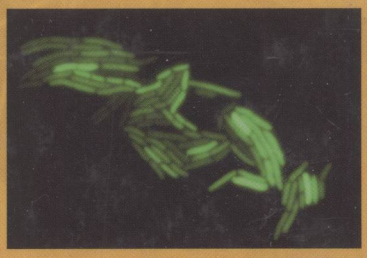
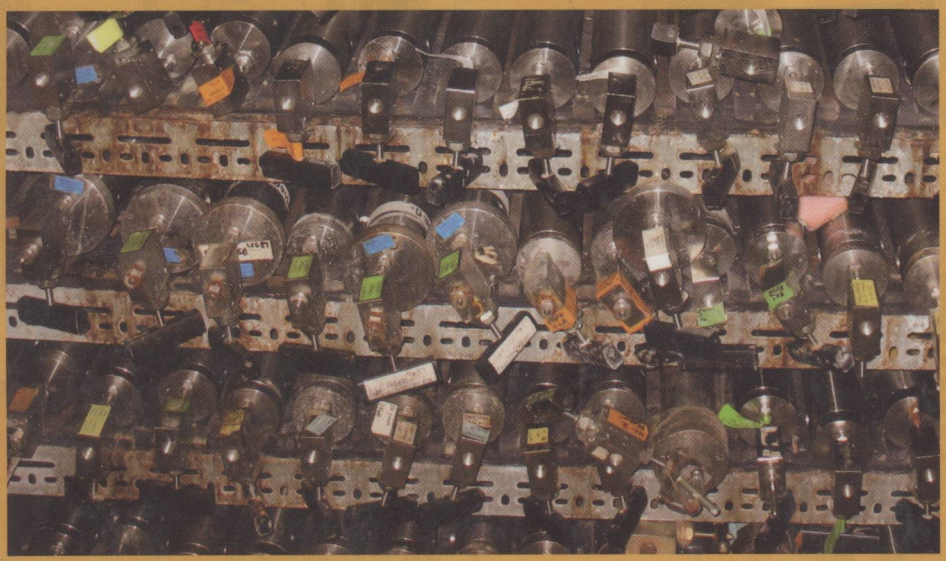
Chris Michiels,
Douglas H. Bartlett,
and **Abram Aertsen**







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Cover figures: (Top left) Phase-contrast microscopy of exponential-phase cells of *Escherichia coli* K-12 grown overnight at 40 MPa and 37°C. Due to inhibition of cell division, cells grow as long filaments. (Courtesy of A. Aertsen and C. Michiels.) (Top right) Epifluorescence microscopy of exponential-phase cells of *E. coli* K-12 containing a reporter plasmid with the promoter of *sulA* fused to the green fluorescent protein gene *(gfp),* 3 h after high-pressure shock (100 MPa, 15 min, 20°C). Cells are bright green and slightly elongated as a result of SOS induction. (From chapter 5, Fig. 1. Courtesy of A. Aertsen and C. Michiels.) (Bottom left) Racks of pressure vessels being used for the high-pressure storage of marine microorganisms collected from various deep-sea locations around the world. They belong to A. Aristides Yayanos (Scripps Institution of Oceanography). (Courtesy of D. Bartlett.) (Bottom right) Transmission electron micrograph of thin sections showing morphological changes in exponential-phase cells of *E. coli* pressure treated at 200 MPa for 2 min (30% survival). Prominent are the unusual conformation of the nucleoid and its fibrillar DNA and the appearance of dark condensed regions that are presumably protein. (From chapter 4, Fig. 6. Courtesy of P. Chilton and B. M. Mackey.) (Background) Some pressure gauges, pumps, and high-pressure tubing from the marine microbiology laboratory of A. Aristides Yayanos (Scripps Institution of Oceanography). (Courtesy of D. Bartlett.)

