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Physical aspects of cryobiology

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Contents

Abstract. Physical phenomena during biological freezing and thawing processes at the molecular, cellular, tissue, and organ levels are examined. The basics of cryosurgery and cryopreservation of cells and tissues are presented. Existing cryobiological models, including numerical ones, are reviewed.

1. Introduction

> *When a physicist for the first time addresses himself to biological structures and biological processes, they seem to be desperately intricate and complicated. But it is an illusion.*
>
> S E Bresler [1]

The reaction of biological objects (cells, tissues, organs) to various physical factors, such as ultrasound [2, 3], electric [4 – 7] or magnetic [8, 9] field, microwave electromagnetic field [10 – 15], ionizing [16] or laser [17 – 21] radiation, high or low temperature, is of interest for understanding natural and technogenic impacts on the living organism and in light of the implications it has for medical diagnosis [22] and therapy. Biological repercussions may be great even if induced by a slight triggering action [9, 14].

Not only is information about processes in biological tissues paramount (e.g., knowledge of the role of hydroxonium H_3O^+ ions in irradiation provided a rationale for glucose administration for the treatment of tumorous cells [16]), but so are the direct effects of physical factors, such as an electric field [6, 23, 24] or ultrasound [25 – 27], used to deliver drugs to their respective targets in the body. The application of physical factors for the destruction of pathologic tissues is a useful noninvasive alternative to classical surgical intervention.

Several factors may act in concert. For example, irreversible electroporation based on disruption of cell membranes by electric pulses [28] is associated with the Joule heating of tissues by a traversing current [24]. Microwave radiation and ultrasound are used for the high-temperature destruction of malignant tissues. At the same time, an electromagnetic field is known to directly affect conformation of DNA [29]. Molecular dynamics (MD) simulation indicates that the field causes the protein dipole moment to reorient, thereby altering the secondary structure of a protein and promoting its denaturation [30]. An intense acoustic field produces nonthermal effects, such as the development of secondary flows in blood vessels and cavitation related to the presence of gas bubbles in the circulation [31]. These processes may mechanically affect endothelial cells lining inner vascular walls via shear stress in the blood flow and induce gene activation [32].

Cryosurgery and cryopreservation. The exposure to low temperatures is widely used to eliminate pathologic tissues [33 – 36] (cryosurgery), inhibit biochemical processes [1] in cells (including stem cells [38]), tissues, and organs for their further implantation after revitalization [35, 39 – 44], test pharmaceutical products [45 – 47], and conserve genetic resources [48 – 50] (cryopreservation). Biological objects are stored at a liquid-nitrogen (sometimes higher) temperature [51]. Based on the examination of samples kept for 11 to 34 years, the shelf life of cryopreserved erythrocytes is estimated at 113 and 276 years if stored at −65 and −196 °C, respectively [52].

The term 'cryosurgery' (*κρύο* + *χειρουργική*) is inexact in its second part, the 'manual work' being restricted to the preliminary stage of the operation, namely, the placement of

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[1] Sometimes, tissue freezing is accompanied by accelerating the rate of bioorganic reactions, attributable to the freeze-concentration effect and/or ice catalytic properties presumably related to high proton mobility in the solid phase [37].

cryoprobes. Wikipedia treats 'cryosurgery' and 'cryotherapy' as synonyms; sometimes they are distinguished by a minimal temperature (above and below the crystallization temperature [53]). Surface tissue operations are performed by spraying liquid nitrogen; the simplicity of this procedure makes it easy to carry out by a family physician [54]. The history of cryosurgery and cryosurgical instruments is expounded in Refs [35, 55 – 57] and [57 – 59], while the development of cryosurgery in Russia in Ref. [60]. Cell freezing experiments date back to the late 18th century [61]. The first successful cryopreservation was reported in 1949 [62].

Artificial tissues. Low temperatures are needed to produce artificial tissues. The porous sponge-like collagen structure of artificial skin substitutes is manufactured by drying up crystallized protein (collagen) suspension and depends on ice dendrite morphology [63].

Cryofixation. Fast freezing is used (sometimes at an elevated pressure [64]) to study biological tissues under a low-temperature electron microscope [65, 66] (cryofracture or cryoetching [67, 68]). A key problem of electron tomography is to preserve the structure of a study object during measurements [69]. Its embedding in amorphous ice secures invariability of conformation and spatial arrangement of macromolecules and elements in the cytoskeleton. A structural survey of proteins and other macromolecules by neutron crystallography [70, 71] or X-ray structural analysis [72, 73] using synchrotron sources [74] requires crystallization of the study objects [75, 76]. The number of details identifiable on the electron density map is determined by the quality of the crystal, including the degree of mosaicity [77]. It is assumed that molecules in the solution (where they are biologically active) and in the crystal have an identical conformation because 'a certain number of water molecules are being embedded in the crystal as it grows' and 'the crystal is analyzed in the aqueous medium'. Indeed, a large number of water molecules (from one-half to two per aminoacid residue, i.e., about 200 molecules for a typical protein [78] or 34 – 39% if expressed in percentage of mass [79]) can fit into the crystal structure. However, some of them become embedded in structures that form in the course of crystal growth [79]. The following needs to be emphasized: (1) in small proteins, 30 – 40% of the solvent-accessible surface (SAS) is normally 'hidden' inside the crystal [80]; (2) protein crystals do not experience cold denaturation probably due to the stabilizing effect of van der Waals forces and hydrogen bonds between the molecules [79], and (3) protein crystals grow in solutions orders of magnitude more oversaturated than those needed for the growth of inorganic crystals [81]. Certain proteins undergo aggregation in a solution for which no energy barrier exists (unlike nucleation) [82]; the aggregates function as 'growth units' [83] (for example, a hen egg lysozyme forms dimers, tetramers, octamers, and 16-mers); dimer formation leads to protein partial denaturation, in particular, to displacement of hydrophobic groups to the surface of the protein [84].

An advantage of cryoelectron microscopy also lies in the possibility of studying not only macromolecules but also heterogeneous structures [85], such as subcellular (and even subnuclear [86]) objects mutually positioned as in a 'living' cell. But fast freezing is critical here [87]. It was demonstrated by the example of several plants that otherwise water redistribution occurs in certain tissues, which is accompanied by the formation of ice deposits in the extracellular space; some cryofixation artifacts are more difficult to detect, for example, six- and seven-member ring structures of water in the protein hydration shell at temperatures below 200 K [80, 88].

Destruction of biological tissues. Tissue reaction to the effect of temperature, unlike that of radiation [89 – 91], depends on the thermal history: the affected area of an organ varies as a function of heat conductivity and heat transfer by blood. The tissue behavior during cryosurgery (in contrast to hyperthermia) is more complicated due to the nucleation and growth of ice crystals, ice recrystallization upon warming [92], mechanical stress generation, and changes in solute concentrations in cells and extracellular space.

Three stages of tissue destruction can be distinguished. The first is cell destruction as a consequence of ice crystal formation upon freezing and associated mechanical disruption of the plasma membrane [93]; another contributing factor is crystal enlargement as recrystallization proceeds [94, 95] under the effect of thawing (free surface energy falls with decreasing surface curvature) and/or due to a rise in the intracellular electrolyte level ('osmotic shock'). The second is microcirculatory disturbances developing within a few hours/days after operative treatment. Finally, cold-induced apoptosis [2] [102] is most clearly manifested in the peripheral regions of an ice ball [103]. Consideration of apoptosis and immune responses of an organism to low temperature [104] is beyond the scope of the present review. Destructive factors should be excluded when performing cryopreservation procedures.

