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The Effect of Hydrostatic Pressure on Biological Systems

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Introduction

The last decade has witnessed a large increase in the number of studies on the effects of large hydrostatic pressure over biological systems. Despite the many different scopes and objectives behind them, three main approaches can be found: the employment of pressure change as a tool for the study of conformational characteristics of biopolymers, the use of high pressure treatments for modification of products of biological origin, mainly foods, and the increasingly intense study of barotolerant and barophilic microorganisms and their macromolecular and genetic mechanisms. These three approaches are highly interrelated, and the understanding of the role of pressure as a variable in physico-chemical processes is a common feature for all of them (Gross and Jaenicke, 1994).

Nevertheless, the effects of hydrostatic pressure had been considered by scientists almost a century ago, when Bridgman and other pioneers started their studies on the topic (e.g. Bridgman, 1912, 1914, 1915). The three mentioned lines have followed a different evolution: the use of pressure as a biophysical study tool has steadily developed at a pace closely corresponding to the availability of instrumentation that has facilitated the production of data under elevated pressure. The industrial application of hydrostatic pressure, by contrast, has had to wait until relatively recent times, a delay due primarily to a lack of appropriate equipment that could guarantee a process economically competitive. The exploration of deep-sea life forms has also had its own progress path during the last century, with the current climax of discovery, research, and even industrial application of some of the microorganisms that have been studied, together with their enzymatic systems.

A variety of review papers have dealt with the different aspects of high pressure. Its effects on protein structure and association behaviour appears the most popular topic

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Abbreviations: FT-IR, Fourier Transform Infra Red; IFN, interferon; NMR, nuclear magnetic resonance; S, Svedberg unit; uv, ultra-violet.

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(Harrington and Kegeles, 1973; Morild, 1981; Heremans, 1982; Silva and Weber, 1993; Gross and Jaenicke, 1994; Balny, 1996; Mozhaev *et al.*, 1996; Heremans and Smeller, 1998; Silva *et al.*, 2001). Other biological systems have also been considered: nucleic acids (MacGregor, 1998), lipid bilayers (Winter and Böttner, 1993; Jonas and Jonas, 1994). The industrial and food related applications of hydrostatic pressure have been the object of detailed reviews (Knorr, 1993; Kalichevsky *et al.*, 1995; Hayashi, 1996; Knorr, 1999; Clark, 2001), as well as the effect of pressure on living organisms from a general perspective (Wong, 1993; Mentre and Hui Bon Hoa, 2001) or different particular points of view: food microorganisms (Tauscher, 1995), barophilic organisms (Jaenicke and Zavodszky, 1990; Jaenicke, 1991; Barlett, 1992; Yayanos, 1995; Abe *et al.*, 1999) and their adaptation and regulation of pressure-related peculiarities (Somero, 1992; Barlett *et al.*, 1995; Horikoshi, 1998; Kato and Qureshi, 1999; Robb and Clark, 1999). The aim of this review will be limited to a summary of the principal lines for the study of the effects of hydrostatic pressure on biological systems at a physical, biochemical and biophysical level, which are, moreover, the basis of the more general effects observed in complex systems such as food or living organisms.

Pressure as a biophysical variable

The effect of moving along the pressure axis in physico-chemical equilibria can be understood as a derivation from the classical Le Chatelier-principle-based equation:

$$d(\Delta G) = (\Delta V)\, dp + (\Delta S)\, dT \tag{1.1}$$

which specifies the dependence of the free energy (ΔG) of a process with pressure (p) and temperature (T), through the increment in volume (ΔV) and entropy (ΔS) of the process, respectively. From this, and considering temperature as a constant, *Equation 1.3* shows a way to obtain ΔV, which gives useful information about the nature of the reaction:

$$-RT\, d(\ln K) = (\Delta V)\, dp \tag{1.2}$$

$$(\Delta V) = -[RT\, d(\ln K)/dp]_T \tag{1.3}$$

Integrating between atmospheric conditions and a given pressure, the constant describing equilibrium in this case can be obtained:

$$\ln K = \ln K_0 - (P\, \Delta V)/RT \tag{1.4}$$

where K is the (two-state) equilibrium constant. ΔV is actually the molar volume increment in the reaction between these two states (see, for example, Harrington and Kegeles, 1973; Silva and Weber, 1993; Silva *et al.*, 2001). Equilibria (both physical and chemical) are displaced as a function of pressure towards the state with a smaller specific volume. All equilibrium effects of high pressure can be understood as derived from this displacement.

Parallel kinetic equations can be employed to describe the pressure dependence of reaction rate constants:

$$(\Delta V^{\#}) = -[RT\, d(\ln k)/dp]_T \tag{1.5}$$

$$\ln k = \ln k_0 - (P\, \Delta V^{\#})/RT \tag{1.6}$$

Table 1.1. Volume increments corresponding to different reactions induced by pressure.

ΔV (cm^3/mol)	Molecule	Process*
#–2/–6	P–450	CO binding [1]
–10/–11	Acetic acid	Ionization [2]
#+3/+21	Haemoglobin	CO binding [1]
#+9/+19	Myoglobin	CO binding [1]
–9.3/–11.8	Lysozyme	Unfolding [3]
–20/–22	Water	Ionization [2]
–27	DPPC bilayer	Phase transition [4]
–25/–45	A monoclonal antibody	Fluorescein dissociation [5]
#–36	P–460	CO binding [1]
–68	Haemoglobin	Dissociation [6]
–70	Apomyoglobin	Unfolding to molten globule [7]
–57/–92	Staphylococcal nuclease	Unfolding [8]
–90/–104	Allophycocyanin	Dissociation [9]
–100/–166	Rubiscio	Dissociation [10]
–168/–189	Tryptophan synthase β-dimer	Dissociation [11]
–200	Lactate dehydrogenase	Unfolding [12]
–199/–337	Glyceraldehyde phosphate dehydrogenase	Dissociation [13]
–377	$_{(-ATP)}$ GroEL	14-mer dissociation [14]
–1388	$_{(+ATP)}$ GroEL	14-mer dissociation [14]

*Volume increments corresponding to different reactions induced by pressure. The sign of ΔV has been altered so that it always corresponds to the dissociation reaction (unfolding, disassembly or unbinding).
#Activation volume. When nothing is indicated, reaction volume.
Data from: [1] Lange *et al.*, 1994; [2] Lown *et al.*, 1968; [3] Samarasinghe *et al.*, 1992; [4] Kamaya *et al.*, 1979; [5] Carrero and Voss, 1996; [6] Silva *et al.*, 1989; [7] Vidugiris and Royer, 1998; [8] Royer *et al.*, 1993; [9] Foguel and Weber, 1995; [10] Erijman *et al.*, 1993; [11] Silva *et al.*, 1986; [12] King and Weber, 1986a; [13] Ruan and Weber, 1989; [14] Gorovits *et al.*, 1995.

where k is the two-state rate constant and $\Delta V^{\#}$ the activation volume. This volume corresponds to the molar volume increment between the initial state and the activated transition state. A selection of reaction volume increments and activation volumes for a number of biomolecular processes is presented in *Table 1.1*.

As the volume increments taking part in the above equations are molar parameters, the effect of pressure over different species will depend on their molecular weight: the molar volume difference between two alternative states of a molecule will, in most cases, be larger for larger molecules, which will be those more affected by pressure variations.

It must not be forgotten that both equilibrium and rate constant depend on the temperature in similar ways to pressure (Gutfreund, 1995):

$$\ln K = \ln K_0 - (\Delta H)/RT \tag{1.7}$$

$$\ln k = \ln k_0 - (\Delta H^{\#})/RT \tag{1.8}$$

For small pressure variations, the associated changes in temperature can be neglected, but when a large pressure change is involved, especially for large sample volumes, or when both variables (P and T) are intentionally changed at the same time, this must not be forgotten. Analytical instrumentation allows the control of temperature but industrial equipment generally does not.

How high pressures are achieved in biological investigation

The progress in the development of appropriate instrumentation has been an important determinant of the pace of high-pressure research. Different experimental

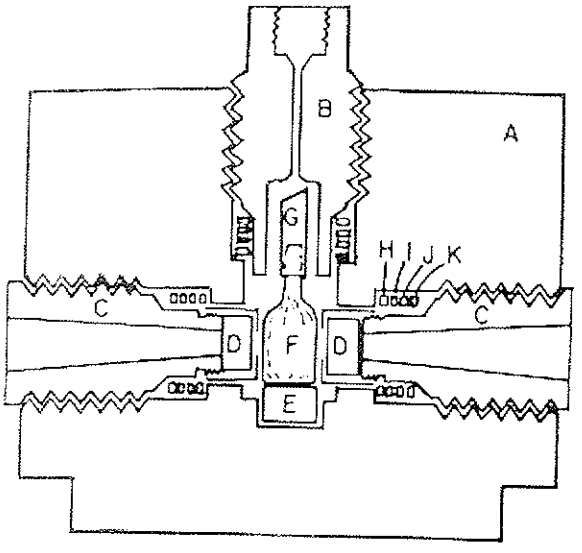

Figure 1.1. Diagram of an experimental assembly for the observation of pressurized samples by fluorescence spectroscopy (reproduced with permission from Paladini and Weber (1981a). Copyright 1981, American Institute of Physics). C, optical windows; F, sample container. See original text for further details.

approaches were followed. The most general one involves different types of vessels connected to pumps, which either directly introduce a pressurization fluid into the vessel or displace a piston to reduce the vessel volume. To ensure the fulfilment of the hydrostatic principle, a pressurization fluid is necessary, even for submitting solid samples to hydrostatic pressure: pressure exerted directly on a solid would be heterogeneously distributed for different spatial directions, giving rise to deformation forces. The fluid can be the sample itself – when this is liquid – or another external fluid, separated from the sample by a flexible wall. A low freezing point fluid is used when low temperatures are going to be used, to avoid ice damage to the equipment. Gas can also be employed but a large amount of energy is stored when compressing a gas. This makes gas-based instruments dangerous.

Different designs on the above lines have been constructed. In many cases, the result of pressure perturbation is fully reversible, so there is need for observation at high-pressure conditions and not just after a pressure treatment. Early work intending to draw conclusions from the latter approach has been largely unsuccessful (Lapanje, 1978). So, to follow the process under pressure, suitable observation techniques are needed. Each one requires making its particular requirements compatible with those of obtaining and maintaining high-pressure conditions. For example, to carry out nuclear magnetic resonance measurements (Samarasinghe *et al.*, 1992; Peng *et al.*, 1993, 1994; Royer *et al.*, 1993; Yamaguchi *et al.*, 1995; Zhang *et al.*, 1995), a non-magnetic metal cell body is needed (see, for example, Jonas *et al.*, 1993). Spectroscopic methods require optical windows, most times sapphire ones. Among these methods, fluorescence has been favoured (Pin *et al.*, 1990; Royer *et al.*, 1993; Bismuto *et al.*, 1996, amongst many others) due to the high sensitivity of this technique to conformational changes. *Figure 1.1* shows an assembly used for this type of study (Paladini

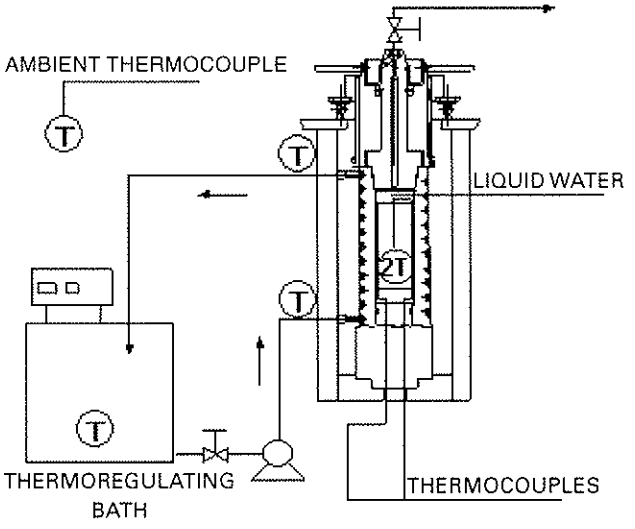

Figure 1.2. Diagram of an experimental assembly for treatment under hydrostatic pressure of relatively large samples (2.3 l) under thermal control (ACB, Nantes, France).

and Weber, 1981a). Techniques as diverse as UV absorption (Zipp and Kauzmann, 1973; Taniguchi and Suzuki, 1983), FT-IR (Takeda *et al.*, 1995), electrophoresis (Masson and Cléry, 1996), light scattering (Gross and Jaenicke, 1990; Patkowski *et al.*, 1990) have all been applied. Other very interesting methodologies, such as size exclusion chromatography, have remained, thus far, out of reach, whilst application of circular dichroism is still struggling against the problems posed by window depolarization under pressure (Balny, 1996; Kato and Horikoshi, 1996). A good control of temperature is always essential as thermal changes will interfere with the perturbations induced by pressure. Currently, this type of equipment has maximum attainable pressures around 1000 MPa.

A similar approach has been used for studies concerning the effect of high pressure in foods (Mertens and Deplace, 1993). Small-scale 'pilot' devices allow the pressurization of larger volumes of sample in conditions similar to industrial equipment. *Figure 1.2* shows a scheme for one such vessel (ACB, Nantes, France), at the author's laboratory, able to submit 2.3 litres of sample to 500 MPa. The larger sample volumes make it impossible to keep a perfect temperature control (Otero *et al.*, 2000b, 2001). The technological difficulties of high-pressure equipment increase 'exponentially' with sample size. Large-volume holders normally lack observation implements, often only allowing the determination of pressure and, possibly, temperature in one or several points inside the vessel. Industrial equipment, such as that illustrated in *Figure 1.3*, belonging to the Spanish food company Espuña (Olot), follows a similar design but at larger scale. Industrial and large-volume instruments currently have a practical pressure maximum at approximately 700 MPa, though progress is very active in this field.

Pressure jump devices allow the observation of a variety of properties in the relaxation process after a quick pressure change (e.g. Heiber-Langer *et al.*, 1992; Zhou *et al.*, 2000). A wide range of pressure amplitudes can be employed, but

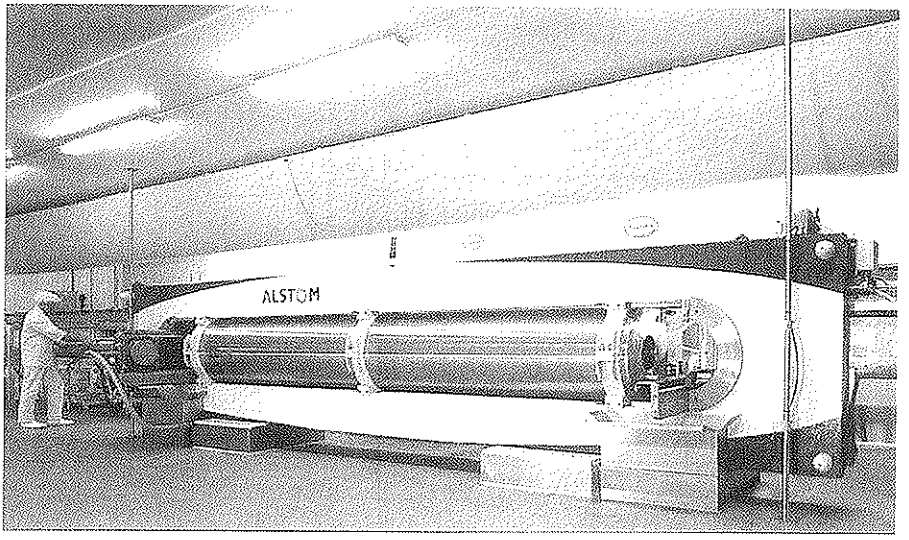

Figure 1.3. Picture of an industrial-scale high-pressure equipment (Alstom, Nantes, France), for the semi-continuous treatment of cooked ham at 400 MPa (Espuña, Olot, Spain).