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PREFACE

Unicellular microorganisms, and in particular prokaryotes, thrive in extremely diverse environments. In fact, the range of physicochemical conditions under which microbial life has been observed continues to expand as microbiologists explore more remote and hostile environments. Today, microorganisms have been described to occur in habitats spanning an extraordinary range of more than 120°C, 10 pH units, and millimolar to molar concentrations of solutes like NaCl. To grow under these extreme conditions, these microorganisms have evolved specific and unique adaptations, and the study of these provides interesting insights not only into microbial physiology but also into the most fundamental properties of living systems as compared to nonliving systems. Temperature, pH, and osmotic pressure are among the most-studied environmental parameters in microbiology, probably because they are easy to manipulate and because they are relevant in our daily life, for example, in food preservation. By comparison, much less is known about microbial adaptation to high pressure, although high-pressure environments are more widespread in nature than high-temperature, high- or low-pH, and high-osmolarity environments. The compartment of the oceans 200 m below sea level constitutes more than 95% of the volume and represents 55% of the prokaryotic cells of all aquatic habitats on earth. Pressure levels vary 3 orders of magnitude, from 0.1 MPa at sea level to more than 110 MPa at the deepest point in the ocean, approximately 11,000 m deep. Besides the deep sea, the deep terrestrial subsurface is another enormous high-pressure habitat, probably the last large remaining unexplored habitat on the earth. Several piezophilic bacteria from the deep sea have been isolated and characterized today, but clearly, the full amplitude of microbial diversity in high-pressure habitats and the physiological adaptations in these organisms are only beginning to be unraveled.

Besides piezophiles, nonpiezophilic microorganisms are an object of study for investigators of high-pressure microbiology. The interest in studying the effects of high pressure on these organisms has been fueled by the development and commercial introduction since the 1990s of a food preservation technique based on high pressure without the need to apply heat. Large amounts of quantitative data on the high-pressure inactivation of food-borne pathogens and spoilage organisms have become available since then. The pressures used in these inactivation studies are typically in the range of 200 to 800 MPa, much higher than the pressures at any depth in the ocean. We have learned from these studies that sensitivity to heat and to high pressure are not necessarily linked and, most remarkably, that vegetative bacteria can acquire extreme resistance to high-pressure inactivation. The findings have, in turn, raised interesting fundamental questions about the actual molecular perception of high pressure, the nature of the cellular damage it causes, and more specifically the adaptations that render these cells high-pressure resistant. Adaptation to stress has always enjoyed a wide interest among microbial physiologists, and several bacterial stress responses are known in great molecular detail. High-pressure

stress has been largely neglected in this field, but in view of its unique effects on biomolecules and biomolecular assemblies, which are different and sometimes even opposed to the effects of heat, high pressure is likely to become a very useful addition to the "toolbox" available to microbial-stress investigators. High pressure has indeed been shown to induce some unique responses in nonpiezophiles, like *Escherichia coli,* and may thus help to uncover novel stress response pathways and adaptational mechanisms in bacteria that have thus far remained cryptic.

It is clear from the above that the field of high-pressure microbiology has evolved along two tracks and that both in the piezophile and in the nonpiezophile tracks, significant progress has been made in the description and understanding of high-pressure effects. Against this background, we endeavored to compile this book, which is probably the first to be entirely devoted to high-pressure microbiology. The objectives are to give an update on the progress in the field and to stimulate a cross-fertilization between the piezophile and the nonpiezophile high-pressure research fields. This approach is reflected in the structure of the book. While the first chapter introduces elementary thermodynamic principles related to high pressure and focuses on biomolecules and biochemical reactions, chapters 2 and 3 look at viruses and bacterial spores, respectively, and form the bridge to the cellular response and adaptation strategies of nonpiezophilic microorganisms, discussed in chapters 4 to 9. Chapter 10 closes the first section with an overview of food- and microbe-related features that affect the efficiency of high-pressure processing. The second section of the book (chapters 11 to 18) highlights different aspects of deep-sea microorganisms and deals both with their isolation, diversity, and ecology and with their physiological adaptations. We hope that this book will appeal to a large readership of microbiologists, not only those actively involved in high-pressure research but also those interested in microbial stress responses or more generally in microbial physiology.

We are grateful to all coauthors for their contributions and to Greg Payne and Susan Birch from ASM for their help in bringing this book to press.