Cryogenic treatment may be combined with traditional procedures, such as immunotherapy [104], chemotherapy [102, 105], and other physical impacts on biological tissues. Employment of cryoheaters [106] and lasers [20] extend the possibility of controlling the freezing zone; injection of solutions with specified thermophysical properties serves the same purpose [107, 108]; cryogenic sprays are applied to protect the epidermis in laser-assisted operations [109, 110]; it has been suggested that pulsed lasers be used in cryopreservation to accelerate freezing [111]; microwave heating in thawing cryopreserved objects [112, 113] improves temperature field uniformity and prevents ice recrystallization. Microwave radiation may also be used to exert some action at the molecular level. The breaking of hydrogen bonds prevents nucleation of ice crystals and thereby decreases the crystallization temperature [114]. This mechanism is regarded as a means of diminishing the amount of bound water inside cells and increasing heat conductivity of the medium and efficiency of cryodestruction [115]. A noticeable (2 – 3-fold) enlargement of the necrotic zone was also reported in Ref. [16].

Cryogenic exposure is essentially different from hyperthermic exposure (ultrasound [2, 117, 118], laser [18, 19, 119] or microwave [12, 13, 118, 120] heating). The latter is

[2] A cell dies under the effect of physical (ionizing radiation, temperature, mechanical injury), chemical, or biological impacts [96]. Apoptosis is 'normal' (programmed) self-annihilation of a cell [97 – 99] differing from necrosis ('accidental' cell death caused by physical or chemical factors) in morphological features (e.g., membrane distortion in the former case, and membrane disintegration in the latter) and the character of biochemical reactions. Apoptosis is initiated by external or internal processes, the latter being mediated through mitochondria [100]. A change in temperature, including cryoshock, is one of the signals 'triggering' apoptosis [101].

based on volumetric absorption of an acoustic or electromagnetic signal. Tissue loses heat only through its surface; hence, the inevitable spatial inhomogeneity of tissue temperature and its variation rates, i.e., different local thermal history of the constituent cells. In the simulation of hyperthermia, the equation of energy acquires a heat source, while the description of cryogenic exposure is contained in the boundary conditions. The purpose of mathematical modeling is to explain (using, inter alia, experimentally unavailable information) and to predict; the latter implies verification of numerical models [121, 122].

This review is concerned with physical (in a broad sense, i.e., regarding chemistry as 'a branch of molecular physics' [1]) phenomena involved in the freezing and thawing of biological objects and with models used to study these processes. Most attention is given to recent investigations; as a rule, publications included in the accessible reviews are not explicitly cited here.

2. Cryogenic exposure in biology

A cryogenic treatment includes one or several (in cryosurgery) freeze – exposure – thaw cycles [33 – 35, 39, 123]. Intervals between them allow for developing circulatory disturbances, thus enhancing destructive processes [124]. Cryogenic exposure is subject to modulation in time ('dynamic cryosurgery') [125].

Vitrification of an object (its changing to the vitreous state without crystallization) [46, 126 – 129] by ultrafast cooling depends in the main on the rate of the process [46]; it is therefore easier to achieve with small objects. A relevant large-scale process is exemplified by the postulated supercooling of back hemisphere of Europa with respect to Jupiter [130]. Vitrification of biological objects is usually conducted using cryoprotectors. The type of cryoprotector, its concentration, and the protocols of tissue saturation and washout [39, 131] influence the outcome of cryogenic treatment.

Two phases of amorphous ice [3] have long been distinguished, one having high density and the other low density (HDA and LDA ice, respectively) [136]. In addition, a peculiar 'very high density' (VHDA) phase of amorphous ice has been reported [133, 138]; it is obtained by isobaric heating of LDA ice under high pressure [137]. All three phases have a tetrahedral structure of the hydrogen-bond net, but HDA (VHDA) ice has one (two) additional molecules between the first and second neighboring shells [137]. Numerical experiments revealed one more (the fourth) phase of amorphous ice [139]. Recent data indicate that HDA ice is a metastable phase, while LDA and VHDA represent stable phases [140, 141]. The possible porosity of amorphous ice (hence, its ability to adsorb gases) is of importance for extraterrestrial objects (comets, satellites of outer planets, interstellar dust) [142, 143]. Various phase transitions of vapor, liquid, and hexagonal I_h ice to amorphous ice, as well as transitions between amorphous phases of different densities, have been considered in Refs [133, 138]. It should be noted that amorphous ice was experimentally obtained by water vitrification half a century after it had been precipitated from the vapor phase on a cold substrate at low pressure by the method resembling modern molecular beam epitaxy used to grow semiconductor heterostructures.

2.1 Crystallization of ice in the biological environment

Ice crystallization in biological objects is essentially different from the growth of semiconductor crystals [144, 145].

Water. To begin with, the liquid and solid phases of water have a number of unusual properties [146 – 150], the list of which includes over 50 points [151], with abnormalities being most strongly expressed in supercooled water [138, 152, 153]. Water also exhibits very specific characteristics as a solvent, for example, nonmonotonic dependence of the mobility of dissolved ions on their radius [154]. These peculiarities of water are due to the presence of the order in its various states (ice crystals, liquid water, gas clathrates [4]) resulting from the cooperative effect of the structurally and dynamically inhomogeneous hydrogen-bond net [136, 150, 157, 158]. The thermodynamic properties of water are first and foremost determined by static inhomogeneities [140], i.e., the local structure with its four-fold net coordination or organization of molecules in the form of tetrahedrons [160, 161] (on the average, calculations with different interaction potentials suggest that many molecules have coordination numbers from 3 to 6 [159]). The tetrahedral short-range order is introduced by sp^3-hybridization of electron orbits [153]. Dynamic inhomogeneities (translational and rotational) are supposed to originate from defects in the hydrogen-bond net [162]. The network structure is substantially modified by steric constraints; for example, the mean coordination number for quasi-one-dimensional water in a single-wall carbon nanotube is below 2 [163]. Liquid-state order is maintained by water hexamers $(H_2O)_6$ structurally resembling ice I_h that give rise to a number of energetically similar forms [164]; clathrates contain pentamers $(H_2O)_5$ grouped into dodecahedrons. A unit cell of 14 molecules is the basic element of an icosahedral structure (280 molecules) that takes one of two forms differing in density, without breaking the hydrogen bonds [165]. The hydrogen-bond net is the cornerstone of several models. Some consider it to be a set of 'space-filling' curves [166], while others [157] represent the temperature dependence of water properties as the expansion in terms of structural functions describing an equilibrium H-bond system (the former gives the mean number of bonds per molecule, while the latter describes the tetrahedrality parameter, and so forth).

[3] Glassy state polymorphism ('polyamorphism' [132]) is characteristic not only of water but also of some other substances (Si, SiO_2, GeS_2) [133]. The structural difference between crystal and amorphous ice manifests itself in the properties of large clusters of water molecules. Clusters comprising $n = 20 - 931$ molecules were examined theoretically, and those of $n = 200 - 10^6$ molecules experimentally in Ref. [134]. Large clusters have a crystal nucleus and disordered outer layers where the number of hydrogen bonds tends to increase in the course of surface reconstruction. Clusters containing $n \leqslant 200$ or fewer molecules are too small to support a stable crystal nucleus; their spectra suggest an amorphous structure. Formal analysis of polyhedral water clusters is presented in Ref. [135]. Based on the 'rules' that dictate the potential number of bonds between oxygen and hydrogen atoms, the author used methods of the graph theory to elucidate the structure and common properties of clusters organized into polyhedrons.