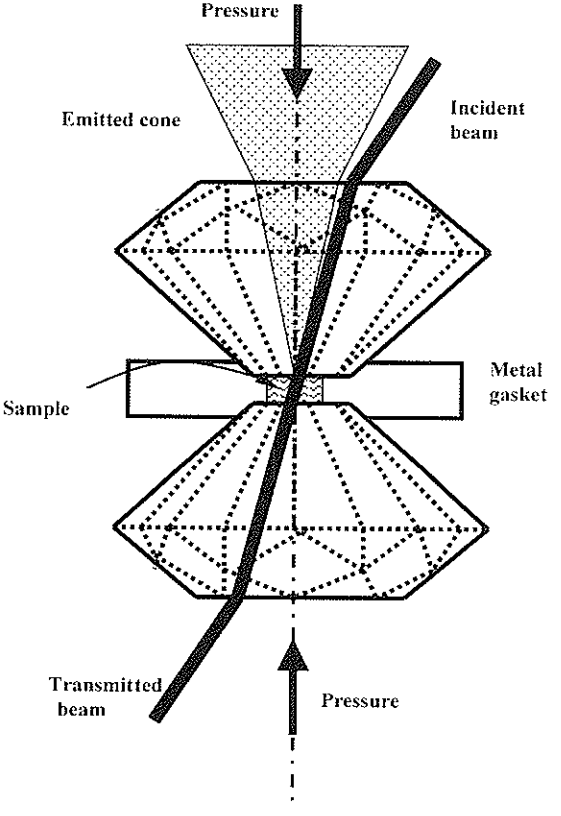

Figure 1.4. Diamond anvil cell designed to permit spectrophotometric observations.

information can be extracted from very small jumps. Pressure jump presents advantages over temperature jump due to the faster relaxation of pressure over that of temperature. Kinetic parameters of protein pressure unfolding and other pressure-driven transitions can be obtained (Gutfreund, 1995; Woenckhaus *et al.*, 2001).

Other completely different approaches, mostly used in the fields of Material and Physical Sciences, have been based on the 'diamond anvil' (Xu *et al.*, 1986; Auger *et al.*, 1990; Reis *et al.*, 1996; Smeller *et al.*, 1999), whereby the sample is compressed between two diamonds. Much higher pressures can be obtained in this way but the thermal control of the sample is more difficult and the number of techniques to follow the process rather limited. *Figure 1.4* presents a general scheme for such a diamond anvil cell that has been prepared for spectroscopic investigations.

Ultracentrifugation has also been used as a source of pressure: the increase in gravitational field developed on a spinning ultracentrifugation cell is so high that the sheer weight of the liquid column generates a significant pressure gradient across the sedimentation axis (*Figure 1.5*) (Harrington and Kegeles, 1973; Molina-García, 1999). Pressure attained in commercial analytical equipment can reach 500 MPa but preparative centrifuges, with similar angular speeds but longer radii and liquid columns, can generate higher values. This has been employed to perform studies where the pressure over a given sample changes as it moves towards the bottom of the cell, or to study the full set of pressure conditions in a single experiment (Infante and Baierlein, 1971; Infante and Krauss, 1971; Marcum and Borisy, 1978; Esman *et al.*, 2000).

A characteristic of the equipment described is that it is mostly at a prototype stage: indeed, most reported studies were carried out 'in-house' using equipment constructed at each author's laboratory. Only a few industrial-scale devices have been commercially available, together with the more basic research equipment. Even these are often custom-made to the design and requirement of the particular customer. This scenario, in spite of favouring both the researchers' initiative and their placement in the 'scientific front line', has had the negative effect of increased equipment prices, lack of availability, and difficulties for data comparison. Ultracentrifuges, primarily designed with other intentions, are probably the only full-scale commercial equipment available for high-pressure research and proper inter-laboratory comparison.

Pressure-associated variations of temperature

The principal technical difficulty for working at different pressures at a spatially and temporally constant temperature has to do with the compression heat or adiabatic heating. Heat is absorbed or released in association with pressure changes, and so the sample temperature varies according to:

$$(\Delta T/\Delta P) = T\ V\ \alpha\ /c_p \qquad (1.9)$$

where c_p is the heat capacity, V the specific volume, and α the thermal expansion coefficient (Otero *et al.*, 2000b). As the values of these properties depend on pressure and temperature, the actual temperature variation must be obtained after integration along the pressure–temperature change. Values for these parameters in a suitable range of pressures and temperatures have been reported and numerically calculated by empirical equations. A convenient source can be found in Otero *et al.* (2002) and

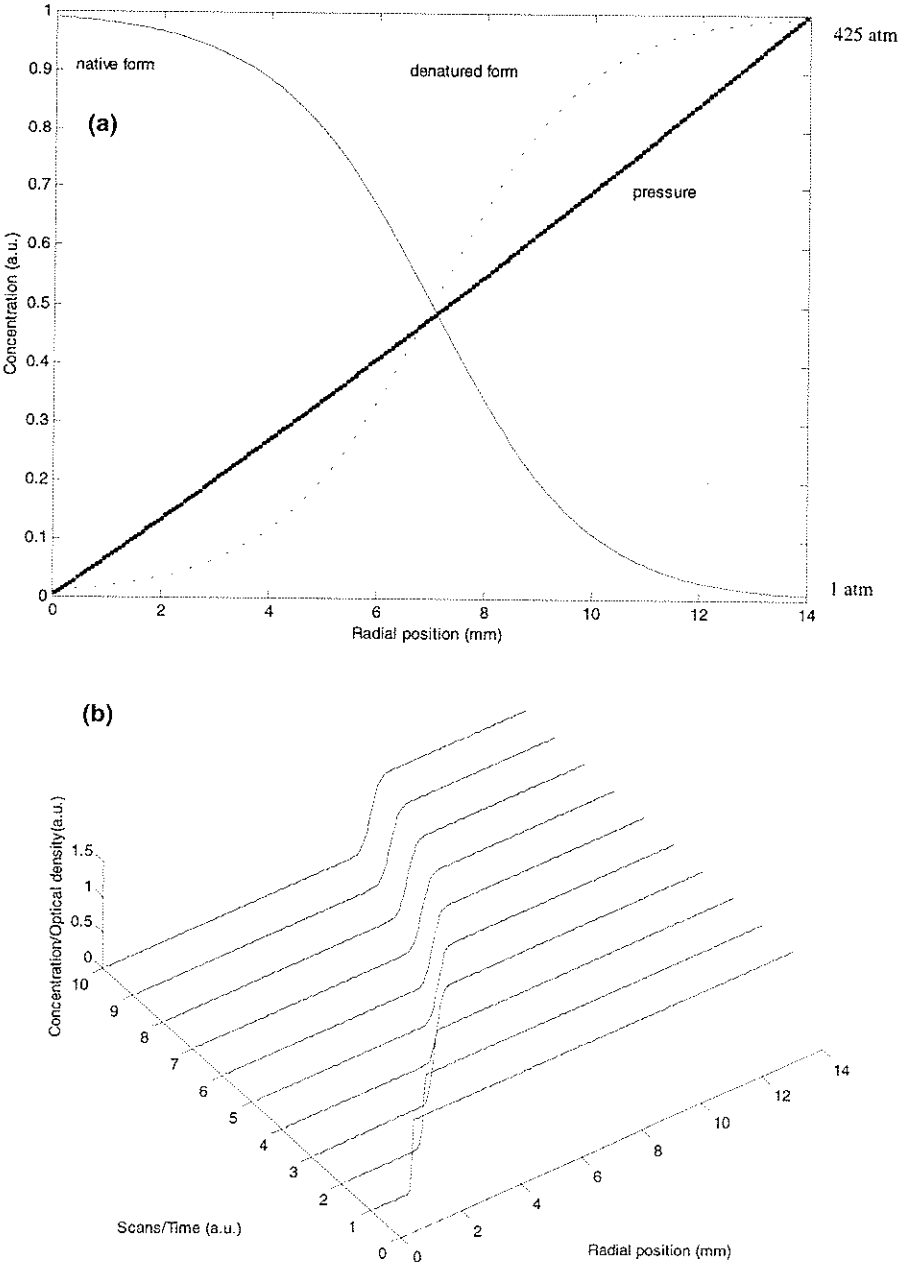

Figure 1.5. Effect of the hydrostatic pressure generated during ultracentrifugation experiments. a) Model of the behaviour of a hypothetical protein that undergoes denaturation in the pressure range attained in the cell. The concentration of native and denatured forms is depicted as a function of the pressure generated in the ultracentrifugation cell, which is itself a function of the radial position. b) Mathematical model of sedimentation velocity absorption profiles for a protein that suffers a conformational alteration so that its sedimenting velocity is reduced upon the change. This could be the case of a denaturing protein with increased hydrodynamic radius. The sedimentation velocity reduction is reflected in the retardation of the sedimenting border in subsequent scans (see Molina-García, 1999).

the public domain routine set there indicated. For largely aqueous samples, the resulting temperature variation can be of several degrees per each 100 MPa increase or decrease. Other substances of larger α, such as fats, suffer an even larger temperature change. This renders difficult the development of isothermal high-pressure processing, especially for large and inhomogeneous samples. A very small size sample will become thermally equilibrated with the vessel mass and surroundings in a very short time, while a larger one will develop a temperature gradient that is slow to equilibrate, especially when stirring implements working under high pressure have not yet been designed. Also, a sample comprising regions of different α will reflect the composition differences in a temperature distribution, which will also equilibrate slowly.

The importance of proper temperature control cannot be stressed enough (Balny, 1996; Otero *et al.*, 2000b, 2001). In many cases, the directions of the effects of pressure and temperature over physico-chemical processes cannot be forecast due to a complex dependence of kinetic and thermodynamic factors, diffusion, different types of bonds, etc. The heat involved in pressure changes and the difficulty for thermo-regulation, especially for large industrial vessels, calls for more careful study. Lack of reproducibility of the effect of treatments in terms of enzymatic activity or microbial counts can be blamed on different thermal histories. The approaches for control vary from attempting the quicker possible thermal equilibration, or to perform slow pressure changes (useful for small cells), to the complete isolation of the equipment (whose temperature will fluctuate with pressure, but will be spatially homogeneous and temporally predictable). Also, knowledge of the temporal and spatial evolution facilitates the modelling of the combined pressure–temperature effects.

Effects through water properties

The central role of water in the behaviour of biological systems makes it necessary to consider the effect of hydrostatic pressure variations on some of the properties of water. Even when the centre of interest of most studies is the solvated part of the system, the very important and more abundant (both in mass and number) solvent must not be forgotten. Water, a surprisingly complex fluid, exhibits an unexpected behaviour for many of its properties, especially at low temperature and high pressure (Lüdemann, 1994). Effects relating to the water ionization constant, physical properties and phase behaviour will now be considered.

CHANGES IN THE WATER IONIZATION CONSTANT WITH PRESSURE

Many of the interactions taking place between water molecules and biological macromolecular systems are strongly dependent on its ionization state. Electrostatic bonds between macromolecular side chains and solvation of free charged groups – which can play a seminal role for structural relations and stabilization energies – are affected by even slight changes in the ionic content of water, that is, in pH. But the ionization constant of water is not a constant with pressure change. K_w, the water ionization constant, grows with hydrostatic pressure as a result of the displacement of the equilibrium of dissociation towards the dissociated state:

$$H_2O \rightleftharpoons H_3O^+ + OH^- \qquad K_w = [H_3O^+]\,[OH^-]/[H_2O] \qquad (1.10)$$

This is not a reflection of the changes in volume in the ionized molecule itself (which are not so significant) but to the much larger action on the whole packing and structure of many layers of solvation water molecules that surround them. This effect takes place even at moderate pressures, as K_w becomes magnified by a factor of 2 at only 100 MPa (Lown *et al.*, 1968) (*Figure 1.6*).

Other equilibria involving charged molecules will be affected, to different extents, but in the same sense. Ionization will always be favoured, the lesser the volume of the solvation water around a charged molecule being the decisive factor. So, it is difficult to foresee how the changes in water ionization constant will affect a given ionic equilibrium. In general, the pH of aqueous buffer solutions varies with pressure. Acid dissociation is favoured with pressure, with the exception of TrisHCl (whose pK is strongly dependent on temperature), which is one of the buffering systems less affected by pressure – a decrease of 0.2 units in 650 MPa (Neuman *et al.*, 1973). Meanwhile, phosphate buffers, rather insensitive to temperature, are among those with a steeper pressure-dependence (2 units in the same pressure range). Another consequence derived from this behaviour of water is that solvation of ionic groups in other (macro)molecules will be preferred to charge compensation between non-water molecules: this has implications for the stability of given macromolecular structures.

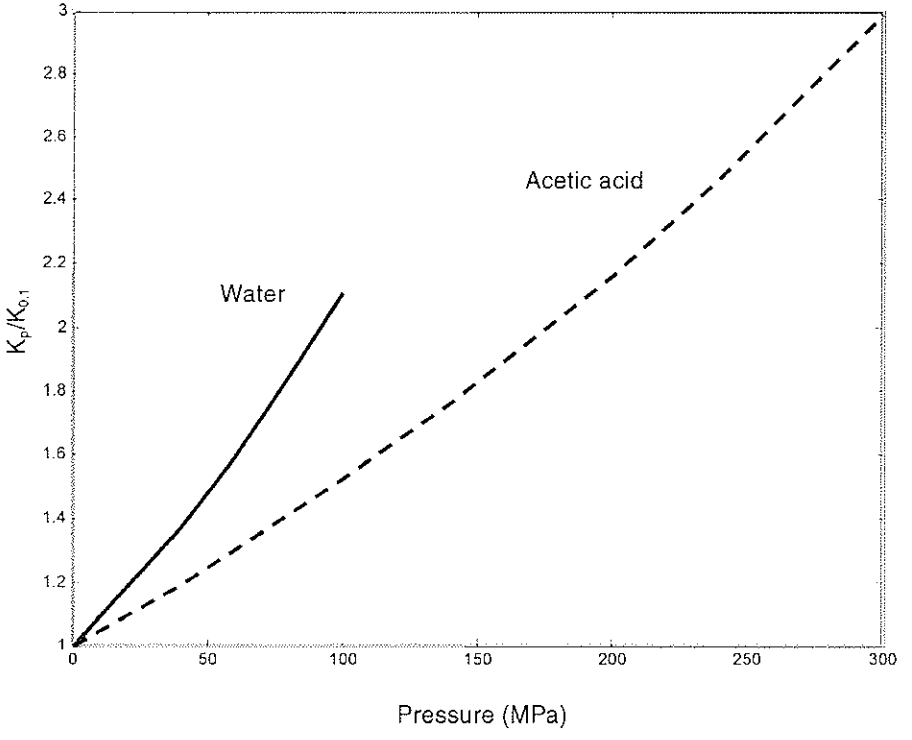

Figure 1.6. Ionization behaviour of water and aqueous solutions of acetic acid under pressure at 25°C. $K_p/K_{0.1}$ is the ratio of the ionization constant at a given pressure over that at 0.1 MPa (original data from Lown *et al.*, 1968).

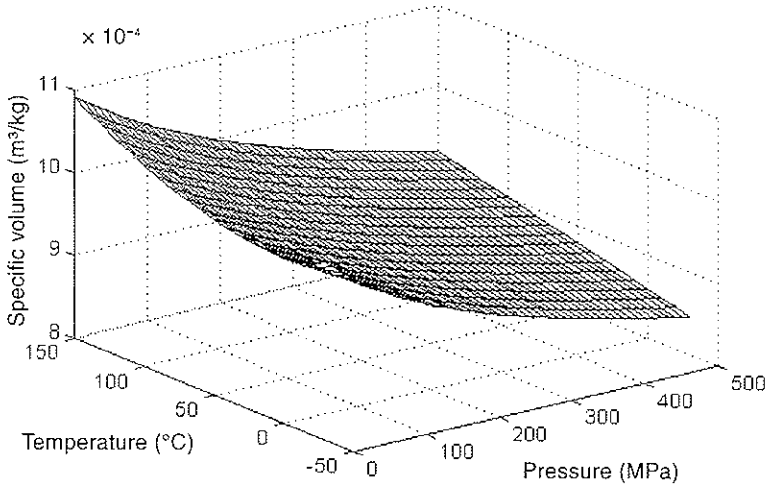

Figure 1.7. Tridimensional plot of the dependence of water specific volume with pressure and temperature (see Otero *et al.*, 2002).