CONTENTS

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Chapter 1

High Hydrostatic Pressure Effects in the Biosphere: from Molecules to Microbiology

Filip Meersman and Karel Heremans

PRESSURE AS A VARIABLE IN THE BIOSPHERE AND THE BIOSCIENCES

The fundamental transformation in the ideas on the possible effects of pressure on living systems can be traced back to the scientific expeditions that were undertaken during the second half of the 19th century. Before 1850 it was assumed that the deep sea would not be suitable for life in general. In 1872 HMS *Challenger* sailed around the globe for about 4 years, and the findings of this expedition were considered as "the greatest advance in the knowledge of our planet since the celebrated discoveries of the fifteenth and sixteenth centuries." During 1882–1883 the French expedition with the *Talisman* recovered a large amount of organisms from a depth of 6,000 m. This drastically changed the opinions on the role of pressure in living systems. It is now well established that a large part of the biosphere is exposed to extremes of temperature and hydrostatic pressure. Approximately 70% of Earth's surface is covered with water. The average depth of the oceans is 3,800 m. Consequently the average hydrostatic pressure is about 38 MPa, with a maximum of about 100 MPa at the deepest point, the Mariana trench. At these depths the temperature is as low as 2°C, except in the vicinity of hydrothermal vents. Even under these conditions thriving microbial communities and invertebrates have been found. Understanding the adaptation of these organisms to this extreme environment requires the knowledge of temperature and pressure effects on the molecules of which they are composed and on their metabolic reactions.

A second, more fundamental reason for studying the effect of pressure on biomolecules is related to the fact that one needs to consider pressure as a variable in order to obtain a full thermodynamic description of a molecular system, i.e., a biomolecule and the solvent. In particular, pressure experiments provide information on the volume changes of a system composed of, e.g., a protein in solution. This can be inferred from the following thermodynamic relationships. The Gibbs free energy *(G)* of a system is defined as

$$G = H - TS = E + pV - TS \tag{1}$$

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where E, V, and S represent the internal energy, the volume, and the entropy of the system, respectively, p is pressure, and T is temperature. The change in free energy as a function of pressure and temperature is given by

$$dG = Vdp - SdT \tag{2}$$

At constant temperature ($dT = 0$) and

$$(\partial G/\partial p)_T = V \tag{3}$$

Thus, for a reversible process, the change of the Gibbs free energy (ΔG) with pressure is given by the volume change of the process (ΔV):

$$(\partial \Delta G/\partial p)_T = \Delta V \tag{4}$$

It is clear from this equation that, according to the Le Châtelier-Braun principle, the system will react on an increase in pressure by shifting towards the state that occupies the smallest volume. A similar quantitative statement can be made for temperature, where a temperature increase will shift the equilibrium towards the state of the largest heat content. Furthermore, equation 4 also shows that pressure affects primarily the volume of the system under study. This is a great advantage of pressure over temperature, which changes both the volume and the internal energy of the system.

The aim of this chapter is to give an outline of the effect of high hydrostatic pressure on proteins, lipids, nucleic acids, and their interactions and to provide a thermodynamic and kinetic framework to describe these effects. The pressure effects of single-component systems (e.g., a protein in solution) are then related to the viability of microorganisms under extremes of high hydrostatic pressure. On the basis of the similarity between the stability curves of proteins and the viability diagrams of microorganisms (in the pressure-temperature plane) (Fig. 1 and 2), it is argued that proteins, and in particular protein-protein interactions, are the main target in the pressure-induced inactivation of microorganisms.