[4] In clathrates ('clathrate hydrates' or 'gas hydrates'), a molecule of a gas is surrounded by water molecules making up a stable cavity maintained by hydrogen bonds. Well known is methane clathrate ('combustible' ice [155]) the amount of which in the seas is believed to exceed the prospected resources of hydrocarbon fuels; such clathrates are supposed to be the source of methane for Titan's atmosphere [156]. Their structure and electronic properties, including H-bond behavior in response to a change in pressure, have been evaluated in Ref. [155] by the density functional method.

The degree of ordering of water molecules increases (decreases) in the presence of kosmotropic (chaotropic) ions. The character of action of ions on water structure depends on their size and charge, manifesting itself, among other things, in the dependence of solution viscosity η on concentration c:

$$\frac{\eta(c)}{\eta_0} = 1 + A\sqrt{c} + Bc\,,$$

where the Jones – Dole coefficient B is positive for kosmotropes, and negative for chaotropes [167]. Experimental studies of the influence of interactions with polar, nonpolar, and charged molecules on water structure are reviewed in Ref. [168]. The ordering of water molecules enhances the heat conduction and brings it closer to the thermal conduction of ice [169]. *Trans*-conformation of hydrogen bonds is more tightly bound than *cis*-conformation due to additional interactions of protons and unshared electron pairs not implicated in the formation of a given bond. As a rule, two OH groups of one water molecule are involved in an H-bond of a different strength [170]. See Ref. [171] for the construction of a model of the continuous hydrogen-bond net taking into consideration strong and weak bonds.

The presence of long-range order in water is confirmed by the form of the radial (pair) distribution function $g(\mathbf{r})$ found theoretically by the MD method and in experiment from the scattering of neutrons or X-rays [158]. The distribution of atoms and interatomic (interstitial) voids is of interest; the *difference* distribution function

$$G(\mathbf{r}) = r^2\big[g(\mathbf{r}) - 1\big]$$

visualizes long-range correlations [172]. Orientational correlation function $g(\mathbf{r}, \Omega_1, \Omega_2)$ [168] cannot be deduced directly from the measured data. The full pair correlation function $g(\mathbf{r}, \Omega_1, \Omega_2)$ includes the polar coordinates of the vector connecting the centers of the molecules [159]. The authors of this work proposed building up correlation functions for spherical layers with $R_{\min} < r < R_{\max}$ when analyzing coordination spheres. A review of structural models of water (both phenomenological and based on integral equations for the Ornstein – Zernike correlation functions) can be found in Ref. [153].

Relying on calculated results, the hypothesis was proposed for the possible occurrence of first-order liquid – liquid phase transition at low temperatures [160, 166, 173, 174], which is analogous to the transitions in structurally similar phosphorus [175], metal – metalloid melts (Ni – P, Fe – P, Ni – B) being amorphized, certain metal alloys (Ni – Zr) [176], and probably several macromolecular solutions [177] and some other liquids [178]. This transition coincides with the violation of the Stokes – Einstein relation

$$D = \frac{kT}{6\pi\eta a}\,,$$

where D is the diffusion coefficient, η is the viscosity, and a is a particle's hydrodynamic radius [179]. MD calculation of viscosity being a difficult task, the authors used time τ_α of the so-called alpha-relaxation [180] that depends on temperature, as does η.

The behavior of disordered media under changes of temperature is attributed to their ability to form structural objects stabilized by directional bonds [176, 181]. It turns out, however, that directional bonds are not at all indispensable because many anomalies of water are reproduced on the assumption of tetrahedral (sp^3) coordination with the use of the spherically symmetric interaction potential between molecules with 'softened' cores (see paper [182] and references cited therein). Detailed analysis of abnormalities of a different nature (structural, thermodynamic, dynamic) is presented in Ref. [183]. The authors employed two order parameters: (1) translational, viz.

$$\Omega_t \equiv \int_0^{s_c} \big|\, g(s) - 1\big|\, \mathrm{d}s\,,$$

where $s \equiv r\rho_n^{1/3}$ is adjusted to scale over the average interparticle distance, $g(s)$ is the pair correlation function, and s_c is the cutoff parameter (assumed to be half the length of the computation region), and (2) orientational (related to ith particle):

$$\Omega_{li} \equiv \left[\frac{4\pi}{2l+1}\sum_{m=-l}^{m=l} |\,\bar{Y}_{lm}|^2\right]^{1/2}.$$

The latter parameter is found based on characterization of particle association with the nearest 12 neighbors by two angles θ, ϕ used to calculate the corresponding spherical harmonics $Y_{lm}(\theta, \phi)$ and the average $\bar{Y}_{lm}$. At $l = 6$, parameter Ω_{6i} is a measure of the orientational order in the system of particles. It turns out that two linear scales in the interaction potential are indispensable for the anomalous structural properties to emerge [183].

Verification of the hypothesis for two water phases was reported in Ref. [184]. The authors made use of differential scanning calorimetry (DSC) and neutron spectrometry to observe two states of supercooled water with different densities and a first-order phase transition between them. Experiments on water molecule dynamics in a single-wall carbon nanotube [185] at 228 K demonstrated a change from the temperature dependence of relaxation time according to the Vogel – Fulcher – Tammann law [186]

$$\tau = \tau_0 \exp\left(\frac{B}{T - T_0}\right)$$

to the Arrhenius type dependence

$$\tau = \tau_0 \exp\left(-\frac{E}{kT}\right),$$

where E is the activation energy. The authors reasoned that this instant of time corresponds to the transition from low- to high-density water. Similar results on changing the temperature dependence of the relaxation time were obtained for water contained in 50-nm pores of zeolyte [187].

The homogeneous nucleation temperature of pure water equals $-39\,^\circ$C; deeper supercooling (to $-92\,^\circ$C [188]) is achieved at high pressure and in nanopores [189] (notwithstanding the fact that pore water may exist in the glassy state at room temperature [190]). Water dynamics corresponding to the liquid state occurs at low temperatures in carbon nanotubes [163, 191]. The action of a 10^6 V m^{-1} electric field on water in a nanometer gap raises crystallization temperature to room temperature [192]. Ice formation is facilitated by restricted water molecule mobility in the direction normal to the walls and by dipole ordering in the field; these promote formation of a stable hydrogen-bond net. This process is reversible since the ice melts as the field strength decreases. The field effect on nucleation of ice crystals has been known for a century and a half [193]. The electric field can be used to grow ice nuclei in an aqueous

solution at a given temperature [194] in order to control the grain size in polycrystals or lyophilization time [195].

Biological water. The behavior of water near hydrophobic and hydrophilic regions of biological molecules is essentially different from that in the bulk. Water is sometimes described as *bound*, *ordered*, or *biological*; in cryobiology, it is referred to as unfreezing, although the term *unfrozen* seems more appropriate since it encompasses nonequilibrium states [196, 197]. Some authors speak about *buried* [29, 198] or *internal* [80] water held by hydrogen bonds in inner hydrophobic regions of biological molecules [199], where it occasionally forms clusters [200]. Such water is present in the majority of globular proteins; it should be regarded as an indispensable component of proteins [78, 80, 198] despite its exchange with extraneous molecules with characteristic times from 10 ns to 1 ms [201]. The importance of water for the stability and function of biological molecules can hardly be exaggerated [202]; some call it the 21st amino acid [203]. In certain cases, the normal behavior of a protein depends on the formation of an infinite-sized cluster of water molecules on its surface (in terms of the percolation theory) [204, 205]. Anomalous water behavior near the surface is reproduced in MD models (see [206–209]).