PHYSICAL PROPERTIES OF WATER

The pressure variation of many physical properties of interest has been measured and calculated by numerical algorithms (Vedam and Holton, 1968; Ter Minassian *et al.*, 1981; Saul and Wagner, 1989; Hill, 1990; Wagner *et al.*, 1994; Wagner *et al.*, 2000). Some of these algorithms can be obtained through Otero *et al.* (2002). Among these properties, α, the thermal expansion coefficient, and β, the compressibility coefficient, are related to the variations in volume with temperature and pressure respectively.

Viscosity is one of the properties of water whose change will have a larger effect on biological systems: the diffusion velocities of reactants and products strongly depend on the medium viscosity, which determines the liquid-phase reaction rate. Water viscosity, which rapidly increases with temperature at low temperatures, does not suffer the expected increase with growing pressure, which is common for other liquids. The weakening of hydrogen bonds, caused by the reduction in intermolecular distance, allows a freer displacement of water molecules. This effect is highly temperature-dependent. At 25°C, the viscosity decrease (by a modest 3%) appears to have a minimum at around 150 MPa, increasing thereafter to follow the classical behaviour for higher pressures (Lüdemann, 1994; IAPWS, 1997b). At sufficiently high pressure (above ~ 200 MPa), water viscosity is effectively increased over its atmospheric pressure value, and so most reactions can be expected to take place more slowly under these conditions. This has to be taken into consideration when calculating the effect of pressure over reaction rate constants (Balny, 1996). Also, heat dissipation will be retarded in pressurized water systems as convection movements will, again, take place at a slower pace. Other liquids present continuous and much larger increases in viscosity with increasing pressure. This must be considered, especially in fat-rich food systems.

The specific volume (*Figure 1.7*) and heat capacity (*Figure 1.8*) of water both decrease monotonously with pressure and temperature as a result of the weakened

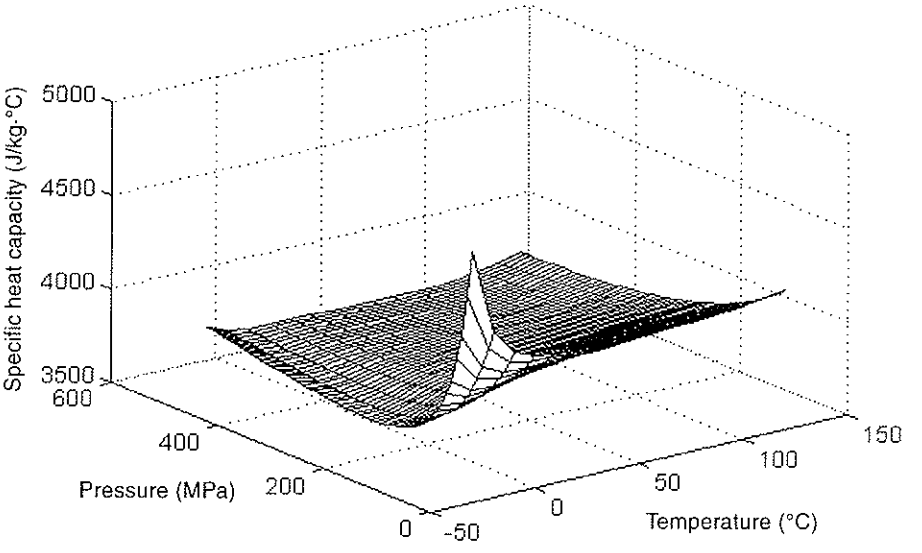

Figure 1.8. Tridimensional plot of the dependence of water heat capacity with pressure and temperature (see Otero *et al.*, 2002).

hydrogen bonds that are its energy storage. Its dielectric constant increases with pressure. This, in association with the increase in density, manifests itself as a reduction in the strength of electrostatic interactions (IAPWS, 1997a).

PHASE CHANGES

Although individual molecules may be small, a crystal formed by many of them can be conceived as a large macromolecule, so that the volume differences between crystalline forms can be significant. The classical pressure–temperature phase-diagram of water (*Figure 1.9*) shows the P and T transition borders between ice forms, with different specific volumes (Bridgman, 1912, 1915). The liquid water region, with 0°C as the lower temperature limit under atmospheric conditions, can be extended to little more than –20°C at high pressure (relatively moderate, around 210 MPa). This can be employed for performing the otherwise impossible studies of biological systems in aqueous solution at these negative temperatures, with the interesting utility of testing cold denaturation of proteins. Other uses are related to low-temperature preservation and the avoidance of damage caused by ice crystals in tissues and structures. Different approaches, referred to below, take advantage of this anomalous and complicated phase diagram.

Effects on biological macromolecules

Biological systems are not structurally static: their many functions and the interrelations taking place among different elements of a living organism would be impossible to conceive with rigid, monotonic structures. All components exist in labile equilibria between states, sensitive to their displacement by slight variations in conditions and/or the action of a variety of effectors. Such is the case of virtually all known proteins

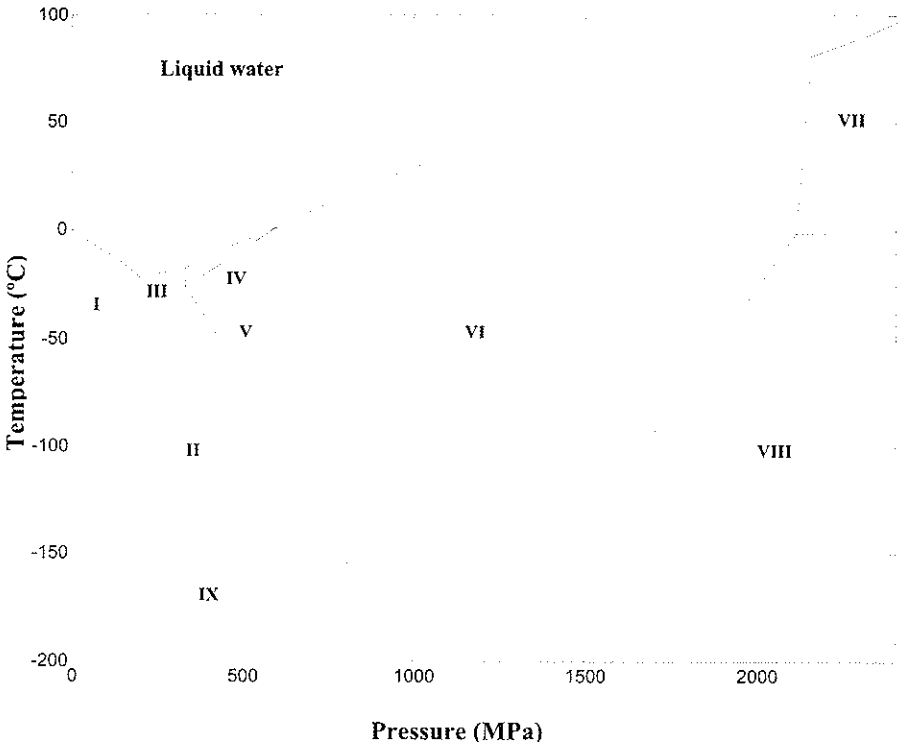

Figure 1.9. Water phase diagram showing the different ice forms and the phase change borders (original data from Bridgman, 1912 and 1915).

of lipid bilayers, of nucleic acids, and of larger and more complex assemblies, which are responsible for so many life functions: protein complexes, nucleosomes, motility systems, receptors, viral particles, etc. This conformational flexibility and complicated landscape of stability are also responsible for the facilities for the adaptation of life forms to extreme conditions (Jaenicke and Zavodszky, 1990; Jaenicke, 1991).

In all these cases, a variety of structures (at least two) can exist, separated by shallow energy barriers. Functionally, this means that a slight change in the surrounding conditions: temperature, ionic strength, etc., or the binding of a signal molecule, can switch a response, which can be modulated by other factors. The practical implication is that, although in given constant conditions we could find a vast majority of the population of a system in a given 'basic' state, many types of perturbation can displace the population totally or partially in one or more different directions, populating other alternative states.

Pressure provides a way of perturbing a system for solely probing the difference in volumes among these different states (Silva *et al.*, 2001). In relation to biophysical experimentation, industrial applications, and the understanding of new life forms, pressure can be considered as an additional variable to those more classically considered, such as temperature, pH, and concentration of key substances. The range of pressures employed in biological studies (less than 1000 MPa) is not enough to cause covalent bond alterations *per se*, though the influence over chemical and

enzymatic reactions can do so indirectly. Pressure over 30 000 MPa is needed to break covalent bonds (Harris *et al.*, 1976).

PRESSURE EFFECTS ON PROTEINS

The driving forces responsible for the stability of a protein native state, or for the aggregation of monomeric units, are of similar character: a negative free Gibbs energy balance. The main contributions to this balance are hydrogen bonds. In both the denatured state and in monomers, some of the possible bonds take place between the protein and solvation water molecules, while in the native/folded state or in aggregates, these are substituted by intra-protein bonds. Another contribution is the destabilization of solvation water. The hydrophobic regions of proteins produce a disruption in the water hydrogen bond lattice, which is minimized if these regions are buried away from contact with water, as happens in folded and aggregated states. Also, the packing of native structures, or the binding of monomers, leaves voids, empty regions not accessible to solvent. Upon denaturation or dissociation, these voids disappear. The three factors are responsible for an overall decrease in volume when a protein denatures or a non-covalently linked structure becomes dissociated.

It is not easy to determine *a priori* the sensitivity to pressure-denaturation for a given protein. For most of the cases thus far studied, denaturation takes place at pressures over approximately 500 MPa, though there are more sensitive cases. Meanwhile, lower pressures, over 200 MPa, can generate perturbations of the aggregation state. As can be appreciated from *Table 1.1*, the ΔV values for denaturation are normally smaller than those for the dissociation of protein aggregates.

Other alterations in proteins can take place on a lower scale than denaturation. The change in activity of a protein (enzymatic or other) can indicate conformational change, sometimes to a large extent: proteins are said 'to breathe'. The energy barriers between these states are small enough for a detectable fraction of molecules to populate each of them. Subtle signals, of physical or chemical origin, alter this population distribution, giving rise to enzymatic reactions, signal transduction, etc. Although the volume differences among these states will often be small, so are the energetic forces stabilizing them. Pressure changes are able to shift the distribution of populations among the different states. These alterations do not imply the complete lack of functionality, the appearance of a state completely absent at native conditions, or the spontaneous formation of aggregates, which are characteristics of denaturation. But the favouring of one or other sub-states can have important consequences for protein functionality. For an enzyme, this could mean the alteration of the ratio of the different protein forms that play a role in the full reaction (activated states, different for each of the three stages: before the binding of the reaction components, during, and after the reaction). The overall result can alter both reaction specificity (again both in terms of reaction type and molecules involved) and its activity. In most cases, since enzymes are especially well tuned for their particular function, this will mean a reduced performance at high pressure (Balny, 1996). But in a few cases, an enhanced activity can be observed, or even a different activity may be created (e.g. Mozhaev *et al.*, 1996). The implications for the engineering of proteins with all ranges of application (biomedical, industrial, etc.) are obvious.

For different protein systems, the physiological/functional meaning of the effect of

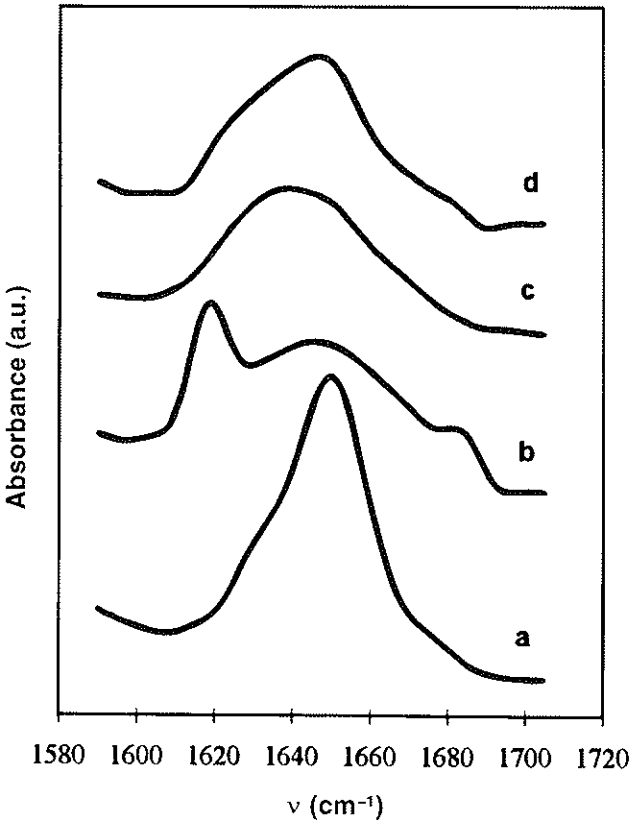

Figure 1.10. Amide region of the infrared spectra of horse myoglobin in different conformational states: a) native form; b) heat unfolded; c) pressure unfolded; and d) at atmospheric conditions after pressure unfolding (from Smeller *et al.*, 1999, with permission).

pressure could be very different. A consequence of the recent interest in genome sequencing is the elucidation of the factors determining the formation of a given protein secondary and tertiary structure. Many of the studies performed on proteins under pressure pursue this aim. The denatured state of proteins produced by hydrostatic pressure has been compared with those obtained by other methods, such as thermal denaturation, pH, or the use of chemicals. The different ways of inducing unfolding are reflected in the characteristics of the unfolded state: thermal denaturation acts on the more energetic interactions, chemical denaturants preferentially lose the bonds for which they compete, and hydrostatic pressure induces those conformational changes involving most of the volume decrease available, sparing, or even enhancing, other interactions (Zhang *et al.*, 1995; Smeller *et al.*, 1999). *Figure 1.10* shows some of the differences between myoglobin denatured states as followed by FT-IR spectroscopy. Some characteristics of pressure-unfolded state (loose tertiary structure, flexibility, preservation of secondary structure elements) allow identification with the molten globule state. The confirmation of the predictions for cold denaturation has been found with the help of water freezing temperature reduction by increased pressure.

The partially unfolded state can conserve all, part, or nothing of the original functionality. Pressure-denatured proteins are often not completely unfolded chains, fully solvated, as the results from thermal denaturation are, but tend to be 'open' structures that have reduced or completely eliminated the voids contained in the native structure and have solvated their charged groups, the major contributions to the volume differences between the two states. Other hydrophobic and internal hydrogen bonds can be maintained, if the overall conformational restraints of the polypeptide chain permit it. The result can conserve different structural patterns that can be manifested, through the different methodologies used to observe the structure, as being more or less close to the native state. Even, in some cases, functional properties can, to some extent, be maintained, including enzymatic activities.

Under ideal diluted conditions, pressure unfolding is largely reversible but, at very concentrated conditions (such as those in real cells), aggregation among the unfolded proteins, or with other proteins or molecules, can take place. After pressure reduction, these aggregates can remain stable. Aggregation will be favoured by the 'open' state of the molecules, exposing interfaces with groups prone to interact (hydrophobic regions). So, the investigation of protein pressure-denaturation is not always easy to keep separate from that of aggregation. The kinetic limits imposed by the low concentration of monomers, the lack of other molecules or structures for alternative binding, and the increase in viscosity can reduce this aggregation tendency.