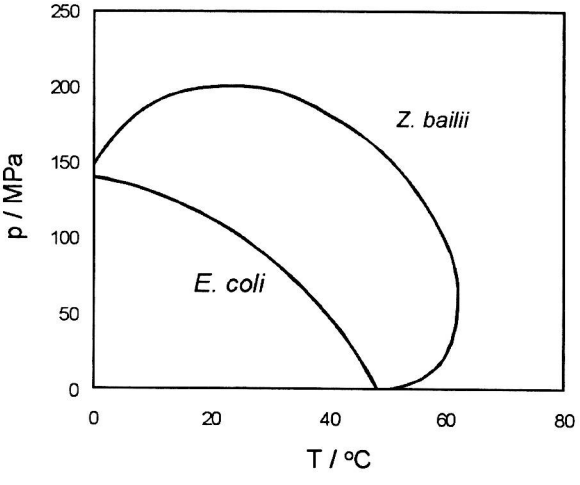

Figure 1. Isokineticity profiles of the inactivation of a bacterium *(Escherichia coli)* and a yeast *(Zygosaccharomyces bailii)* as a function of a combined pressure and temperature treatment. For first-order reactions the decimal reduction time (D) is inversely proportional to the inactivation rate (k). Similar differences in stability have been observed for proteins (30, 46). (Redrawn after references 24 and 38.)

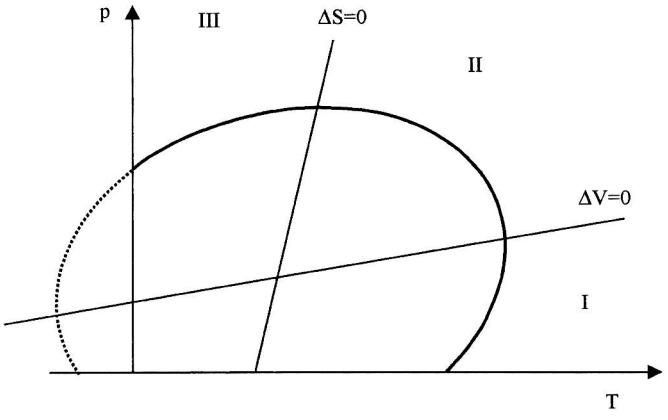

Figure 2. Pressure-temperature phase diagram of proteins. In zone I ΔV and ΔS are positive, in zone II ΔV is negative and ΔS is positive, and in zone III both ΔV and ΔS are negative.

PRESSURE VERSUS TEMPERATURE EFFECTS: FROM WATER TO MACROMOLECULES

A temperature increase will cause a volume expansion, and an increase in pressure will cause a reduction in volume. If, however, as in the case of water, the forces are strong, then an increase in temperature might actually decrease the volume, as is observed between 0 and 4°C, where it reaches its maximum density under ambient pressure conditions.

Under compression all liquids will invariably decrease their volume, although the effect is much smaller in the case of water than in the case of hydrocarbons. How can a system shrink under compression? This can only happen by pushing the molecules closer together. In the pressure range that is of interest here, one can safely neglect the pressure effects on covalent bonds. When strong intermolecular forces are also highly directional, as is the case for hydrogen bonds in water, then these forces will oppose a further closer approach of the molecules under compression.

The above-mentioned general principles become even clearer when we consider the effect of pressure on the melting temperature (dT_m/dp) of solid hydrocarbons and ice. Since the volume of the solid phase of hydrocarbons is smaller than that of the liquid phase, pressure will increase the melting point of hydrocarbons. The dT_m/dp value is about 15°C/100 MPa and is almost independent of the length of the hydrocarbon chain. The value is higher for unsaturated hydrocarbons. From the Clapeyron equation one can estimate the volume and the enthalpy change:

$$dT_m/dp = T_m \, \Delta V/\Delta H \tag{5}$$

As melting is an endothermic process ($\Delta H > 0$), the volume change on melting is positive. The opposite is true for the melting of ice, where the T_m decreases with increasing pressure down to about −20°C ($dT_m/dp = -20$°C/100 MPa). In contrast, the T_m of other ice phases increases with increasing pressure, as can be seen from the phase diagram of water (46).

It is of particular interest to consider briefly the effect of pressure and temperature on mixtures of small molecules, lipids, and proteins in aqueous solutions. The liquid-liquid phase separation that occurs at ambient pressure in the case of water-alcohol mixtures disappears at high pressure. On the other hand, pressure can also induce phase separation in water-soluble-synthetic polymer systems. Its antagonistic effect to temperature has been nicely demonstrated in these systems (32). In the case of aqueous suspensions of lipid vesicles, formed by the insolubility of the lipid molecules in water, the general outcome is a phase diagram which shows pressure dependencies of the phase transition temperature of the order of that found in pure hydrocarbons. The Clapeyron equation (equation 5) can be used to describe the pressure dependence of the phase transition temperature.