Water properties near a biological surface change as a result of hydrogen bond rearrangement favoring better ordering of water molecules [210]; this process is governed by both the surface nature (polar or nonpolar) and spatial restrictions on the hydrogen-bond net structure (cutoff of linear correlation scale [211]); the mobility of water molecules and dielectric constant of water markedly decrease [127, 128], while viscosity increases [219]. The wall proximity effect on crystallization temperature is known from studies of soil freezing [220, 221] and the behavior of pore water in minerals [222]. Crystallization of water in a narrow (several diameters of a water molecule) plane gap was investigated by the MD technique in Ref. [223] for an inert surface with which water molecules do not form hydrogen bonds. A change in the gap width at room temperature resulted in a sharp change (three to four orders of magnitude) in the diffusion coefficient, interpreted as transition to the crystalline state which the authors called confinement-induced freezing. They observed two phases of liquid water in the gap and synergism between steric constraints and the electric field. The effect of molecule–wall interaction potential was evaluated in Ref. [224] for a gap containing two or three molecular layers; the authors focused on the Lennard–Jones and purely repulsive potentials. Elevation of pressure led to the displacement of molecules toward hydrophobic surfaces and increased the tetrahedrality parameter. The pressure effect was inapparent for hydrophilic surfaces [225].

MD calculations in the framework of the density functional method revealed the presence of a thin water layer of elevated density near a hydrophilic surface [226] and marked strengthening of intermolecular bonds between first-hydration-shell water molecules near a nonpolar surface compared with the molecules of neighboring shells [227]. An ultrafast electron crystallographic method was employed in a study on the evolution of water structure at a model amphiphilic surface (specially treated silicon) [228]. It was shown that characteristic times of decomposition of hydrogen-bonded structures in the surface layers and in the bulk differed by an order of magnitude. At the same time, measurement of the rotational mobility of water molecules in the protein hydration shell and in the bulk by magnetic relaxation dispersion method demonstrated only a two-fold decrease in mobility [80].

Proofs of the existence of specific dynamic properties of *biological* water were obtained by terahertz absorption spectroscopy in experiments with water around a lactose molecule (with a fraction of a picosecond resolution [217, 229]) and water at the protein surface (with a femtosecond resolution [230]). Also, a few layers of water molecules ordered by virtue of their interaction with the heads of lipid molecules were found close to the biological membrane [231]. Studies on water molecule dynamics in micelles revealed no significant changes in mobility in the core and its considerable decrease in the Stern layer comprising polar groups of amphiphils and counter-ions [232]. Both experimental results and MD calculations suggest the existence of three types of water molecules, viz. (1) molecules bound only to other water molecules (*free* water), (2) molecules having one or (3) two hydrogen bonds with polar groups of a macromolecule (*bound* water) [233].

Reduced mobility of water molecules near surfaces is qualitatively similar to the behavior of supercooled water. Some authors even report the magnitude of 'equivalent temperature shift' and estimate this parameter by MD simulation as equaling 40 K for water confined in a nanospace between two smooth *hydrophobic* walls [211]. On the other hand, simulation for the case of *hydrophilic* surfaces of $Mg(OH)_2$ hydroxide indicates that the restriction effect is more akin to elevation of pressure [234]. A fall in temperature results (as in the aforementioned nanotube experiments [185]) in a change of temperature dependence of relaxation time in approximately the same temperature range (222 and 220 K for water at DNA and lysozyme surfaces, respectively [235]).

The discrepancy among data on the dynamic properties of biological water, obtained by different methods, can be accounted for by the difficulty in distinguishing between the effects of motion of water molecules and macromolecules in individual experiments [80]. The author of Ref. [80] emphasizes that the reduced mobility of macromolecules in solution compared with that of an equivalent (in the hydrodynamic sense) body (sphere, ellipsoid of revolution) is also related to marked surface irregularity.

Finally, any biological medium has a complicated microstructure. In the absence of a seed surface, ice formation in a supercooled solution is initiated by nucleation, either homogeneous [236] or heterogeneous. The latter predominates when the inequality $\sigma^{vs} - \sigma^{vl} < \sigma^{ls}$ ('wettability criterion') holds, where σ^{ij} is the surface tension at the i/j interface, and the indices v, l, and s stand for the vapor, liquid, and solid phases, respectively [237]. Nucleation occurs either on the cell membrane [238] or cytoplasmic structures; its contribution to ice formation on subcellular objects increases with decreasing temperature [239]. Heterogeneous nucleation is absent in cells of certain woody plants [240] where ice and supercooled water may co-exist [241]. Phase transition in a cooled biological medium occurs over a certain temperature range [242, 243] (biological tissues are not unique in this respect — the same is true of soil freezing [221, 244]).

2.2 Cryogenic effects

The severity of cell injury depends on the freezing and thawing rates, exposure time, and environment [isolated cell; densely packed cell ensemble in a tissue sample (*in vitro*), and cells in a living organism (*in vivo*)].

2.2.1 Cellular processes under hypothermia. The normal body temperature of warm-blooded animals (and even certain cold-blooded organisms due to efficient behavioral thermoregulation) ranges from 35 to 42 °C. Human brain temperature varies within one-tenth of a degree [245], while some organisms die from overcooling before they are frozen [246]. At the same time, many types of cells tolerate cooling to above a respective freezing point (e.g., erythrocytes survive for 1.5 months [247]). Nature has developed two cold adaptation strategies for living organisms [248 – 250].

One is prevention of crystallization. During acclimation [251], organisms accumulate oligosaccharides [252, 253], other soluble carbohydrates [254], glycerol [255], and free amino acids [240] that interfere with ice formation [256]. Antifreeze proteins (AFPs), or thermal hysteresis proteins (THPs) act with the same effect by lowering the crystallization temperature of a solution without altering melting temperature [257]. The hysteresis amplitude depends on the AFP concentration [258] (see papers [259, 260] for molecular and genetic cold adaptation mechanisms in psychrophilic organisms).

The other is control of crystallization in space (ice formation outside cells) and time (to be precise, in a temperature range, for example, by minimizing supercooling ΔT and thereby limiting the nucleation rate $J \propto \exp(B\tau)$, where $\tau \propto (\Delta T)^{-2}$ [238]). In this case, the ice formation process is modified by 'nucleators', i.e., proteins facilitating the birth of ice nuclei of a critical size[5] at a required temperature (as a rule above -10°C [262]) in those parts of the body that tolerate ice presence (extracellular space where nucleators-proteins were found *in vivo*). Ice formation control ensures dehydration of vital organs [255] and protects them from injury. Organisms of this group also comprise antifreeze proteins that preclude recrystallization [263].

The strategy changes depending on the environment [264]. The lizard *Lacerta vivipara* uses both strategies [265].

Certain animals are able to recover all physiological functions after prolonged freezing of 50% (up to 70% according to [266]) of their total body water (e.g., 65% in the tree frog *Rana sylvatica* for two weeks [253, 267, 268]). Some organisms tolerate intracellular ice [269]. Insects survive after a fall in temperature to -70 °C [249]; the same is true of a number of woody plants (to -50 °C [252, 270]). Desiccated seeds and invertebrates are preserved at temperatures close to absolute zero [271]).