Most calculations on the effect of pressure on equilibrium and velocity constants assume a reaction between two, well-defined states. The validity of this assumption must be checked, as the existence of a number of intermediate, relatively stable states can invalidate this theoretical approach (Masson and Cléry, 1996). Also, the volumes of the two states are considered as independent of pressure. This is basically untrue. Proteins are slightly, but still finitely, compressible. Also, temperature, through effects on the different interactions stabilizing a protein structure, has an influence on those volumes.

Protein unfolding

Many proteins have, to date, been studied by hydrostatic pressure-induced denaturation. The alteration of the molecular dimensions, which change upon denaturation, can be employed to follow the process. Small-angle X-ray scattering under pressure was used to determine the radius of gyration of staphylococcal nuclease at different temperatures and pressures and to obtain information on its size and shape. This protein becomes reversibly pressure-unfolded. At high pressure (300 MPa), refolding is slower than at atmospheric conditions, due to the large positive activation volume for the rate-limiting step of the process. Information about the folding mechanism and characteristics of the intermediate states can therefore be obtained (Woenckhaus *et al.*, 2001).

A very productive strategy for investigating the factors contributing to protein stability is the generation of different mutants with tailored amino acid substitutions. Studies of pressure-denaturation on mutants obtained for staphylococcal nuclease, followed by fluorescence and NMR, yield information on the stabilization forces acting upon the native protein. This nuclease exhibits conformational heterogeneity in its native, wild form, and the amino acid substitutions that control the distribution

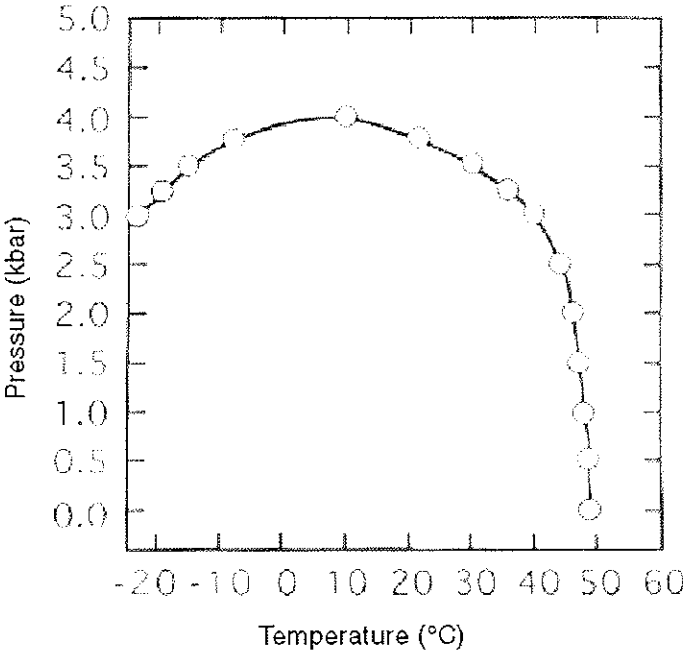

Figure 1.11. Temperature–pressure phase diagram for RNAse A (at pH 2.0) showing the region of stability of the protein ('below the line') (from Zhang *et al.*, 1995, with permission).

of population among the different states are also involved in determining the pressure sensitivity of this protein (Royer *et al.*, 1993).

The study of ribonuclease A unfolding under high pressure has yielded information on the influence of temperature on pressure-unfolding. It was found that the negative unfolding ΔV decreases with increasing temperature. So, at higher temperatures pressure-denaturation becomes favoured. Close monitoring of the process by NMR has permitted the calculation of a full set of thermodynamic parameters, which in turn has facilitated the elaboration of a hypothesis on the driving forces behind unfolding of this protein (Yamaguchi *et al.*, 1995). Meanwhile, Zhang *et al.* (1995), using NMR on the same protein, found significant remains of native structure in pressure-denatured ribonuclease A. A pressure–temperature stability diagram can be drawn for this protein (*Figure 1.11*). Fourier transform infrared spectroscopy yields information on secondary structure alterations from shifts in the spectral frequency bands. Pressure-induced secondary structure changes of reversible character were observed by FT-IR spectroscopy in ribonuclease A (Takeda *et al.*, 1995).

The denaturation of α-chymotrypsin by pressure was followed by the evolution of its enzymatic activity and its dependence with temperature, up to 500 MPa. The results in terms of volume increments were fitted to models for protein stabilization, which take into consideration the different volumetric contributions (Taniguchi and Suzuki, 1983).

The high-resolution proton NMR study of lysozyme pressure unfolding in different states reveals that the ΔV measured when different protons are employed as probes differ. This information will help us to understand the mechanism of pressure-

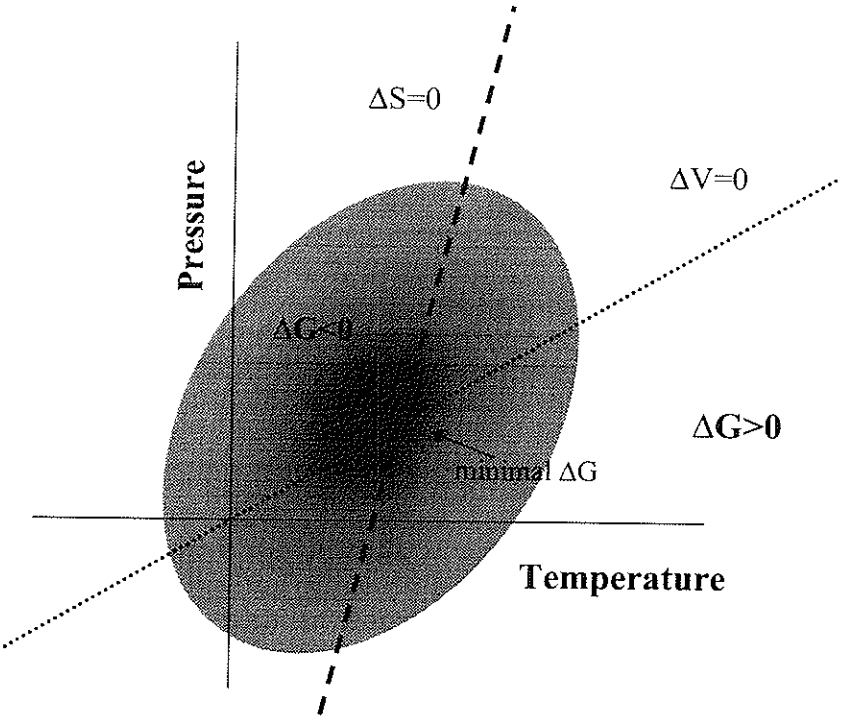

Figure 1.12. Cartoon showing the stability domain of a protein versus pressure and temperature.

denaturation (Samarasinghe *et al.*, 1992). The unfolding of specially compact proteins, such as the dimeric yeast prion Ure2, can employ the combined efforts of pressure and denaturing agents. The mechanism of unfolding appears to be similar to that induced by guanidine hydrochloride (but at higher concentrations). The unfolded state conserves part of its original structure (Zhou *et al.*, 2001).

Cold denaturation

The study of the dependence of ΔH and $T\Delta S$ for many proteins shows that both terms yielding ΔG are very similar in value. That is to say that the enthalpic and entropic contributions to protein stability almost cancel each other, and so protein stability is only marginal. ΔG is negative at lower temperatures (but always above 0°C) and reaches zero at the denaturation temperature. The shape of these curves allows the extrapolation at negative temperatures, predicting another zero of ΔG in that area (*Figure 1.12*). But this is difficult to corroborate in an experimental way, as water (or a dilute solution) freezes at 0°C (or a little below this temperature).

As has been mentioned above, incremented pressure can be employed to lower the water freezing point so that protein denaturation can be detected. As the pressures inducing a sufficient degree of freezing-point depression are relatively low (approx. 10 MPa for each 1°C of depression), normally pressure-induced denaturation does not interfere with cold denaturation. So, the high and low temperature denaturation

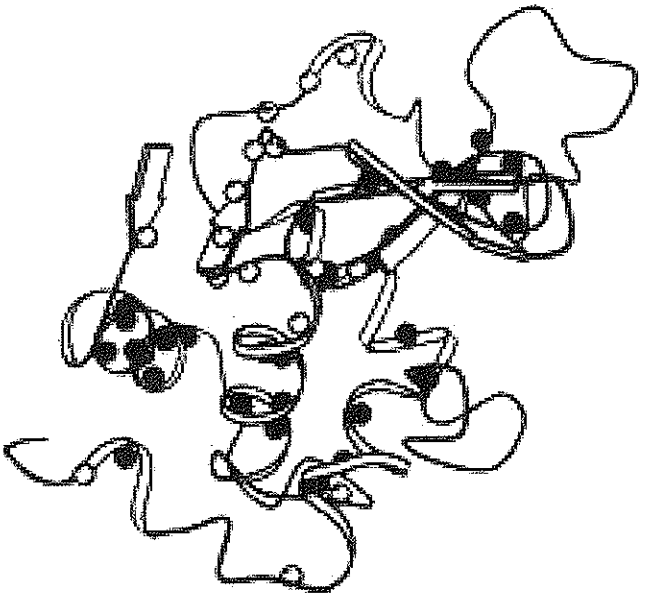

Figure 1.13. Drawing of the tertiary structure of lysozyme showing (as dots) the regions that are protected against proton interchange in the pressure-assisted cold denatured form (from Nash and Jonas, 1997, with permission).

processes have been compared for β–lactoglobulin, ribonuclease A and troponin C apo-regulatory domain (Griko and Kutyshenko, 1994; Zhang *et al.*, 1995; Tsuda *et al.*, 1999). The structural characteristics of cold-denatured proteins may differ from those of other types of process (Griko and Kutyshenko, 1994), and the mechanism of denaturation following dissociation of oligomeric structures presents similarities to pressure denaturation (King and Weber, 1986a,b). Both (cold- and pressure-induced) unfolded states retain part of the native structure (Zhang *et al.*, 1995). Lysozyme (pressure-assisted) cold denaturation has been followed by NMR. The exchange proton-deuteron in the amide position allows the generation of a map of protection of the different residues (*Figure 1.13*). The persistence of a protected area in cold-denatured lysozyme is evidence for the limited degree of unfolding (Nash and Jonas, 1997).

The pressure-induced cold denaturation of specific protein–DNA complexes points to a way in which specific and non-specific DNA sequences can be distinguished. The entropic contribution is seen to vary as the degree of apolar interaction changes (Foguel and Silva, 1994).

Molten globule

In the search for the determinants leading from sequence to protein secondary and tertiary structures, the study of folding intermediates has been central. Among them, a conformer was postulated that would preserve most of the secondary structure features, but the tertiary structure would be missing, its general conformation being less tight than for native proteins and much more fluctuating: this is the so-called

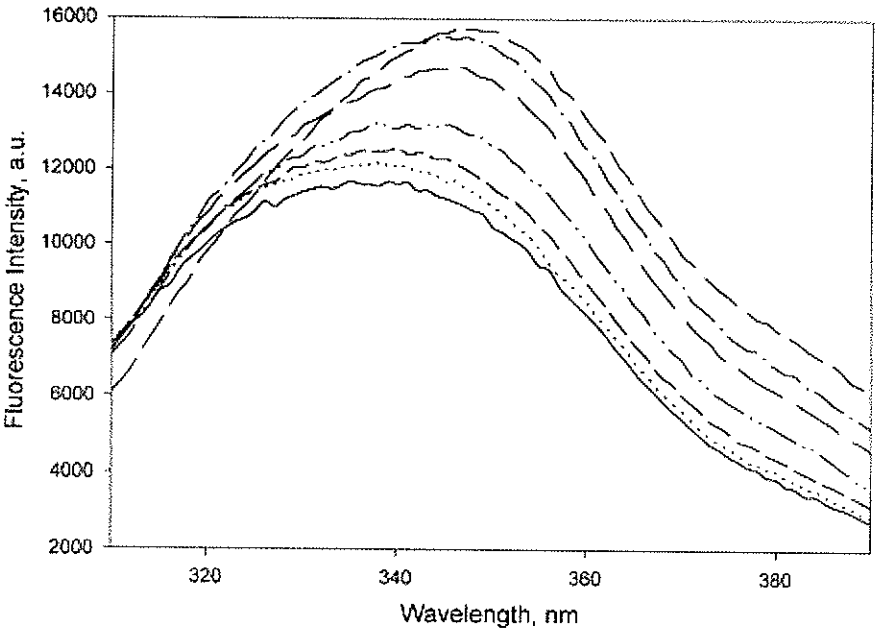

Figure 1.14. Dependence of the intrinsic fluorescence spectrum of native apomyoglobin (at pH 6.0 and 21°C) on pressure: 0.1 MPa (solid line); 15 MPa (dotted line); 60 MPa (small dashed line); 100 MPa (dash-double dotted line); 120 MPa (large dashed line); 140 MPa (dash-single dotted line); and 260 MPa (medium dashed line) (from Vidugiris and Royer, 1998, with permission).

molten globule (Peng *et al.*, 1995; Ptitsyn *et al.*, 1995; Masson and Cléry, 1996). Its significance as an indication of the driving forces of protein folding eclipses its concept as a mere instrumental intermediate for mathematical models. A decade after its postulation, the first of these structures was experimentally reported: as an intermediate, it is a naturally unstable structure and it is difficult to perturb protein structure so that a molten globule is formed for enough time to be detected by biophysical methods.

Pressure has been reported as a good way to induce the formation of stable molten globules. Its moderate effect over factors stabilizing preferentially tertiary structure motifs, and the stabilization by water interactions of the partially unfolded state, allows the persistence of this intermediate state at moderate pressures: 50–150 MPa. So, the structural properties of molten globules corresponding to diverse proteins and induced by incremented pressure have been reported. For example, apomyoglobin at 250 MPa (Vidugiris and Royer, 1998), whose tertiary structure gradual alteration can be followed by intrinsic fluorescence data (*Figure 1.14*), or Arc repressor monomers studied by high-pressure ^{1}H NMR spectroscopy at up to 500 MPa (Peng *et al.*, 1993, 1994). An important retention of secondary structure elements was detected, which was not found in chemically or thermally denatured proteins. NMR allows the identification of the residual elements of secondary structure persisting in the molten globule state. Fluorescence also can be employed to characterize the product of partial pressure unfolding of this protein as a molten globule (Foguel and Silva, 1994).

Small changes in protein structure and conformational drift

In many natural systems, pressure does not fluctuate as often as temperature. Consequently, the minimum of conformational potential energy selected by evolution has not been optimized towards pressure changes. So, a number of close, local, free energy minimums can be found, and relatively small variations in pressure will distort the native fold in a fully reversible way, even preserving most of the tertiary structural elements (not to say the secondary ones) and the functional capacities, though, may be altered. For example, the structure of apomyoglobin is affected by pressure, as followed by fluorescence techniques, yielding two relatively stable, conformationally different forms (Bismuto *et al.*, 1996). Enzymes such as cytochrome P-450_{cam} exist in a very sensitive equilibrium between the forms involved in the catalytic process. Low levels of pressure (Primo *et al.*, 1995) easily displace this equilibrium, without further structural changes being originated.

Some proteins are stabilized by interactions with other molecules or monomers to yield an oligomer. Once they have been dissociated (by increased pressure or by dilution), the more distant domains and chains are not anchored in their positions by so many intermolecular bonds. So, the potential barrier between many different sub-states is reduced and the initial structure slowly migrates to other sub-states. This is, sometimes, a very slow process, taking hours or even days (Ferreira and De Felice, 2001). This process, termed 'conformational drift', has been observed after pressure-induced dissociation for lactate dehydrogenase (King and Weber, 1986a,b), tryptophan synthase (Silva *et al.*, 1986) and glyceraldehyde phosphate dehydrogenase (Ruan and Weber, 1989). The general picture is that of the existence of a variety of close, conformational states whose distribution is controlled by factors such as protein concentration, temperature, pressure, and specific or non-specific ligands.