In contrast to the rather simple thermodynamic description of the phase diagrams for small molecules and lipids, the stability diagrams of proteins dissolved in water cannot be described by simple thermodynamic considerations. Kinetics may become very important in the unfolding and aggregation processes. The latter is often the cause of the irreversibility of protein denaturation. The molecular details of the denaturation of a protein are also much more complex than the melting of water or hydrocarbons. Experiments and computer simulations make it clear that water-water, water-solute, and solute-solute interactions are equally important in the behavior of the system.

PROTEINS AND ENZYMES: FOLDED, UNFOLDED, AND AGGREGATED

The pioneering work in high-pressure protein research is that of Bridgman, who observed that a pressure of 600 MPa will give egg white an appearance similar, but not identical, to that of a cooked egg (3). Here we describe the mechanism of pressure-induced denaturation, the role of water in this process, and how the pressure-denatured state relates to other denatured states as induced by heat or chemical denaturants. Finally, the effect of pressure on protein assemblies and the pressure-temperature phase diagram of proteins are discussed.

Mechanism of Pressure Denaturation

Suzuki (48) found that at temperatures below 30°C the kinetics of the pressure denaturation of carbonylhemoglobin and ovalbumin were characterized by a negative activation enthalpy. Such negative activation energies have also been observed for the urea-induced denaturation of proteins. To explain his observations, he proposed the following mechanism:

$$P + n\mathrm{H_2}0 \leftrightarrow P(\mathrm{H_2}0)_n \rightarrow P_D \tag{6}$$

where P is the native protein, $P(\mathrm{H_2O})_n$ is the hydrated protein, and P_D is the denatured protein. Thus, he suggested that pressure induces the penetration of water into the protein in a strongly exothermic step, thereby causing denaturation. A similar conclusion was reached by Silva and Weber (45) on the basis of experimental data. Computer simulations on the association of methane in water (19) and on bovine pancreatic trypsin inhibitor (54) have provided further evidence for this water penetration mechanism. The former simulation demonstrated that high hydrostatic pressure disrupts hydrophobic contacts in favor of the

solvent separated apolar partners, whereas the latter showed that under pressure, protein-protein hydrogen bonds are replaced by protein-water hydrogen bonds. Pressure also affects other noncovalent interactions, mainly electrostatic interactions (Table 1). Note that the volume change associated with hydrogen bond formation is close to zero, implying that pressure will not strongly affect this interaction.

Protein denaturation is associated with volume changes on the order of -10 to -100 ml·mol^{-1}. What is the origin of the volume decrease? The volume of a protein in solution, V_i, is the sum of

$$V_i = V_{\text{atom}} + V_{\text{cavities}} + \Delta V_{\text{hydration}} \tag{7}$$

where V_{atom} and V_{cavities} are the volumes of the atoms and the cavities (that originate from imperfect packing of the atoms in the native conformation), respectively, and $\Delta V_{\text{hydration}}$ is the volume change resulting from the interactions of the protein with the solvent (17). Upon protein denaturation the volume of the atoms will not change, so the volume change accompanying the denaturation can be written as

$$\Delta V = \Delta V_{\text{cavities}} + \Delta\Delta V_{\text{hydration}} \tag{8}$$

Contributions to $\Delta\Delta V_{\text{hydration}}$ are summarized as follows. Exposure of charged and hydrophobic groups to water will cause a volume decrease (Table 1). The former is due to a phenomenon called electrostriction: upon the formation of an ion in solution, the nearby water dipoles will be strongly attracted by the Coulombic field of the ion. The solvation of a monovalent ion is accompanied by a volume decrease of ~10 ml·mol^{-1}. In the case of hydrophobic groups, the mechanism underlying the volume change is not fully understood. Presumably the contribution to the volume change arising from the compressibility of the hydrophobic hydration layer plays an important role (28). Solvation of polar groups, on the other hand, also results in a volume decrease (Table 1).