Lowering of temperature initiates the synthesis of heat shock proteins (sometimes called heat stress proteins [272] or cold shock proteins [256]) [274, 275, 277, 278] that contribute to maintaining cell homeostasis, e.g., compensate for the decreased rate of metabolic reactions [240]. That these proteins are so confusingly misnamed reflects the history of their discovery: they are actually synthesized in response to a variety of physiological stress factors, such as heating, cooling, anoxia, ischemia, and the presence of toxic substances. Their universal nature is manifested in the fact that they are expressed in all living organisms, from bacteria to humans [273, 274]. An objection against the usage of the term 'stress' proteins [275] (their being produced under normal conditions, too) equally applies to the term 'heat shock' proteins. Specific and nonspecific reactions of plants to cold, lack of moisture, and elevated salinity at the molecular and cellular levels are dealt with in Ref. [276]. It should be recalled that nonspecific reactions to adverse impacts at the organism level are explained by Selye's theory of the general adaptation syndrome, which is widely known.

Notwithstanding the aforesaid, cooling may kill the cell [34, 279] by changing protein tertiary structure, by disturbing polypeptide constituents of proteins composition, or by protein denaturation [257, 280]. The stability of native conformation depends on the difference between the Gibbs free energies of native and denaturated states [281]. As a function of temperature, these energies have a parabolic form with a maximum in the 15 – 25 °C range [282]. The presence of H-bond net in the system of water molecules interacting with proteins plays an important role in the denaturation process [203] (see also Ref. [238] for a comprehensive review of protein stability).

Denaturation of protein. 'Cold' protein denaturation (distortion of the secondary structure maintained mainly by hydrogen bonds [284]) at $0 - 20$ °C [285] may be either reversible or irreversible, in contrast to usually irreversible thermal denaturation [278]. This process is interpreted as a sharp cooperative transition [286] of the 'all or nothing' type [287]. Such a definition is not very rigorous (see Ref. [288] for a review of intermediate protein states), but the single-stage model described by the first-order irreversible reaction $N \xrightarrow{k} D$, where N and D are native and denaturated proteins, provides a fairly good approximation to the solution of many problems. The amount of denaturated protein is given by

$$F_{\mathrm{d}} = 1 - \exp\left(- \int_0^t k \, \mathrm{d}t \right),$$

where the temperature dependence of the reaction rate obeys the Arrhenius model:

$$k(T) = A \exp\left(- \frac{\Delta E}{RT} \right),$$

where ΔE is the activation energy. There is little doubt as to the value of high-order kinetic models into which intermediate protein states between the native and the denaturated ones are explicitly introduced [278], provided the post-unfolding (reversible) state U is distinguished from the final (irreversible) denaturated state resulting from aggregation, chemical modification of amino acid residues, or other causes [289]. The process is described as comprising two stages (Lumry – Eyring model): $N \Longleftrightarrow U \longrightarrow D$. More complicated protein denaturation models were considered in the aforecited work. The protein denaturated state is not necessarily unique [288].

Membrane behavior. The cell membrane constitutes a bilayer of amphiphilic (i.e., having hydrophilic and hydrophobic sections) lipid molecules of three types, viz. phospholipids, glycolipids, and steroids. A membrane contains up to one hundred types of lipid molecules [68], along with peripheral and integral proteins. Membrane lipids have a small polar head and a long nonpolar (hydrocarbon) tail. Membrane structure, stability, and properties depend on the interaction

[5] Those organic molecules are efficacious that have a characteristic linear size commensurable with that of the ice lattice constant; they serve as crystallization nuclei for ice growth from a gaseous phase (for example, $C_nH_{2n+1}OH$ monolayer raises nucleation temperature to -8 °C for even $n \geqslant 22$, and to -1 °C for $n = 31$). Then, the critical nucleus size is only 20 Å which corresponds to some 50 water molecules and compares with the critical nucleus size in homogeneous nucleation at -39 °C [261].

between membrane molecules and water. Part of the water is osmotically inactive (hydration shells around the polar components of lipid and protein molecules, together with individual water molecules in the hydrocarbon moiety of the membrane). Surfaces of many membranes are on average negatively charged [290]. Different mesophases are realized, depending on water content in the system and concentration of electrolytes [291]. The normal state of a biological membrane is that of a lyotropic liquid crystal [292].

The classical liquid-mosaic model [293] implies a single type of lipid molecule. Generalization to the case of two different lipids [294–296] appreciably extends the range of issues that allow rigorous consideration; in particular, it makes possible the correct description of membrane strain [296] and phase separation [295, 297]. Heterogeneity and the dynamic character of the membrane structure are indispensable for its functioning [272]. Membranes are sensitive to temperature variations (thermotropic mesomorphism [68]). Heating results in the formation of hexagonal structures in the lipid bilayer, while cooling induces transition of constituent lipids to the gel phase ('liquid–solid' transition, the *main phase transition* [245]). This transition is associated with a sharp size change [298], causes creation of defects between membrane proteins and lipid layers [299, 300], and enhances membrane permeability to ions [301]. The critical transition temperature depends on water content in the system and drops to a minimum as soon as it exceeds the amount of water capable of binding by lipid structures [68]. It also depends on the membrane composition (saturated to unsaturated phospholipid ratio, a well-known example being a change in the fraction of unsaturated lipids in the cells of a reindeer's leg from the hooves upward with varying characteristic temperature by dozens of degrees. Cooling decreases the muscle membrane potential and excitability of myofibrils; this effect is ascribed to membrane disintegration [257] and its altered chemical composition (primary removal of hydrophilic lipid components [302]). Fragmentation of a membrane may be preceded by the loss of proteins contained in it [240]. An impaired mechanical strength of the membranes is confirmed in a cooled cell centrifugation experiment [303]. A drop in temperature causes depolymerization of the cytoskeleton [51] and impairs the ability of integral proteins to regulate intracellular electrolytes [304] that may also lead to denaturation of protein.

2.2.2 Treatment of cells in aqueous solutions. The behavior of a single cell being frozen as a function of the cooling rate (see, for instance, Refs [39, 40, 305, 306]) can be accounted for in the framework of the 'saltwater sack' model. The process starts from crystallization of a supercooled solution and causes its concentration to increase. The rate of nucleation first increases with an increasing degree of supercooling, then reaches its peak and thereafter drops, because the mobility of molecules decreases [238]. Primary and secondary nucleations are distinguished in polycrystal growth, the latter being interpreted as processes at grain boundaries [307]. The morphological features of a growing crystal (including fractal dimensionality in the case of dendrite growth, if any) is first and foremost determined by the degree of overcooling [303, 308].

Cell dehydration. Slow cooling destroys cells via dehydration (water leaves the cells both by the agency of diffusion across the membrane and through the pores in it [309]); this leads to a rise in intracellular solute concentration and cell shrinkage. In the approximation of an ideal solution (with the difference between the chemical potentials of cytoplasm and water proportional to $\ln(1-\chi)$, where χ is the molar fraction of dissolved substances), the drop in crystallization temperature is given by $\Delta T \propto \chi$ [310]. This colligative effect is determined by the *amount* of dissolved substances rather than their chemical nature; an additional effect for macromolecules is related to their surface properties [196]. Notice that the influence of the dissolved substances on the hydrogen-bond net and crystallization temperature is in many respects analogous to elevated pressure effects [311]. The prevention of ice formation within a cell plays a protective role [252], but the growing concentration results in 'osmotic' shock, i.e., protein denaturation, membrane lysis, and injury to the nucleus and mitochondria [35, 312]. Membrane behavior is susceptible to the temperature triggering crystallization of the solution [285].