The chaperonin cpn60 (GroEL) forms a tetradecamer (see Walters *et al.*, 2000) that can be dissociated by pressure of around 175 MPa. The binding of ligands, such as MgATP, increase its pressure susceptibility. The dissociated monomers differ in properties from the urea-denatured products and re-associate in a slow way, which is considered a conformational drift case (Gorovits *et al.*, 1995). The extension of this drift has been related to the maximum pressure attained in experiments on allophycocyanin dissociation (Foguel and Weber, 1995). Weber (1986) has attempted a detailed explanation of this behaviour, according to several models.

A related (and possibly complementary) observation reports both elastic and inelastic pressure-induced perturbations in the structure of alcohol dehydrogenase and alkaline phosphatase dimeric proteins. Reversible alterations take place at lower pressures (150 MPa) than those causing dimer dissociation (Cioni and Strambini, 1996). A similar observation has been reported for creatine kinase, studied by stopped flow up to 200 MPa and fluorescence up to 600 MPa. Zones of the hydrophobic protein core become accessible to probes at pressures around 300 MPa, which are insufficient to separate the monomers, presumably strongly hydrogen-bound (Zhou *et al.*, 2000). Another protein suffering denaturation prior to dissociation (but with the help of denaturing agents) is the Ure2 prion (Zhou *et al.*, 2001). The study of the unfolding mechanism does not detect monomers or intermediate states.

Effects on protein ligand bonding

Structural alterations in proteins are reflected in all their functions, such as their ligand bonding behaviour. This is a very wide phenomenon, related to enzymatic activity, allosteric control, transport, signalling, etc. Haem proteins bind a number of ligands with the roles of transported molecules and/or allosteric effectors. The protein environment has a strong influence in the haem-binding behaviour (Frauenfelder *et al.*, 1990; Jonas and Jonas, 1994; Lange *et al.*, 1994). This influence has been studied with the help of pressure perturbations. The $\Delta V^{\#}$ of O_2 binding appears especially sensitive to the amino acidic distribution in the ligand path. Also, the electron transfer process has been shown to be sensitive to solvation effects, when studied in several haemoproteins by pressure-stopped-flow (Heiber-Langer *et al.*, 1992). However, the CO-bound form of several haemoproteins appears to show little difference compared with the unbound state.

Hydrostatic pressure has also been employed as a probe for the study of protein-model ligand interactions: the temperature and pH dependence of fluorescein binding to the active site of an antibody has been followed upon pressure-perturbation (Carrero and Voss, 1996).

Pressure activation of enzymes

Pressure can enhance thermal stability and/or enzymatic activity for given proteins. This is the case for α-chymotrypsin (Mozhaev *et al.*, 1996), where activity multiplies by a factor of 6.5 at 450 MPa. As the protein activation volume grows with temperature, an increase in both pressure and temperature has a synergetic effect over activity (more than 30-fold). Pectinesterase is also a pressure-activated enzyme. Its thermal denaturation is retarded by pressure and its maximum activity can be found at 200–300 MPa, in regions of temperature which cause its denaturation at atmospheric pressure (Van den Broeck *et al.*, 2000).

Butyrylcholinesterase becomes irreversibly denatured by a combined pressure (100–400 MPa) and temperature (50–65°C) treatment in a multi-step process (Weingand-Ziade *et al.*, 2001). The intermediate state possesses enhanced catalytic efficiency as a result of rearrangements in the active site or in the water network surrounding it. After return to standard conditions, the denaturation process continues by evolution of the formed intermediate states.

Simulation of pressure effects on proteins

The effect of pressure on protein conformation can be simulated by techniques of molecular dynamics that take into consideration a number of interactions with the solvent for a limited (but considered significant) time of evolution. Bovine pancreatic trypsin inhibitor (BPTI) and other proteins have been studied in this way (Kitchen *et al.*, 1992; Brunne and Van Gunsteren, 1993; Van Gunsteren *et al.*, 1995). BPTI shows a small degree of perturbation in simulation experiments to 500 MPa. A denser protein packing and the reduced mobility of side chains are the more noticeable effects. This peptide may be too small (60 amino acids) for effects of 500 MPa to be noticed. Studies simulating short times at higher pressures (1000 MPa), in good

agreement with practical observation, show no protein denaturation. The effects detected include those resulting from the compressibility of protein (its radius decreases by 2%) and those compressibilities of the different water populations.

PROTEIN AGGREGATION

Protein aggregation usually implies an important increase in specific volume if the contribution of the change in ordering the degree of the surrounding solvation water is considered (see *Table 1.1*). This increment has been estimated as 10–20 cm^3/mol per bond (Harrington and Kegeles, 1973). As the factor to be considered is molar volume, and the aggregates have a very large molecular weight, the total volume change becomes significant. A consequence is that the rupture of protein aggregates takes place at pressures significantly lower than the denaturation of monomers, often between 100 and 500 MPa. But pressure denaturation is also the cause of aggregation phenomena. Several oligomerizing proteins have been studied in terms of pressure dissociation, observed through different techniques. The Arc repressor, by NMR (Peng *et al.*, 1994); recombinant human IFN-γ, by co-solute studies (Webb *et al.*, 2001); haemoglobin probed by fluorescence (Pin *et al.*, 1990), are three examples. The studies of lysozyme crystallization show how pressures up to 200 MPa (Gross and Jaenicke, 1991) can inhibit the aggregation process to form crystals. As for enolase dimers, they dissociate upon 100–300 MPa pressure application, in a largely reversible way (Paladini and Weber, 1981b).

Pressure denaturation often ends with the aggregation of the unfolded monomers, even when these may be only partially destabilized. Myoglobin denatures at elevated pressures (pressurized in a diamond anvil cell) and, on returning to atmospheric conditions, forms aggregates which are similar to those thermally induced. Combinations of pressure and temperature perturbations give rise to aggregates of different stability (Smeller *et al.*, 1999). Extracellular haemoglobin is also capable of forming irreversible aggregates after extreme degrees of pressure dissociation (Silva *et al.*, 1989). In heterogeneous protein aggregates, some of the monomers can depend on the others for stabilization. This can be a suitable strategy for sequential folding of a protein complex (the first synthesized monomer stabilizes the rest) or for control (if one element is missing, the whole activity would be lost). So, the α-subunits act over the high-pressure stability of (apo and holo) β_2-subunits of the di-enzyme complex tryptophan synthase from *Escherichia coli* (Sindern *et al.*, 1995).

The presence of ligands modifies the association equilibrium of proteins, and so its dependence with pressure. Also, the binding of such ligands to specific positions in the protein molecule can help us to follow the dissociation process, for example by monitoring the hydrophobic environment of a probe (Erijman *et al.*, 1993).

NUCLEIC ACIDS AND PRESSURE

The number of studies on the behaviour of nucleic acids under pressure has been relatively small, despite the interest in understanding the role of macromolecular hydration in all kinds of structural and functional phenomena (MacGregor, 1998). Nucleic acids are generally less sensitive to hydrostatic pressure changes. This is due to the smaller volume change associated with their conformational transitions: nucleic acids do not

form, like proteins, a globular structure with deep buried areas where voids are generally present. Also, the ionization state remains constant in conformational changes. Anyhow, both changes in their structures and in the interaction with protein systems have been found. Under most conditions, the helical structures formed by DNA or RNA are stabilized by pressure, but the effect is small (MacGregor, 1998). Pressure increases induce significant alterations on the tertiary structure of supercoiled DNA (Tang *et al.*, 1998). Meanwhile, the helix-coil transition temperature for DNA oligomers appears to undergo an increase with higher pressure of 0.7–3.2°C/MPa^{-1}, and is a function of chain length (MacGregor, 1996). Model DNA molecules (poly[d(G–C)]) show pressure dependence of their helix-coil transition. The ΔV for this transition varies with the concentration of sodium ions. The difference in volumes between the two states is justified by the release of counter-ions upon structural reorganization to yield the coil form (Wu and MacGregor, 1995).

Kryżaniak *et al.* (1991) reported pressure-induced reversible B–Z transitions in DNA. The same effect is achieved by increased ionic strength or DNA chemical modification. The high pressure applied (1000 MPa) can have an additional influence on the dynamics of the DNA molecule by restricting its movement freedom.

Protein–nucleic acid interactions often obey a characteristic sequence specificity. This specificity is related to a balance of relatively weak interactions, which includes the role of solvation water: it is reasonable to expect that such weak interaction will be susceptible to pressure perturbations. The endonuclease enzyme *Eco*RI binds and cuts DNA molecules at sequence-specific sites. This specificity has been shown to depend on water-bound molecules and not other water-related effects, such as viscosity, dielectric constant, or water concentration. These molecules are released by increased ionic strength, while hydrostatic pressure, reversing this effect, allows the recovery of specificity (Robinson and Sligar, 1994). The specificity of the interaction of DNA and a human papillomavirus protein has also been studied using pressure changes as a tool and observed by intrinsic fluorescence. This enzyme becomes dissociated into monomers by pressure, and DNA protects from this pressure dissociation. While both specific and unspecific DNA confer protection against the pressure effect (the ΔV of association is equivalent), the ΔG for dissociation increases when the ligand presents a specific palindromic sequence (Lima *et al.*, 2000). On the other hand, the pressure sensitivity of DNA–protein complex has been seen to dramatically depend on its specificity (Foguel and Silva, 1994).

CARBOHYDRATES: STARCH

The effects of hydrostatic pressure on polymeric carbohydrates can be expected to follow the same general rules observed for other macromolecules. Temperature–pressure phase diagrams for starch gelatinization, for example, seem to mirror the effects of studies on the unfolding of proteins (Douzals *et al.*, 2001). We will consider this further in the 'Food section' below.

EFFECT OF PRESSURE ON MACROMOLECULAR ASSEMBLIES

As has been mentioned, larger aggregates, bound through weaker interactions, are more sensitive to hydrostatic pressure. The information that pressure-perturbation

studies can provide on these systems is very rich, and a large number of articles have been published on several of these systems. We focus on just a few here.

Viruses

Viral particles are assemblies of a diverse degree of complexity, composed mainly of highly organized protein subunits and nucleic acid molecules, and enriched with a number of functional properties. A system so described can be expected to be sensitive to alterations in physical conditions, especially when its main functional activity is self-disintegration once it has allowed the viral genome to make its way inside the host cell. So, perhaps unsurprisingly, a clear effect of hydrostatic pressure on simple virions has been demonstrated. Bacteriophage R17 is a T3 icosahedral capsid RNA virus. The capsomers for $T > 1$ have been shown to follow the principle of 'quasi-equivalence' (Rossmann and Johnson, 1989), so that their structures must be slightly distorted to fit in the icosahedral capsid. This implies an additional degree of flexibility. Pressures of 250 MPa promote completely reversible disassembly of this particle (Da Poian *et al.*, 1993). Reversibility of a viral particle assembly and disassembly is not unexpected as particles are also formed *de novo* inside infected cells. The synergy of urea and pressure perturbations changes the dissociation characteristics.

The coat protein of bacteriophage P22 is pressure destabilized at 50 MPa. Structural changes lead to protein denaturation, yielding a partially unfolded end-state. The assembly into the icosahedral shell has the effect of conferring on the protein additional pressure resistance up to 250 MPa. In these conditions, virions become cold labile. This effect can be related to conformational drift as the monomer stability suffers upon dissociation, but it is a rare example of a protein undergoing denaturation at lower pressures than for dissociation. Important conclusions about the viral assembly and disassembly mechanisms can be drawn (Prevelidge *et al.*, 1994). Another relatively simple viral particle, brome mosaic virus, has been pressure dissociated at moderate intensities: 60–160 MPa (Silva and Weber, 1988). Smaller, empty capsids are formed in the dissociation process. The pressure sensitivity is modulated by ions and pH and can give rise to conformational drift in the monomers, which can aggregate in an unspecific way.

The particles of another plant virus, namely cowpea severe mosaic virus, disassemble reversibly at 250 MPa but, while fluorescence and hydrodynamics show dissociation, the ribonuclease digestion protection conferred to RNA implies that the protein–nucleic acid interaction has not been broken. Reduction in temperature to −10°C produces irreversible disassembly and RNA digestion. The coat protein has been found to exist in at least four stable conformational states (Gaspar *et al.*, 1997). The larger and relatively more complex tobacco mosaic virus can also be disassembled by the combined use of pressure and low temperature. At moderate (250 MPa) pressures, no denaturation of capsomers took place. When the effects of urea and pressure were tested, a higher degree of dissociation was found and the same pressure level induced monomer denaturation (Bonaface *et al.*, 1998).

Bursal disease virus, an animal pathogen, was pressure dissociated at 240 MPa and 0°C. The immunogenic capacity was found to be maintained, or even enhanced (Tian *et al.*, 2000). Other lipid bilayer-enveloped and much more complex viruses, such as

simian immunodeficiency virus and vesicular stomatitis virus, have been inactivated by hydrostatic pressure (Silva *et al.*, 1992; Jurkiewicz *et al.*, 1995). Dissociation did not involve complete monomer migration, due to the membrane holding together the loose elements, but morphological changes were observed that coincided with loss of infectivity. The 'conservative' conformational alterations caused by hydrostatic pressure on the virion structure have been considered as a suitable method to yield inactivated viruses for its use as vaccines, even with enhanced immunogenic activity, due to the higher degree of chain exposition and accessibility to antibodies of pressure-unfolded proteins (Silva *et al.*, 1992; Tian *et al.*, 2000).

Ribosomes

Ribosomes are fairly conserved structures comprising several different proteins and nucleic acid chains. Their function comprises two delicate tasks: enzymatic activity for protein synthesis and recognition of the different nucleic acids implied and their control signals. Hydrostatic pressure up to 100 MPa induces protein subunit dissociation when followed by light scattering (Infante and Baierlein, 1971; Infante and Krauss, 1971; Gross and Jaenicke, 1990). Though the binding of other components of *E. coli* ribosomal system, such as t-RNA and spermidine, fails to stabilize the assembly, its different conformational states show different susceptibilities to pressure.

The microtubular system

Aspects of cellular physiology as diverse as the maintenance of shape differentiation, intra-cellular transport and the mechanical control of membrane receptors and cell division, are all mediated by microtubules. This extremely dynamic and delicate structure is formed by the polymerization of tubulin, a single protein (with two subunits) to yield long, hollow tubes that grow on one end and shrink on the other. Their organization, movement, control, and specificity is facilitated by a number of other minor proteins. The flexibility of this assembly is, again, a source for its sensitivity to relatively mild perturbations. In this way, modest pressure increases (20–40 MPa) induce microtubule dissociation, both *in vitro* (isolated microtubules) and *in vivo* (cellular mitotic spindle) (Salmon, 1975; Robinson and Engelborghs, 1982). Microtubule stabilizers, both natural (MAPs) and extrinsic (dimethyl sulphoxide), have been found to increase their resistance to dissociation by pressure.

A large number of regular tubulin aggregates has been obtained. Some of them have biological significance, some are laboratory generated, but they are always useful for investigating microtubule behaviour. 30S tubulin rings were depolymerized by the pressure generated in an analytical ultracentrifuge (under 18 MPa) in which an oil column had been employed to create additional pressure (Marcum and Borisy, 1978). The entire microtubular system in cells is susceptible to the effect of tenths of MPa. The most evident effect is the change in the cell shape (loss of differentiation, rounding, recession of protrusions). Both direct effects on tubulin polymerization and indirect actions through regulatory pathways can be considered (Crenshaw *et al.*, 1996).