The elimination of cavities upon denaturation is also expected to contribute to the observed negative volume change. This has been confirmed experimentally. Mutants of

Table 1. ΔV associated with specific biochemical reactions (25°C)

Reaction	Example	ΔV (ml·mol^{-1})
Protonation	$H^+ + OH^- \rightarrow H_2O$	21.3
	Imidazole + $H^+ \rightarrow$ imidazole·H^+	−1.1
	Tris + $H^+ \rightarrow$ Tris·H^+	−1.1[a]
	$HPO_4^{2-} + H^+ \rightarrow H_2PO_4^-$	24
Hydrogen bond formation	Poly(L-lysine) (helix formation)	~0
Hydrophobic hydration	$C_6H_6 \rightarrow (C_6H_6)$water	−6.2
	$(CH_4)_{\text{hexane}} \rightarrow (CH_4)_{\text{water}}$	−22.7
Hydration of polar groups	*n*-Propanol$\rightarrow$(*n*-propanol)$_{\text{water}}$	−4.5
Protein dissociation	Lactate dehydrogenase ($M_4 \rightarrow 4M$)	−500
Protein denaturation	RNase A (at pH 2)	−46

[a]The small ΔV for Tris-HCl indicates that the pH of this buffer is pressure insensitive. It is therefore the ideal buffer for pressure studies near physiological pH. A phosphate buffer, in contrast, will have a pH shift of approximately 0.4/100 MPa. In practice the effect may be less pronounced, since the ΔV of ionization becomes smaller at high pressure. It should also be pointed out that pressure-insensitive buffers often show a large temperature dependence (large ΔH) and vice versa.

RNase A in which the mutations created additional cavities are characterized by a larger negative ΔV upon denaturation (50).

Summation of the above contributions would result in a large and negative ΔV. However, experimentally only small, negative volume changes are observed. This suggests that there is also a positive contribution that, at least in part, compensates for the above negative contributions. The origin of this contribution is still the subject of debate (40).

Water as a Reaction Partner

From the above-described mechanism it is clear that water should be considered a reaction partner rather than an inert background. This is supported by other observations. For instance, the presence of water is required for enzyme activity, and it strongly affects the temperature stability of proteins. Fujita and Noda observed a decrease in the denaturation temperature from ~137°C for dry lysozyme to ~67°C at a hydration level of ~0.4 g of H_2O/g of protein (10). This observation has been made for several proteins, and it applies also to the pressure denaturation (12, 35). The resistance of bacterial spores and small organisms, such as tardigrades, and the stability of amyloid fibrils are other illustrations of the importance of water in the effects of pressure on organisms and molecules (27, 43, 47).

Water availability is also one of the crucial parameters for the growth of microorganisms. The effect of water activity on bacterial growth is more pronounced than the effect of water activity on protein denaturation. Hence, other parameters, such as osmotic pressure, play a role in the water stress response of bacteria.

Nature of the Pressure-Denatured State

It is now well recognized that the denatured state of a protein is not a random coil but that there are still some persistent native-like long-range contacts. However, the degree of conformational change depends on the denaturation method. Pressure-denatured proteins are often considered to be molten globule-like structures (49). The molten globule state of a protein is generally characterized by a loss of tertiary contacts, whereas the bulk of the secondary structure is maintained. This results in a more hydrated and more expanded conformation. In contrast, the heat-denatured state often shows a more disordered conformation of the protein in which both tertiary and secondary structures are lost (28). The above-described pressure denaturation mechanism provides a basis for the observed differences between the heat- and pressure-denatured states (Fig. 3) (30).

The different characteristics of the heat- and pressure-denatured states affect properties such as the aggregation propensity and the gelation of proteins. The latter is being exploited in the food industry to produce foodstuffs with novel properties using high-pressure treatment.

Pressure Effect on Multimeric Proteins and Aggregates

So far we have considered the effect of pressure on monomeric proteins, which generally become denatured between 400 and 800 MPa. Moderate pressures, of 200 to 300 MPa, are also known to dissociate protein oligomers into their monomers (45). The latter can maintain their native conformation or may denature in this process. Of particular interest is the pressure-induced depolymerization of larger protein assemblies, such as cytoskeletal