The two-chamber model of cell dehydration and shrinkage (a change in its volume, to be precise) in a freezing solution [313] was proposed over 40 years ago and still provides a basis for the majority of contemporary models. It is underlain by the Kedem–Katchalsky equations (derived from the principles of linear thermodynamics of irreversible processes) for the nonelectrolyte transfer through a permeable membrane separating well mixed diluted ideal solutions [314]:

$$J_v = L_p \Delta P - L_p \sigma \Delta \Pi\,, \qquad j_s = \omega \Delta \Pi + (1-\sigma)\,\bar{c} J_v\,,$$

where J_v is the bulk flow, j_s is the solute flow, L_p is the hydraulic permeability (filtration factor), σ and ω are coefficients of reflection and permeability, respectively, ΔP and $\Delta \Pi$ are the differences between hydraulic and osmotic pressures on the membrane, and $\bar{c}$ is the mean concentration. The 'mechanistic' derivation of equations for transmembrane transport proposed in Refs [315, 316] offers a simple interpretation of coefficients entering these equations. The same publications consider membranes with pores of different radii.

Osmotic transport of water from the cell [317] is limited by temperature-dependent membrane permeability

$$L_p = L_{p0} \exp\left[-\frac{E_a}{R}\left(\frac{1}{T_0}-\frac{1}{T}\right)\right]$$

and is the sole parameter of the model; here, E_a is the activation energy. Extension of the model to the case of a cryoprotector present in the solution and able to penetrate into the cell [39, 318] adds membrane permeability to the cryoprotector and the parameter describing solute–water interactions. Generalization of the model to the case of concentrated nonideal solutions is reported in Ref. [319]. The most comprehensive formulation of the problem for a cell in a multicomponent solution [320] takes into account the effects of membrane stress and nonunit distribution coefficients of the dissolved substances. Membrane stress is considered on the assumption of cell sphericity and the threshold character of the membrane response (surface stress is supposed to vanish when the membrane surface area is smaller than a certain specified value).

An arbitrarily shaped cell was considered in Ref. [317]. Although cytoplasm has a nonzero hydrodynamic velocity (unlike that of a spherical cell), its scale is given by the velocity of membrane motion, and the quasistationary analysis holds

as before. A numerical solution of the equations is possible in the general case. The authors reported approximate results for the practically important case of cylindrical cells.

This zero-dimensional model neglects diffusion processes in cytoplasm. Such an assumption is true, for example, for leukocytes and lymphocytes, but does not hold for erythrocytes [321]. A one-dimensional model including diffusion [322] allowed dehydration regimes to be categorized into those limited by membrane transit and those constrained by intracellular diffusion.

Intracellular ice. Rapidly cooled water fails to leave the cell and the resulting intracellular ice [323] causes its death from membrane disruption and cytoskeleton disintegration [324] (see paper [325] for more details about mechanical cell injury).

The probability of spontaneous nucleation depends on the cytoplasm volume, degree of supercooling, solute concentration, and fluid viscosity. The relationship between intracellular ice formation temperature and the fraction of unfrozen water has been experimentally investigated in Ref. [323]. The thermodynamic model of intracellular ice formation taking into consideration heterogeneous nucleation at the cell membrane and cytoplasmic particles has been developed in work [326]. Based on the classical theory of nucleation [6] as a sequence of bimolecular reactions $\alpha + \beta_m \Longleftrightarrow \beta_{m+1}$, the authors estimated the homogeneous nucleation rate I^s as a function of two parameters — thermodynamic Ω, and kinetic $\varkappa$:

$$I^s = \Omega \exp\left(-\frac{\varkappa}{\Delta T^2 T^3}\right),$$

where

$$\Omega = 2(v^\beta)^{1/3}\frac{(\sigma^{\alpha\beta}kT)^{1/2}}{\eta v^\alpha N_v}, \qquad \varkappa = \frac{16\pi(\sigma^{\alpha\beta})^3 T_f^4}{3kL^2}; \qquad (1)$$

here ΔT is supercooling, v^α is the molecular volume of phase α, $\sigma^{\alpha\beta}$ is the free energy at the ice – water interface, L is the crystallization heat, and η is the cytoplasm viscosity approximated as

$$\frac{\eta}{\eta_w} = \exp\left(\frac{5}{2}\,\frac{\phi_m}{1 - Q\phi_m}\right),$$

where η_w is the viscosity of pure water, ϕ_m is the total volume fraction of the solutes (e.g., salts and proteins), and Q is the interaction parameter; either the Vogel – Fulcher formula

$$\eta \propto \exp\left(\frac{E}{T - T_0}\right)$$

or the power approximation is used for the temperature dependence of viscosity. Also, the authors of Ref. [326] proposed modification of parameters Ω and $\varkappa$ for heterogeneous nucleation, which was reduced to the appearance of factors $[f(\theta_0)]^{1/6}$ and $f(\theta_0)$, respectively, in Eqns (1), with $f(\theta) = (1 - \cos\theta)^2(2 + \cos\theta)/4$ and θ_0 being the contact angle between the ice cluster and the membrane inner surface that linearly depends on solution concentration. This model is most sensitive to $\varkappa$ and η values. It was later coupled to the trans-membrane water transport model [327] and applied to a water – NaCl – glycerol mixture.

At the end of freezing, ice crystals may be so small that cell organelles remain intact; however, recrystallization upon thawing leads to crystal enlargement which is deleterious to the cell [328]. A decrease in temperature reduces the efficiency of active ion transport across the plasma membrane in the course of metabolic reactions [329]. Eutectic crystallization also injures the cell [330]. The role of osmotic shock was studied in Refs [303, 331] by mimicing the evolution of cell environment during freezing [an initial rise in solute concentration ('crystallization of the solution') followed by dilution ('warming')]. Substituting NaCl with a lithium salt exhibiting a stronger lyotropic effect results in more serious cell injuries [303]. Cell resistance to modulation of its volume and cytoplasmic composition at the molecular level remains to be elucidated; it is known to depend on activation of ion channels and transport mechanisms, as well as on regulation of protein synthesis [44, 276].

There is an optimal cooling rate for cell survival [332]. It is given by the relationship between characteristic times of heat withdrawal during ice crystallization and of water diffusion across the membrane. In this context, the size of the cell (or the volume-to-surface ratio for cells whose shape is far from spherical) and hydraulic conductivity of its membrane are the most important parameters [333]. By way of example, the optimal cooling time for disk-shaped erythrocytes having high membrane permeability is up to three orders of magnitude higher than that for other cell types [111]. Cells also differ in terms of sensitivity to the thawing rate [334], the response being more pronounced in the presence of a cryoprotector than without it [335]. They benefit from rapid thawing.

Cryoprotectors. The optimal cooling rate during cryopreservation is modified by cryoprotectors [33, 128, 247, 336 – 338] categorized into two groups depending on their ability to penetrate a plasma membrane. One group comprises intracellular (endocellular) agents [339] that lower electrolyte concentration and preclude reduction of the cell volume in a hypertonic solution. Extracellular (exocellular) cryoprotectors stimulate the loss of water by the cell and inhibit intracellular ice formation at a high cooling rate. Certain cryoprotectors (glycerol, succharose) prevent partial denaturation of proteins in a freezing solution [340] and inhibit eutectic crystallization [330]. Cryoprotectors include both low-molecular compounds [dimethylsulfoxide $(CH_3)_2SO$ (DMSO), glycerol, disaccharides, etc.] and high-molecular-weight polymers [129]. In addition, metal nanoparticles are utilized to modify cryogenic exposure [341]. One of the parameters accounting for the efficiency of cryoprotectors is the amount of water they are able to bind [342].