LIPID BILAYERS

The effect of hydrostatic pressure on functional and structural aspects of lipid bilayers

has been investigated, both by means of pressurized vessels and of diamond anvils. As a consequence, the range of pressures examined has been extensive, and reaching examples up to 2000 MPa in a study by Reis *et al.* (1996), and up to 2500 MPa by Auger *et al.* (1990). Fourier transform infrared spectroscopy (FT-IR), NMR, and extrinsic fluorescence have been the favourite observation techniques. Effects on the self-association behaviour, binding and functionality of membrane-associated proteins have been studied, as well as those effects taking place in the actual lipid bilayer. A general observation for the latter case has been the pressure-induced increase in the order of lipid molecules, especially in the vicinity of protein molecules, a phenomenon driven, as usual, by the smaller volume associated with a more ordered, tighter packing (Hubner *et al.*, 1990; Peng and Jonas, 1992; Reyes Mateo *et al.*, 1993; Teng and Scarlata, 1993; Barshtein *et al.*, 1997). In this way, changes in the intermolecular distances and dimensions of bilayers have been reported, indicating a reduction of the separation between lipid chains and an increase in membrane thickness. These alterations resulted in a total volume decrease (Braganza and Worcester, 1986; Hubner *et al.*, 1990).

A complex series of phase changes has been described in pure or mixed lipid bilayers, involving fluid liquid crystal states and more ordered and static gel phases. Access to the more mobile phases is given by the increase in temperature, which augments the kinetic energy of the lipid molecules, or by the addition of drugs that, intercalating themselves inside the lipid phase, have a general effect of decreasing the degree of order and impairing the more perfect lipid head group and chain packing that is possible for the mono-component case. Hydrostatic pressure tends to impair the transition to gel states, having the opposite effect to that of temperature (Reyes Mateo *et al.*, 1993; Jonas and Jonas, 1994), or to the insertion of drugs, such as cholesterol (Reis *et al.*, 1996; Bernsdorff *et al.*, 1997). It has been found that, in the same cellular system, those membranes richer in cholesterol (cellular) resist higher pressures, without acquiring rigidity, than those poorer in this molecule (from endoplasmic reticulum) (Mentre *et al.*, 1999). Regulation of the cholesterol content is considered as a potential mechanism for the adaptation of membrane behaviour towards high-pressure conditions, in a similar way to its role towards temperature adaptation.

The formation of inter-digitated phases is favoured by pressure (Peng and Jonas, 1992; Jonas and Jonas, 1994). The effect of pressure can be significant, as in the case of DMPC and DPPC unilamellar vesicles, where 500 MPa was sufficient to increase the phase transition temperature by 11.5°C (Reyes Mateo *et al.*, 1993). Other effects observed in human red blood cell membranes have been demonstrated at mild conditions, such as a pressure of only 1.5 MPa (Barshtein *et al.*, 1997).

Among the substances that can become intercalated in the bilayer, the case of anaesthetics is of considerable relevance. Pressure has been a fundamental tool in understanding the mechanism of anaesthetics: anaesthetic power appears to be reversed by hydrostatic pressure (Auger *et al.*, 1990). The action of many anaesthetics and similar substances, such as lidocaine, tetracaine, ethanol, etc., has been studied under high pressures (Kamaya *et al.*, 1979; Tonner *et al.*, 1997). The mechanism involves expulsion of the anaesthetic from the bilayer to the aqueous phase, and takes place at different pressures which characterize the interaction between lipids and anaesthetic molecules (Auger *et al.*, 1990).

It goes without saying that membrane proteins are of enormous physiological relevance, ranging from chlorophilic function and mitochondrial respiration to nervous transmission and signal recognition. Pressure effects on them can be similar to those generally considered for other protein types and, due to their special situation of insertion, to those in apolar surroundings. The effect of pressures up to 200 MPa on protein association with membranes has received considerable attention. Melittin association with membranes of different composition appears to be relatively unaffected, reflecting similar compressibilities of lipid and protein phases (Teng and Scarlata, 1993). Meanwhile, in the same conditions, haemoglobin is released from human red blood cell membranes (Yamaguchi *et al.*, 1993). (Na, K)-ATPase shows reversible inhibition below 150 and 250 MPa, depending on the species. Further pressurization causes irreversible effects. Decrease in membrane fluidity is involved in this membrane protein behaviour (Chon *et al.*, 1985). Other ATPases are irreversibly inactivated, even at the low pressures achieved by ultracentrifugation, and factors such as ions, pH, and other solutes modulate the process (Esman *et al.*, 2000).

Food systems

High hydrostatic pressure constitutes a new technological approach to food processing with many applications for food safety, texture control, and enhanced quality, particularly in its connection with other processes (Knorr, 1993). Food processing can make use of hydrostatic pressure to find new and better production methods. The areas of current interest include enzyme activity modulation, microorganism deactivation, texture modification, and general improvements in quality and nutritional properties of food. Often, hydrostatic pressure processing is compared, both on economic efficiency and process performance, with thermal treatments. A number of advantages for pressure treatments have been described.

Both the high cost of equipment and technical difficulties have impaired and discouraged the earlier practical application of pressure treatments in the food industry (see, for example, Hayashi, 1996). Recently, two factors have increased the interest for high pressures: the technological advances allowing a significant reduction in the production costs of pressure equipment and the current consumer's trend for minimally processed food, favouring physical over chemical treatments.

A reduced energetic expense has also been named among the benefits of pressure treatment over other methods. The 'ideal' energy involved in the process (W) (i.e. ignoring to a first approximation the consideration of friction or other constraints imposed by the equipment) and for a given constant temperature, can be calculated as the product of the strength (f) exerted by the displacing piston (liquid jet or other way to increase pressure) and its displacement (l). This can be related to pressure and the decrease of volume, which, as the compressibility factor changes with pressure, must be integrated between the two extreme pressure values:

$$W = f\,l; \quad f = P\,s; \quad l = \Delta V/s; \quad W = P\,\Delta V$$

$$W = \int_{P_1}^{P_2} \beta\, V\, dP \tag{1.11}$$

Equation 1.11 is an over-simplification of reality as this compression energy (after *Equation 1.9*) is transformed into heat, and so the temperature variations affecting β will also have to be considered and integrated. As a rough approximation, and for water (larger volume changes are associated to other food components such as fat), a pressure increase of 500 MPa will only cause a ΔV of around 15%, and an energy expense of 7.5×10^7 J/m^3. A 100°C thermal treatment from a start point at 25°C will require four times more energy.

Additional advantages are the reduction in pressure losses, once the target has been achieved, so that no additional energy expense is needed to keep a sample under pressure indefinitely. Meanwhile, thermal treatments always need a constant energy supply to compensate for the heat dissipated to the surroundings. The speed of pressure rise or release is also relevant. To pressurize a large volume vessel can take between 1 and 3 minutes, while to heat a large mass to boiling temperature takes a much longer time and the involvement of complex heat diffusion engineering. To release pressure is even faster: a few seconds, while to cool down industrial volumes is a difficult task. If a short, high intensity treatment is required – perhaps because longer treatments at intermediate intensities are deleterious to some sample properties – pressure can be much easier to apply than heat.

The instantaneous transmission of pressure is also worth mentioning: at any particular moment, the value of pressure (allowing for very minimal fluctuations) will be the same in the entire vessel content. Thermal treatments generate gradients that are often problematic: to heat a large mass by thermal exchange with a heater element set at the target temperature is very slow as heat diffusion depends on the thermal difference. And if a larger temperature than the target is employed, the sample regions next to the heater will become over-heated, which can produce unwanted effects. Treatment parameters will always be heterogeneous. Heated liquid samples can have this problem minimized by stirring, or the use of capillary heating, at the cost of increased energetic expense. But, for solid food samples, there is no easy solution.

Substances of relatively low molecular weight confer many of the interesting characteristics of food. Taste, flavour, colour and nutritional characteristics are often due to simple substances such as salts and mono- or dimeric sugars, vitamins and amino acids. The absence of deleterious effects on small molecules and on these organoleptic and nutritional properties of food can be regarded as among the advantages ascribed to hydrostatic pressure over other treatments, especially the thermal ones (Knorr, 1999). For the same reason, this technology would not lead to the generation of new and potentially harmful substances. On the other hand, most pressure effects take place on high molecular weight species, and here more careful consideration of the effects of pressure are required, as is evident in the earlier parts of this review.

High pressure can cause two main types of alteration in foods: those related to protein functionality and those to the food system microstructural characteristics. The main interest of protein pressure-denaturation in food treatments is, very likely, the selective inhibition of certain enzymes whilst allowing a proper function of others. As in most foods, the concentration of protein and other substances is very high: this favours all kinds of aggregation phenomena. On the other hand, the increased viscosity perhaps impairs such phenomena. Specific and unspecific macromolecular associations mainly have a structural role in this context. Specific protein aggregates

(alone, or with other components such as fat) and storage polysaccharides form structures as important as milk casein micelles and fat globules, myofibrils, and starch granules. Depending on the particular case, the main focus of attention will be to conserve or eliminate these interactions. Other associations are created during food processing through (mainly unspecific) aggregations of denatured proteins and hydrated polysaccharides. These man-made associations can be 'wanted', as in the case of protein or starch gels, increasing the textural properties of food, or 'unwanted', for example, if they produce the precipitation of a soluble protein.

STRUCTURAL MODIFICATIONS

Starch

Among the food-related and biological-interest polysaccharides, the most studied in any context is starch. This chemically simple polymer, a mixture in different proportions, depending on the biological origin, of branched and linear glucose chains, possesses a very complex structural organization that conceals the clues for the management of the energetic resources by that part of the plant for which it provides storage. The main implications, from the food point of view, are related to food-energy aspects. Most of this energy provided by food comes in the form of starch. There are two aspects to its use: nutritive needs of consumers depend on its easy assimilation and, at the same time, the growing interest in low-energy food represents an opposite interest – its reduced bioavailability. Starch is hardly digestible in its native form, and an irreversible hydration process that involves major changes in starch structure, called *gelatinization*, is needed before assimilation. Thermal treatments are usually employed for this purpose. Another way to use the energy trapped in the form of starch is to wait for (or to induce) the starch-producing plant to degrade the starch by its natural metabolic mechanisms, to lower molecular weight sugars. That is essentially what we all do when we wait for fruits and vegetables to ripen.

The other role of starch in the food realm has to do with structure. Gelatinized starch forms a spatial network that links other food components and retains large quantities of water. Starch is related to the characteristics of food structure, and efforts, including chemical, enzymatic and genetic manipulations, have been made to modulate its structural powers. Additionally, the polysaccharide chains suffer a process of recrystallization called *retrogradation* that, favoured by time and low temperature, generates unwanted structural characteristics.

The effects of hydrostatic pressure on starch have many parallels to those on proteins with regards to the tendency to saturate all the possible water-binding sites with aqueous molecules. So, the many voids generated in the spinning of the starch granules' large and complex structure are filled with water, and interactions with the groups capable of hydrogen bonding are created. This results in the gelatinization process taking place at reduced temperatures. The use of an expensive technology for a process that is easy to carry out by simple thermal treatments may, however, be considered as not economically interesting. But, interest may increase when such a thermal treatment is not desirable because of other deleterious effects on the properties of the whole food. Additionally, to gelatinize a large quantity of starch will require

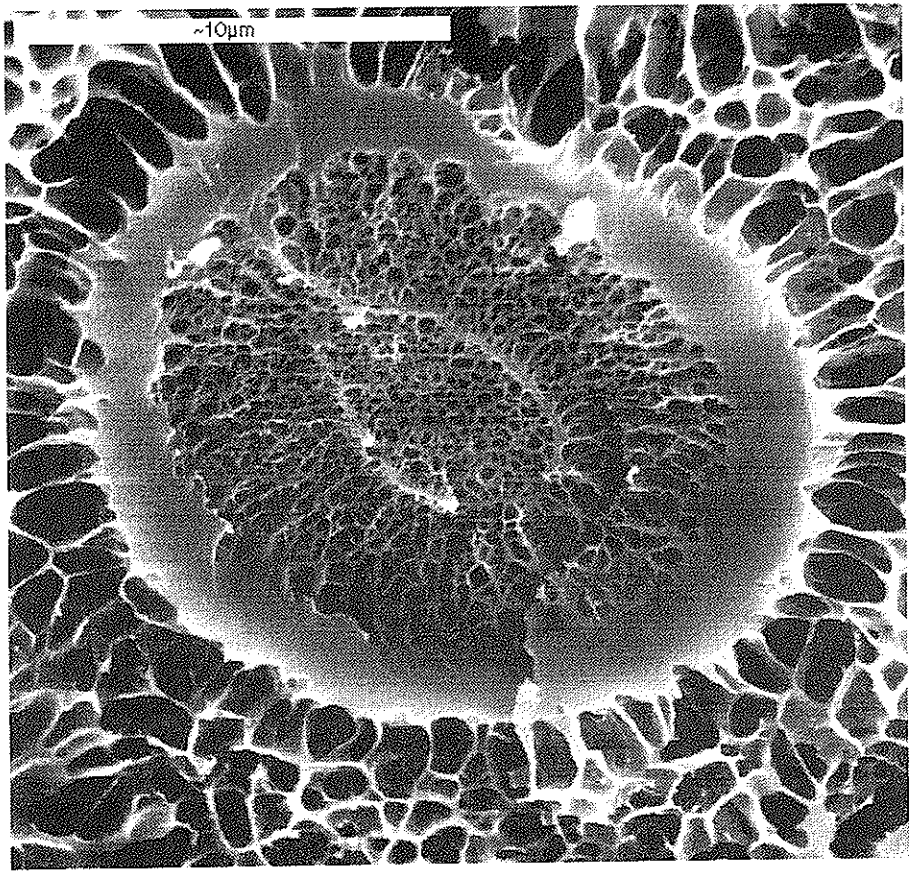

Figure 1.15. Cryo-electron scanning micrograph of an acetylated tapioca starch granule partially gelatinized after treatment for 30 min at 400 MPa and 20°C. The bar corresponds to 10 µm.

large quantities of energy and considerable time. Meanwhile, to do it by means of pressure can take place at a lower input of energy and as quickly as desired, provided that pressure is sufficiently high.

Starch, depending on its biological origin and/or chemical modifications, has different susceptibilities to pressure-gelatinization. *Figure 1.15* shows a tapioca starch granule partially gelatinized by 400 MPa pressure. In spite of this pressure-induced gelatinization process, the shift of thermal-gelatinization temperatures towards higher values by pressure up to 150 MPa has been reported (Thevelein *et al.*, 1981).

A special interest for starch gelatinization is related to its use as substrate for enzymatic treatment. Pressure (500 MPa)-treated starch has been reported to possess increased digestibility by amylase. The exposure of suitable enzymatic attack points has been inferred, by analogy with protein behaviour (Hayashi and Hayashida, 1989). The susceptibility of amyloglucosidase is different for pressure gels than for the thermal ones. In a similar way to protein pressure-unfolding, pressure-gelatinized starch might be different, in its hydration degree, from the temperature-gelatinized

form (Selmi *et al.*, 2000; Douzals *et al.*, 2001). Also, rheological differences between the products resulting from both types of gelatinization have been reported. Again, following the protein model, the granular structure was better conserved when compared with heat treatment (Stolt *et al.*, 2001). Other differences in the process can arise from regular or irregular ruptures in the polysaccharide chains, or effects involving minor starch components, such as lipids. The inter-relations between starch gelatinization, water activity and the presence of other substances is an interesting field, open for most promising research.

Other structural macromolecules

Many foods rely on other polysaccharides, such as agar, xanthan, guar gum, etc., for generating or maintaining a given structure. Protein gels also possess these properties and, in addition to giving 'texture' to food, contribute to water and nutrient retention. Protein gels can be enhanced, generated, or even dissociated by pressure treatments, and some of the studies that have been carried out with the aim of using pressure in an industrial context consider their properties. Among them, the gelation of β-lactoglobulin has been one of the more visited topics, due to the high added value of this dairy industry sub-product.

β-lactoglobulin dimers can be dissociated and denatured by the effect of several perturbing agents, such as guanidine hydrochloride, temperature (both high and low) and pressure (see, for example, Griko and Kutyshenko, 1994). The recovery of native state properties can be followed easily when pressure is the perturbing agent, as depressurization is a very fast process. A slow hysteresis type of renaturation has been observed, and recovery of the native form is never complete, due to the formation of non-native disulphide bonds. The association behaviour appears not to depend on protein concentration and is favoured by cold temperatures (Valente-Mesquita *et al.*, 1998). This slow recovery process eases the formation of gels once the atmospheric conditions have been recovered.