Cryoprotector molecules interact with the cell membrane even at room temperature, changing the conformation of integral proteins in the lipid bilayer [343]. They reduce membrane fluidity [344], main phase transition temperature [254, 345], and the temperature of transition from the lamellar liquid-crystal phase to the inverted hexagonal one [346], and also change the membrane's hydraulic conductivity [347], its temperature dependence (activation energy) [61, 348, 349],

[6] In this theory, the nucleation rate $J = J_0 \exp(-W^\star/kT)$, where the prefactor J_0 is related to molecular mobility, and the excess energy $W^\star$ of critical cluster is computed on the assumption of its sphericity and constant density ('liquid drop model' in which energy is represented as $E = -4/3\pi R^3\Delta F + 4\pi R^2\sigma^{\alpha\beta}$, where ΔF is the free energy difference between phases per unit volume, and $\sigma^{\alpha\beta}$ is the specific surface energy). The accuracy of the analysis decreases for very small critical nuclei and polar molecules. A more rigorous model based on the density functional method takes into account the density distribution $\rho(r)$ along the radius [236].

the thermophysical properties of the solution [350] (including a decrease in crystallization temperature [351]), and the morphology of the growing crystals (for example, an elevated DMSO concentration facilitates the formation of secondary dendrites [352]). Cryoprotectors lower intracellular ice formation temperature [353].

Oligosaccharides and other low-molecular-weight compounds prevent too close a contact of plasma membranes under strong hydration conditions; this slows down their transition from the liquid phase to the gel [254]. The effect of the chemical nature of cryoprotectors (DMSO, sorbitol, succharose, trehalose) on the freezing of the lamellar phase [354] of the model lipid membrane was investigated by the nuclear magnetic resonance (NMR) technique which measures the fraction of unfrozen water; heavy water (D_2O) was used as a solvent in work [355]. It was shown that all the above substances strengthen hydration of the lamellar phase even though a purely osmotic effect predominates at low succharose and trehalose concentrations. The proposed thermodynamic model of the system under consideration disregards the size of water and cryoprotector molecules with respect to the distance between the lipid layers.

Antifreeze proteins [37, 258, 356–359] show their worth in two ways. On the one hand, they inhibit ice growth and recrystallization during thawing [250, 360–364]. These molecules are fitted into the growth steps at the crystal surface and cause multiple fragmentation of the crystallization front. Greater front curvature results in a rise in the surface free energy and front deceleration. A large area of the protein surface is involved in the interaction with the ice [258]. Studies with a scanning tunneling microscope have shown a system of grooves or protein adsorption sites, the depth of which (2–10 nm) is a measure of the curvature of the crystallization front [363]. Moreover, MD calculations suggest a raising of the energy barrier of homogeneous nucleation in the presence of an antifreeze protein [364]. On the other hand, these proteins affect the morphological structure of extracellular ice crystals and promote cell destruction [365]. The latter property is used in cryosurgery [366]: at a sufficiently high antifreeze concentration, crystals assume a needle-like (bipyramidal) shape and mechanically disrupt the plasma membrane [34, 270]. Crystals formed by two hollow hexagonal pyramids may also grow in pure water if supercooling exceeds 2.7 °C [367]. The temperature T_I at the water–ice interface can be represented as

$$T_I = T_m\left[1 - d_1(\theta,\phi)\,k_1 - d_2(\theta,\phi)\,k_2 - \beta(\theta,\phi,V_n)\right],$$

where T_m is the melting temperature, θ,ϕ are the angles defining the orientation of the boundary with respect to two orthogonal axes, d_1, d_2 and k_1, k_2 are the corresponding values of capillary lengths and interfacial curvatures, and V_n is the normal interface velocity. Kinetic supercooling $\beta(\theta,\phi,V_n)$ describes the deviation from equilibrium for a given orientation and normal velocity. Morphological distortion of the crystallization front is determined by the dependence of surface tension and the supercooling kinetic coefficient on the crystallographic orientation of the surface. More complicated structures can be seen at supercooling above 5.5 °C.

Cell interaction with the crystallization front. Cells in a freezing solution are injured mechanically and when they enter channels between ice crystals growing in the form of 'fingers' [40]. A crystallization front propagating in a supercooled solution is unstable [368]. Its instability in the presence of cells shaped like hard spherical particles was considered in paper [369]. The volume fraction ϕ of the particles in solution is described in the coordinate system associated with the front by the mass conservation equation

$$\frac{\partial\phi}{\partial t} - V\frac{\partial\phi}{\partial z} = \frac{\partial}{\partial z}\,D(\phi)\,\frac{\partial\phi}{\partial z},$$

where V is the crystallization front velocity, $D(\phi) = D_0\hat{D}(\phi)$ is the diffusion coefficient, $D_0 = kT/6\pi R\mu$, R is the particle's radius, μ is the dynamic viscosity of the liquid, $\hat{D}(\phi) = (1-\phi)^6\,\mathrm{d}(\phi Z)/\mathrm{d}\phi$, and the compressibility parameter $Z(\phi)$ for the hard sphere model is the fourth-order rational function [369]. The diffusion of particles in the solid phase is neglected, and the mass conservation condition at the front takes the form $(\phi_l - \phi_s)\,V_n = -D(\phi_l)\,\nabla\phi_l\,\mathbf{n}$, where V_n is the normal front velocity, and concentrations ϕ_l in the solution and ϕ_s in the solid phase are related by the distribution coefficient k_s: $\phi_s = k_s\phi_l$. The linear analysis of stability shows that enlarged particle size stabilizes the front, while an elevated concentration strengthens the instability.

The behavior of cells with respect to the crystallization front depends on the front velocity (cells are rejected from the interface if the velocity exceeds a threshold value, V_c, and are trapped in the ice if the velocity is lower than that [370]). The problem contains two linear scales, besides the particle size [371]. One is $\Gamma = T_m\sigma^{sl}/\rho_s LRG$ (σ^{sl} is the surface energy, ρ_s is the solid phase density, R is the particle's radius, and G is the temperature gradient); it defines the effect of the crystallization front curvature. The other is related to scale λ and the molecular interaction pattern: $l = (\lambda^v T_m/G)^{1/(v+1)}$ ($v = 3$ for van der Waals forces, and $v = 2$ for electrostatic interactions). The critical radius R_c is given by the condition $\Gamma = \lambda$. The analytical solution $V_c \propto R^{-1}$ is possible for large particles ($R \gg R_c$), and $V_c \propto G^{1/4}$ for van der Waals forces. In the opposite case ($R \ll R_c$), V_c is independent of the temperature gradient and $V_c \propto R^{-4/3}$ [371].

A further rise in the cooling rate again promotes cell survival. In Ref. [372], where this parameter was varied from 5 to 30,000 °C min^{-1}, the fraction of viable cells preserved after freezing grew *monotonically* starting from 5000 °C min^{-1}. The authors offered two explanations: (1) the formation of small ice crystals in the cell (characteristic size decreases with increasing cooling rate [373]) that do not injure the plasma membrane, and (2) vitrification.

One mode of vitrification is via extra fast cooling (a cooling rate of 130,000 °C min^{-1} was achieved for volumes of roughly one nanoliter using supercooled (−200 °C) liquid nitrogen [374]; the cooling rate above 300,000 °C min^{-1} is predicted as attainable with the aid of special systems maximizing the heat-transfer coefficient [375]). Another method is based on the use of concentrated cryoprotector solutions (up to 50% by mass) [127, 128]. In this case, a sample may contain both crystal ice and amorphous ice regions [376]. One effect of high cryoprotector concentration consists in enhancing viscosity of the solution, thus facilitating vitrification [377].