CELL WALL

The cellular structure of foods such as fruits and vegetables can be diversely affected by pressure, as a number of different effects (maybe even opposite) will take place. The permeability and compartmentalization of these tissue types is generally lost, because the different compressibilities deform the cellular network, which is especially fragile at elevated pressures. Any air-filled voids, frequent in seeds, fruits, etc., will collapse. Cellular vacuoles are also susceptible to volume contraction. A secondary effect will be the migration of solutes and enzymes from the different compartmentalized regions (Butz and Tauscher, 1998). This migration may give rise to exudates that cause losses in water, food weight and nutrients; meanwhile, dehydration has deleterious effects in the structure of cells, which tend to shrink. Enzymatic activities take place once the enclosed enzymes reach regions with suitable substrates, which may be at different ionic strength and pH conditions. Proteolysis and self-digestion take place if those enzymes responsible have not become inactivated during the pressure treatment.

FREEZING

'Pressure-assisted freezing' is a general term for a serial of methodologies that take advantage of the anomalous phase diagram of water to modify the properties of freezing processes. The possibility of having water in the liquid state at sub-zero temperatures is used in different ways to increase the preservation capacity, to improve the efficiency of the process, and to reduce the damage to tissues caused by ice crystals. For a review on the possible uses of these technologies, see Kalichevsky *et al.* (1995). These uses include acceleration of thermal exchange and freezing and control of the kinetics of the freezing process by the generation of a high degree of supercooling (quickly expanding a water-based sample held at 210 MPa and −20°C in what is called 'pressure-shift freezing') and so a massive and uniform ice nucleation (e.g. Le Bail *et al.*, 1997; Sanz *et al.*, 1997). Other related methodologies make use of high pressure to maintain samples at temperatures between 0° and −20°C, without ice formation.

Though the main developments have been carried out in the realm of food science, other fields are also taking advantage of these processes, especially, as we have just mentioned, pressure-shift freezing. For example, pressure-shift is currently applied in the preparation of samples for electron microscopy to accelerate freezing and reduce the damage caused by ice crystals. This damage reduction is of special interest for living cell preservation. Experiments are under way to study the effects of these technologies in the transplant of human tissues and plant seed cryopreservation at the author's laboratory. The advantageous use of the multiple forms of ice and the transitions between them can be employed to increase the specific volume differences (in order to damage unwanted microorganisms) or to minimize them (to reduce the deterioration of food or other biological origin tissues). 'Ice VI freezing' is currently being studied as an ice phase that does not cause distortions in the tissue structure, because the ice VI freezing volume increment is negative and, additionally, it can exist at room temperature. The effect of exploring different ice forms (all but ice I with negative volume increment) has been generally positive for the maintenance of the structural properties of foods (Fuchigami *et al.*, 1997).

Another fruitful topic that takes advantage of the reduction in the size of ice crystals is the production of ice slurries for its industrial use as a secondary refrigeration media. Mixtures of ice and water are circulated between the primary refrigeration system and the element to be cooled. Their heat exchange and fluidity properties depend on the size of the ice crystals originally formed. The author's laboratory is involved in the application of pressure technology for this novel application.

The efficient use of pressure and low temperature requires careful study on the behaviour of thermal diffusion and ice formation. A number of researchers have modelled this process (Denys *et al.*, 1997; Otero and Sanz, 2000; Sanz and Otero, 2000) as a way of controlling this process. The texture of food samples that have been pressure-shift frozen has been studied, both for vegetables such as eggplant, peach and mango (Otero *et al.*, 1998, 2000a; Sanz *et al.*, 1998, 1999b) and meat (Otero *et al.*, 1997; Martino *et al.*, 1998; Sanz *et al.*, 1999a; Fernández-Martín *et al.*, 2000). Whilst vegetable commodities have, after thawing, shown better textural and water retention characteristics than ordinary frozen samples – as reflected in the preservation of the cellular organization (*Figure 1.16*) – by contrast, meat structure is highly distorted by

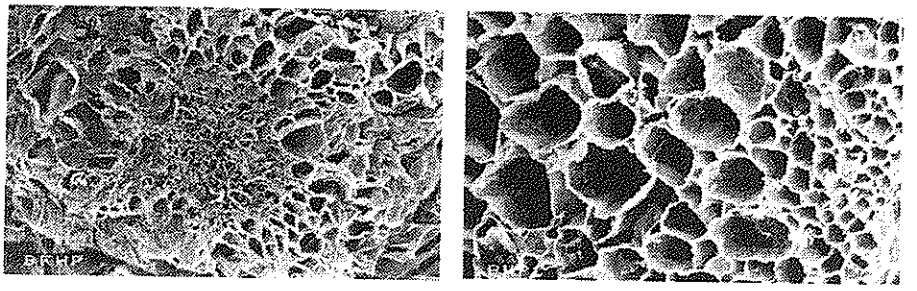

Figure 1.16. Scanning electron micrographs of eggplant tissues. Left: untreated. Right: pressure-shift frozen. The tissue organization is preserved.

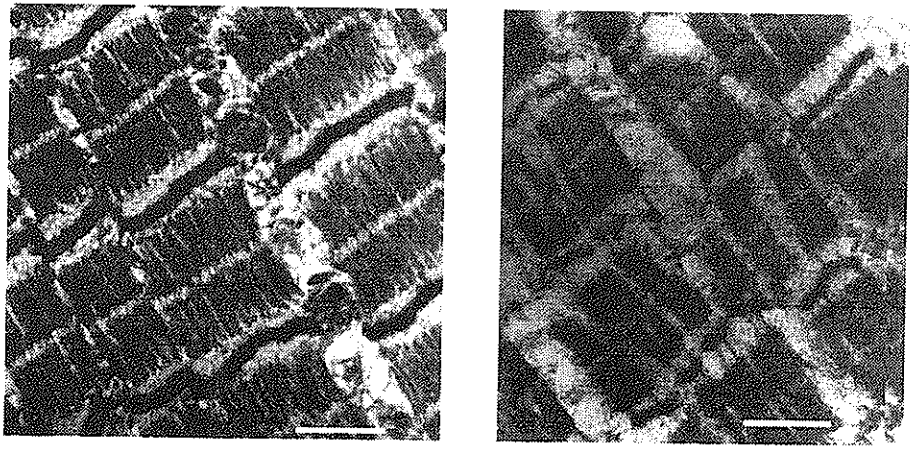

Figure 1.17. Transmission electron micrographs of pork meat tissues. Left: pressure treated (200 MPa). Right: pressure-shift frozen. The sarcomeric structure is damaged in both cases.

this process. The sarcomeric distribution is grossly altered not only by pressure-assisted freezing but also by the application of pressure alone (*Figure 1.17*).

BIOCHEMICAL CHANGES

Many biochemical changes, both desired and unwanted, take place in food during processing, storage, cooking, etc. Pressure treatments can contribute to the inhibition of some of these changes, such as vegetable and shellfish browning, lipid oxidation, vitamin C decomposition, or to the promotion of others, such as cheese ripening and enhanced enzymatic reactions. Many of these processes are mediated by enzymes, and the mechanism of activation of pressure is the usual one. Food, nevertheless, presents peculiarities in its heterogeneous composition, reduced water activity, and high molecular crowding. Proteins can behave quite differently from their expected 'ideal' behaviour, and care has to be taken when extrapolating results from other conditions.

Vitamins and other small size compounds

These small molecules are generally understood to be unaffected by hydrostatic

pressure at the levels practically employed. But indirect effects involving enzymatic or metal-catalyzed reactions can take place. Vitamins of the B group have been shown not to be significantly affected at pressures up to 600 MPa. The content in ascorbic acid is reduced during treatments, but pressure level does not appear to be the responsible factor (Sancho *et al.*, 1999). Other researchers have found that the pressure effect on vitamin C is significant but dependent on the accessibility to oxygen and the presence of other substances (Butz and Tauscher, 1998).

Vitamin K at high temperature (70°C) and pressure (600 MPa) suffers a specific polymerization reaction. The degree of vitamin alteration can be significant at these extreme conditions, but not at lower temperatures. The effect of the protein matrix could induce or retard the reaction (Butz and Tauscher, 1998). Other compounds, such as co-enzyme Q and isothiocyanates, can also react at high temperature and pressure. Vitamin A is also degraded at these extreme conditions, while temperature alone does not affect it. Other substances of nutritional or organoleptic interest (e.g. chlorophyll) can suffer degradation processes. Short pressure processing times and moderate temperatures can help to minimize the unwanted effects. Only in the cases when (either by the formation of toxic substances or by the very strong influence over an organoleptic parameter of small quantities of others) even traces of product should be avoided, this type of processing would represent a risk. But most, or all, of the reactions enhanced by pressure take place also (although maybe much more slowly) at atmospheric conditions. No new undesired substances can be expected to be generated. Nevertheless, the effect of the many compositional and structural factors of real food will have a very decisive influence on the actual global behaviour.

ENZYMATIC CHANGES

The pressure response of enzymatic systems of interest for the food processing industry is complicated by the many interactions taking place in real food, a semi-solid, multiphasic and microstructured system. Pectinesterase is among the enzymes of interest. It is responsible for the 'cloud destabilization' of fruit juices. It also has deleterious effects in the texture of vegetable products. Though it is relatively temperature-labile, this protein is very pressure resistant. High (900 MPa) pressure treatments, combined with denaturing (at 0.1 MPa) temperatures have slower deactivating kinetics than at atmospheric conditions. The enzymatic activity is increased with pressure to a maximum reached at 200–300 MPa, 65–70°C and the presence of Ca^{2+} ions (Van den Broeck *et al.*, 2000). Contrarily, the inactivation of myrosinase from broccoli was easily (200–450 MPa) carried out by pressure but, again, pressure protected this enzyme from thermal inactivation (Ludikhuyze *et al.*, 1999).

Polyphenoloxidases, a family of enzymes responsible for oxidative food browning, are a common target of inactivation food processing. The factors affecting their pressure-deactivation in mushroom and avocado have been studied (Weemaes *et al.*, 1997, 1998).

Metal release and oxidation

Oxidation is a phenomenon general to high pressure conditions, and can be related to diverse causes. Heavy metal atoms released from metal-proteins is a very likely cause

in meat products for the generation of unsaturated lipid oxidation, which in turn gives rise to rancid flavours. In a pressure-induced conformational change, myoglobin structure would partially unfold so that the steric constraints over the iron atoms are reduced and may become free. As for the case of thermal treatments, lipid oxidation in meat treated by hydrostatic pressure can be ascribed to the catalytic effect of iron atoms released from haemoproteins and other sources. When metal chelators were added to samples treated up to 800 MPa, lipid oxidation levels were found to be reduced (Chea and Ledward, 1997). Meat colour intensity reduction upon pressure treatment has been ascribed to this phenomenon. It currently impairs the application of high-pressure treatments to fresh meat, which is limited to pre-cooked products, the colour of which has already been altered. The discovery of mechanisms for avoiding the destruction of the haem group should be of practical interest.

Other causes for oxidation could be the interference with the natural mechanisms of elimination of highly oxidized species and free radicals, such as the mitochondrial systems. In 'alive' cells or, at least 'physiologically active', this can be a major cause of redox equilibrium displacement, as it is known that a variety of stresses cause this cellular malfunction. Other natural mechanisms of free radical detoxification, working without need of the cells to have an active metabolism, are scavengers. The equilibrium binding of ligands to these molecules can also be displaced by pressure. Gaseous oxygen, dissolved or otherwise included, can also have a role in promoting oxidation. Hydrostatic pressure favours reactions that reduce perturbations in water structure as those caused by gaseous molecules. So, gaseous oxygen would react to form chemical species with less contribution to the total volume. In any case, since the energy per bond that high pressure can yield is small, it is generally agreed that covalent bonds are too strong for disruption to occur at the levels of hydrostatic pressure encountered in biological work. So, species that are especially reactive or have already been destabilized by one or other means would need to be involved. Study of the oxidation of linoleic acid reveals that the reaction is thermodynamically unaffected by pressurization, but that the free radical diffusion process becomes locked under conditions of increased viscosity (Butz and Tauscher, 1998).

The oxidation of lipids, with rancid flavour development and the possible toxic role of free radicals, is also a problem requiring solution, possibly by the addition of extrinsic substances (natural additives, as one of the main values of pressure treatments is the 'natural', 'non-chemical' character of its action). De-gasification of samples or the pre-treatment with low oxygen atmospheres could also help minimize the problem. The role of temperature in this context has also to be studied: metal release, its migration through the sample and the effect on other molecules, can also be controlled by this factor, which leads – again – to a high level of interest in the knowledge and regulation of the temperature increase during high-pressure processing.

Effect on living systems

Living cells can be affected by pressure in a variety of ways. The effect of pressure on cells is a combined result of the effects on its different macromolecular components. Observations on this point are very different for the diverse cell type and origin. Cultivated human cells respond to moderate pressure with cellular death and induced

apoptosis. Other cells, from barophilic microorganisms but also from common food bacteria (in their spore forms) or from pluricellular organisms such as plant seeds and nematodes, are able to survive to much higher pressures.

Membrane-based processes are among the phenomena that are suspected to cause cell death. Pressure alters, either directly or indirectly, the lipid bilayer functionality, inducing rigidity and even fracturing the membrane. It also acts through the structural alteration of the many membrane-bound systems, such as receptors, ionic pumps, and many others. Macromolecular assemblies playing central roles for cell function can also be affected by pressure, as has been seen above. Ribosomes and the omnipresent microtubular network are altered at low-pressure levels. An important oxidative stress has been detected. This could be the result of the dysfunction of mitochondria, or just chemically induced by pressure.

The maximum pressure levels to which living organisms are naturally subjected in the deepest sea basins amount to approximately 100 MPa. Cultured bacteria can grow at this pressure level and 2°C. Life at 100°C reduces the pressure limit to 40 MPa (Yayanos, 1995). So, natural defences towards pressure will have been developed just to this pressure level and would only be present in 'abyssal' organisms. But, responses to pressure treatments have been observed in other life forms, and a common feature of these is that the underlying mechanism is identical or similar to that for heat-shock and other types of stresses. The investment by an organism in directed efforts for the preservation of native protein structure and the prevention of unspecific aggregation looks like a sensible cellular defence strategy.

From the natural point of view, the task is to survive in these extreme conditions. But, from the human perspective, the interests are more diverse, from easier and more efficient elimination of pathogenic microorganisms, to the manipulation of living cells' behaviour through induced stress, reduced activity, etc., and to the preservation of the cellular integrity and function of pressure-assisted frozen organisms.

MICROORGANISMS

Barotolerance, barophilia, and high-pressure resistance are the three concepts that define the metabolic constraints of microbes that face (usually often, or just once) large hydrostatic pressures. Deep-sea life forms develop different strategies for normal growth at 'moderate' pressures. Also, other microbes, normally not living in those conditions, resort to general or specific defence mechanisms against pressure, which can then be understood as a stress condition.

Food microorganisms

Most vegetative microorganisms common in foodstuffs and other realms of human activity can be eliminated, or significantly reduced in number, by treatments at 'attainable' pressures (500–700 MPa). But, as in other types of disinfectation procedures, spore-forming organisms have found the way to survive. These bacterial forms, so resistant to heat, desiccation and chemicals, also present, perhaps unsurprisingly, a remarkable endurance towards pressure. With a very limited metabolism and a series of in-built mechanisms to preserve the latent activity of their biomacromolecules, spores are scarcely damaged by very high pressures.

Early studies indicated that, while 200–800 MPa was sufficient to inactivate *Bacillus* and *Clostridium* spores, lengthy treatments were required (1 hour) and a fraction of spores remained intact. Under these conditions, high temperatures contributed to spore destruction, but extremes of pH and ionic strength decreased inactivation percentages. The need for this long period was interpreted as an indication of the germination of spores, prior to their destruction (Sale *et al.*, 1970). Most approaches to spore destruction involve the induction of its germination or the association of other disinfecting agents. Spores germinate as a response to a number of signals, which include many types of shock or brisk changes in conditions. So, spores can be treated by a preliminary process (a heat shock or even a moderate pressure shock). In only a few minutes, spores will have reverted to vegetative cell forms and then a second high-pressure treatment will find them in a much more susceptible state. A drawback of this conception is that spores germinate sequentially: rarely will all spores in a sample germinate at the same time. So, by this bi-phasic treatment, only a reduction (though significant) and not eradication in the bacterial count can be achieved; as always, a small number would be able to survive. The treatment is therefore considered to be equivalent only to what is generally termed as pasteurization (Tauscher, 1995). The comparison with traditional pasteurization techniques is satisfactory for a number of food products (e.g. Drake *et al.*, 1997).

The combined use of other bacteria-killing agents, such as chemicals like nisin, oxygen peroxide, etc., or heat (Mallidis and Drizou, 1991; Hayashi, 1996) or ultrasonic treatment, has been relatively more successful. For example, combination of pressure (400 MPa), chemicals (sucrose, laurate) and mild heat (45°C) achieves a significant reduction in the number of *Bacillus* and *Alicyclobacillus* spores (Shearer *et al.*, 2000). However, the initial aim of obtaining a simple and scarcely invasive sterilization method is thereby compromised.

Even if pressure treatments of food (and other commodities) can only yield pasteurized products, there can be a place for this methodology for particular processing needs. Those cases where a thermal treatment is not advisable, often for its destructive effects on sensitive components or the general structure, would be good candidates for this type of technology. The disease or food spoilage capacity of some organisms would be seriously compromised if their number and optimal state were reduced, without the need to resort to a complete degree of sterilization. This approach, frequent in present-day food technology, may be potentially dangerous. Not only is there a danger of the application of sub-lethal injuring stresses giving rise to selection of the microorganisms more resistant to the injuring stress, but there is evidence relating the organisms' responses to the different stresses, so that resistance capacities appropriate for one type can actually confer protection towards other ones (Hauben *et al.*, 1997; Benito *et al.*, 1999). Additionally, the virulence of some microbes is enhanced by sub-lethal stresses. Attempts at enhancing not only our knowledge of the microbial response to stresses, including this stress-association response, but also the factors determining spore behaviour, may be a wise strategy for the future (Abee and Wouters, 1999).

The effect over non-sporulating organisms is more marked, but the time needed for a satisfactory count reduction is normally too high for industrial interest. Treatments of 350 MPa for 20 min were needed to considerably reduce the counts of Gram-negative bacteria (*Figure 1.18*). Gram-positive bacteria, more resistant, were not

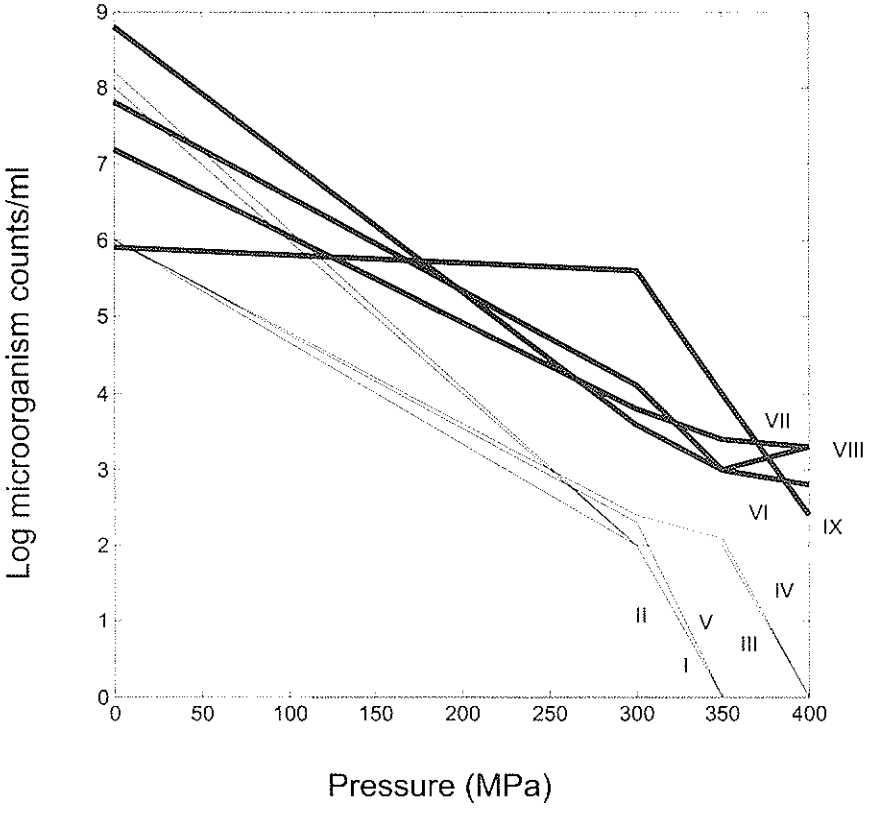

Figure 1.18. Inactivation of food Gram-positive (thick lines) and Gram-negative (thin lines) bacteria after treatments at 10°C for 20 min. I: *Escherichia coli*; II: *Salmonella typhimurium*; III: *Yersinia enterocolitica*; IV: *Pseudomonas aeruginosa*; V: *Aeromonas hydrophila*; VI: *Bacillus cereus*; VII: *B. subtilis*; VIII: *Staphylococcus aureus*; IX: *Micrococcus luteus* (data from Arroyo *et al.*, 1997).

satisfactorily damaged at 400 MPa (Arroyo *et al.*, 1997). Even in combination with lactoperoxidase, *Listeria innocua* required 20 min at 400 MPa. *E. coli* exhibited no synergism with lactoperoxidase and was only sub-lethally affected at 600 MPa (García-Graells *et al.*, 2000). On the other hand, *E. coli* exhibits ease of mutation, giving rise to pressure-resistant strains. Some of these strains resist significantly at pressures of 800 MPa and have also reduced thermal sensitivity (Hauben *et al.*, 1997). Especially virulent *E. coli* O157 heat-resistant spores also possess significant pressure resistance (Benito *et al.*, 1999). Nisin appears to be a more effective co-treatment for hydrostatic pressure. In a synergistic process, suggested to be mediated by an increase in pressure susceptibility upon nisin binding to membranes, 150–200 MPa pressures are considered as sufficient for achieving a more than 6 log reduction in *Lactobacillus plantarum* and, to a lesser extent, in *E. coli* (Ter Steeg *et al.*, 1999).

Fungi and yeast

Other organisms, such as fungi, cause food-related contamination problems. Aerobic fungi of vegetable food were reduced by 1 log at 300 MPa in conditions that

significantly alter the food properties, especially its texture (Arroyo *et al.*, 1997). The fungus *Mucor plumbeus* can be inactivated by 400 MPa pressure treatments. When pre-treated with the enzyme chitinase, which acts on the chitin structures in the fungus, only 300 MPa are required (Fenice *et al.*, 1999).

The growth of *Saccharomyces cerevisiae* is arrested by modest increases in hydrostatic pressure (40–60 MPa). This process is believed to be mediated by vacuole acidification, which is related to the function of a membrane ATPase pump (Abe and Horikoshi, 1998). The kinetics of inactivation of the fruit contaminant yeast *Zygosaccharomyces bailii* was studied at much higher pressures (around 500 MPa) and a relation with water activity was found (Palou *et al.*, 1997). A barotolerant mutant *Saccharomyces cerevisiae* has been compared with the wild-type strain and, while no difference in heat-shock proteins was found, trehalose content was increased in the mutant. Also, the behaviour of the membrane was different, with increased rigidity (Fujii *et al.*, 1996). Higher pressure levels (300 MPa) completely inactivate *S. cerevisiae* (Arroyo *et al.*, 1997). Meanwhile, a combined nisin–pressure (200 MPa) treatment is reported to have only slight effects (Ter Steeg *et al.*, 1999).

Barotolerant and barophilic bacteria

The microorganisms found in recent years in deep-sea basins can be divided in two types. Those that can exert all their life functions at elevated pressure, but not under atmospheric conditions: *barophilic*. And those that can grow under both conditions: *barotolerant* (Kato and Horikoshi, 1996; Horikoshi, 1998; Kato and Qureshi, 1999). Non-adapted organisms respond to pressure with morphological anomalies, growth inhibition and death (Gross and Jaenicke, 1994).

A remarkable coincidence exists between the capacity of microorganisms to resist different stressful conditions, such as temperature and pressure (Hauben *et al.*, 1997; Benito *et al.*, 1999; Robb and Clark, 1999). Nevertheless, barotolerance, understood as the ability to grow at moderate pressures, is not always correlated with pressure inactivation resistance (Hauben *et al.*, 1997).

In some cases, elevated pressure is a key condition for the thermal stability of a given organism. This is the case for some strains of *Pyrococcus*. One of the more important enzymes for its growth, DNA polymerase, is stabilized towards thermal denaturation by pressure. Moderate ranges of pressure (3–89 MPa) are sufficient to decrease this enzyme inactivation rate significantly (Summit *et al.*, 1998). Pressure-regulated operons have been found in barophilic bacteria and relate to genes implied in the respiratory chain. The evidence thus seems to point to the ability of such bacteria to adapt their respiratory system according to the prevailing pressure (Horikoshi, 1998; Kato and Qureshi, 1999). The involvement of specific lipids in barotolerance has also been investigated using gene-defective mutants of *Photobacterium profundum* (Allen and Bartlett, 2000). The mesophilic bacterium *E. coli* does not, however, exhibit the ability to adapt to growth at elevated pressure and even complementation with *P. profundum* genes does not allow its growth under pressure.

FOOD-RELATED MULTICELLULAR ORGANISMS

Other living organisms of higher complexity than microbes are sometimes present in

food and other products. Their destruction, without deleteriously affecting the properties of the food product, is always desirable. This is the case of parasites, such as the fish nematode anisakis, meat trichina, or insects in vegetable products, seeds, etc. We could include here unwanted germination of seeds. High-pressure technology has a major role to offer in all of these cases, as the higher complexity of these organisms would presumably make them more susceptible to pressure death.

Studies on *Anisakis simplex* larvae show that, at pressures of the order of 180 MPa, all nematodes are destroyed by a short treatment at conditions causing only minor damage in the host fish meat structure (Molina-García and Sanz, 2001). The treatment duration was found to be interchangeable with pressure level: longer treatments also have a deleterious effect at lower pressures. However, for its practical implementation for industrial application, the treatment time should be the shortest possible.

It is worth stressing that some of these organisms can be hard to kill. Preliminary treatments with alfalfa seeds show that only a partial reduction in germination is achieved after treatments at pressures as high as 400 MPa (Gonzalez-Benito and Molina-García, unpublished results).

ANIMAL AND PLANT CELLS

The study of the effect of pressure on animal and plant cells and full multicellular organisms is of practical importance when their viability and physiological function needs to be maintained, as for the germination of seeds and induction of given metabolites in cell cultures. Also, pressure is an interesting and relevant tool for the investigation of diving physiology or mechanical stresses in human body tissue. In some cases, the resistance of these systems to pressure is surprising: vegetable cell cultures yield 50% of viable cells after treatments of up to 100 MPa. Perhaps unsurprisingly, the causes for cellular death involve more parameters as cellular complexity increases. Cellular membranes have an important role in the adaptive capacity towards hydrostatic pressure. The fatty acid composition of barophilic bacteria has been found to vary with pressure, the content of unsaturated fatty acids increasing with environmental pressure, in a similar way to temperature adaptations observed in other organisms (De Long and Yayanos, 1985). As for murine cells, they can remain viable up to 110 MPa, different perturbations taking place in their membrane systems (Mentre *et al.*, 1999). When the order parameters of myelin membranes belonging to fish from different environments were compared, it was found that the degree of order was conserved, once it had been corrected to the corresponding ambient pressure and temperature conditions for each species (Behan *et al.*, 1992). These adaptive differences in pressure sensitivities for proteins and membrane systems are common for organisms living at different pressures (Somero, 1992).

The direct relation between some of the factors protecting cells against temperature and pressure has also been proven for eukaryotic cells. The increase in the amount of Hsp70 heat shock protein, regulated at mRNA stabilization level, has been observed in pressure-treated chondrocytes at low (30 MPa) but continuous pressure (Kaarniranta *et al.*, 1998). The threshold for what constitutes an unusually high pressure varies for different physiological systems, but is generally constant for different species. Enzymes such as adenylyl cyclase are perturbed by modest pressure of 5–10 MPa,

while membrane ATPases and self-associating actin are sensitive to 20 MPa. This implies differences in the pressure-optimum habitats, at depths of only 500 m. The plethora of strategies for pressure adaptation is an example of convergent evolution for different species (Somero, 1992). The resistance to 200 MPa pressure haemolysis of red blood cells has been related to Ca^{2+} and K^+ ionic fluxes and intracellular viscosity (Harano *et al.*, 1994).

Conclusions

The general panorama of research under high hydrostatic pressure is one of wide expansion. As in other cases when a new technology enters into the biophysical world, as soon as the technical difficulties can be solved, virtually all the systems already under study by other means are introduced to the 'virtues' of the new approach. Such was the case, for example, for differential scanning calorimetry and nuclear magnetic resonance. All of a sudden, scientists do not know how they could have done without it before. The number of articles employing high pressure for different studies is increasing each year, as is the number of laboratories with facilities for its study. A parallel development of commercial equipment is also expected and desirable.

Industrial uses of pressure treatments are also being developed. To date, although the uses in USA and Japan are more numerous, there are only two or three pressurized food producers in Europe. This has to do not only with the advantages or disadvantages of the technique, but also with the technical and economical industrial structure of Europe and, more than anything else, with the current state of European legislation about novel food acceptance. The recent cases of food-related illnesses and food poisoning cases, some of them even related to innovations in the food chain (e.g. the BSE syndrome), have provoked a very restrictive and defensive legislation. And indeed, many aspects related to the safety of pressure-treated foods remain to be solved: the factors determining pressure-induced oxidation and its actual level, the possible modifications of bacterial resistance and virulence, the affects on substance migration and contamination of containers under pressure, the appropriate conditions for pressure sterilization, and others. Initiatives targetted at globally addressing these questions will be most welcome, and would set the basis for a wider implantation of pressure technology.

Economical and logistic factors are also challenging this new technology. The addition of a new and (even just for being new) expensive technology to the pre-existing production lines is, of course, a cause of increased expense and reduced effectiveness. The time accepted as maximum for economically viable pressure treatments is 5 minutes. This period is, in most cases, too short for many effects of pressure to be manifest. A way to make a short treatment effective may often (but not always) be to increase pressure, which increases expense in equipment and maintenance in an exponential way. Pressure treatments are, in our current conception, intrinsically discontinuous processes. This introduces delays and irregularities in the modern continuous production lines. Efforts for designing more suitable equipment are also encouraged. But a major need arises: to integrate the new process into the global context of production, instead of merely 'adding a tail wagon to the train'. In this way, some treatments could be substituted or performed at the same time or in

association with pressure treatment. This can solve some of the problems related to treatment and discontinuity. Also, the combination of pressure, temperature and other factors for a treatment can be expected to yield a better overall result, from the food quality, security and economy points of view together.

Baromicrobiology is also coming of age, and the flux of information encompassed by it, and also biophysical pressure research, is allowing us to address many questions of relevance to the atmospheric pressure 'common place' world. In addition, micro-organisms resistant to pressure, either naturally or after engineering, and their enzymatic elements, are new tools for the chemical industry and are expected to allow newer, better, and less polluting ways of production.

The world of high pressure is getting organized in a number of specialized, regional and global groups, and many meetings, workshops and schools have taken place in the past ten years on pressure-related topics. The common consideration of the different challenges and questions posed by pressure for scientists working in different areas is of the utmost interest.

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