Cryoprotectors have the disadvantage of being highly toxic [378]. Their toxicity becomes apparent after transplantation [379]. Another drawback is the difficulty of saturating tissues during cryosurgery and in the cryopreservation of large objects (the microinjection technique [362] only mitigates the problem). The toxicity has either a chemical or an

osmotic nature [46]; moreover, intracellular cryoprotectors can directly affect cell organelles [44]. The toxicity of these cryoprotectors largely depends on the strength of hydrogen bonding between their polar moiety and water [380]. To reduce toxicity, cytoplasm perturbation should be minimized by the use of a mixture of cryoprotectors [380 – 382] containing trehalose and glucose in highly concentrated solutions [381, 383] or by preliminary saturation of the tissues with an ethylene glycol solution [129].

The effect of different cryoprotectors on amorphous ice formation and stability was evaluated in Ref. [384] by the example of erythrocytes. Vitrification temperature proved to be the main variable determining the behavior of a glassy specimen during storage (it is a highly viscous metastable liquid above this temperature, and an amorphous solid below it). Comparison of different models for the determination of vitrification temperature was undertaken in Ref. [385] using a water – trehalose mixture. It was found, in particular, that the usual approach to the estimation of the weighted mean value of the parameter Y_{mix} from the values Y_i for components: $Y_{\text{mix}} = \left(\sum Y_i f_i\right) / \left(\sum f_i\right)$, where f_i is the parts of fractions in appropriate units (molar, mass, or volume), was impracticable. The authors considered the Kochman – Karasz and Gordon – Taylor models for binary solutions and their generalization to a mixture of cryoprotectors of a close chemical nature (e.g., low-molecular hydrocarbons). In earlier studies, the vitrification temperature of water was assumed to be 136 K; however, recent authors report it to be higher (from 145 K [190] to 160 K [386] or 165 K [387]). Thawing needs to be carried out so as to avoid devitrification [388], i.e., the nucleation and growth of ice crystals. In concentrated solutions, both hexagonal ice I_h and cubic ice I_c undergo nucleation [389]. Nucleation in aqueous ethylene glycol solutions was investigated in paper [390] based on the – Johnson – Mehl – Avrami – Kolmogorov model wherein variations in the fraction x of crystallized ice with time are described as $x = 1 - \exp\left[-(Kt)^n\right]$, where the parameter n depends on crystal morphology. The authors proposed the expression $K \propto D(T)\, N^{2/3}$ for the reaction rate constant, where $D(T)$ is the diffusion coefficient of water molecules at temperature T, and N is the number density of crystallization nuclei.

A cryomicroscope [391] was used to watch intracellular ice formation, cell deformation in the course of dehydration and interaction with ice crystals, as well as some other phenomena [93, 392].

2.2.3 Cryogenic exposure *in vitro*. Freezing in tissues and freezing in solutions have much in common, even though the distances between cells in the former are much smaller than in the latter [34]. The main differences are the higher resistance of close-packed cells to dehydration compared with suspended cells [393] and the influence of intercellular contacts on the intracellular ice distribution. This last phenomenon was explored in papers [394 – 398]. In particular, the possibility of ice front propagation through membrane pores was demonstrated experimentally and confirmed in Ref. [399] by the regularity of time delay between the appearance of intracellular ice in neighboring cells. Ice was seen traveling along the channels formed by aquaporin [400, 401].

This protein was found in a variety of organisms, from plants and bacteria to mammals [402]. In membranes, it forms tetramers in which monomers make channels permeable to water and impermeable to charged ions or molecules. The fifth channel in the center of a tetramer permits the passage of both water and charged particles [403]. Chemical blockade of the pores impairs ice propagation in the neighboring cells but fails to stop it completely [404]. The intensity of the process is temperature-dependent; ice propagation is totally arrested at temperatures above a certain threshold level [399] as a result of lowering crystallization temperature in the capillary [397]. It should be noted that analysis of crystallization front propagation in pores 0.8 – 2.5 nm in diameter in the framework of the continuous medium model provides only a qualitative description.

The predominant nucleation mechanism of intracellular ice depends on the cell type and membrane properties. The authors of Ref. [405] considered three mechanisms, viz. homogeneous nucleation in cytoplasm, ice propagation through membrane pores, and ice propagation through membrane injuries inflicted by an electric field [406]. The field is generated due to the interactions of ions of the solution with the crystallization front. This process gives rise to a double electric layer at the phase boundary and the resultant potential difference between the liquid and the solid phases (freezing potential). This effect, known as the Workman – Reynolds effect [407], is believed to be responsible (together with charge transfer during collisions of ice particles [408]) for charge separation in thunderclouds [409] and generation of electromagnetic radiation by growing ice [410]. The probability of electric breakdown in the membrane increases with decreasing temperature due to enhanced surface tension in the lipid bilayer.

The problem of pore formation in plasma membranes was considered both in the framework of the continuous medium model [411, 412] and by MD simulation [413]. The growth of extracellular ice was shown to affect the transmembrane potential and cause membrane proteins having a dipole moment to reorientate [414]; this led to altered membrane permeability.

It turned out that none of the above models explains the totality of experimental data on intracellular water crystallization at different cooling rates and concentrations of cryoprotectors. The authors of Ref. [405] proposed a new mechanism of membrane injury that includes an intense water flow caused by osmotic pressure difference and its mechanical action (friction) on the lipid bilayer constituting membrane. The friction force per water molecule is proportional to the drift velocity: $F_f/n = fv$ defined via the mass flux as $v = (J_w h)/n$, where h is the depth of the membrane hydrophobic region. Then, the overall force acting on the membrane is $F = f J_w h$. The coefficient of friction is estimated from the analog of the Einstein relation $f = kT/D_w$, where D_w is the water diffusion coefficient. Then, the mean pressure on the membrane, $P = (kT/AD_w)\, J_w h$ (A is the membrane surface area) [415], is comparable to the maximally permissible one. This hypothesis is confirmed in Ref. [309]. It is also supposed in work [416] that the state of the membrane depends on a set of parameters, including water flow intensity, osmotic pressure difference and the value of pressure.

Important factors influencing ice propagation are the cell interaction with the extracellular matrix [417] and the amount of tissue water [418]. The behavior of individual cells differs from the behavior of a cell system; it was illustrated by the example of spherical cell (70 to 140 μm in diameter) aggregates surrounding a charged polysterol particle [419]. The authors do not discuss the role of interactions with the

surface, but it may be very important even for a single cell [420]. Analysis of the propagation of water and cryoprotectors in fragments of biological tissues containing thousands of cells indicated that cellular membrane properties are of primary importance even though interstitial diffusion effects should be taken into consideration, too [421]. Under certain conditions, a frozen sample displays a network of ice crystals in the extracellular space [422].

The osmotic effect and ice crystallization cause irreversible changes in biological tissues. Damaged intercellular links fail to recover after thawing. Therefore, the microstructure and the mechanical and thermophysical properties of the tissue remain distorted [418, 423–425]; it may contain cavities [426] that are sometimes (e.g., in the liver) arranged into regular structures. It was proposed to use this fact in forensic medicine to determine whether a tissue was exposed to freezing in the past [427].

Another mode of ice front propagation in tissues is by traveling in blood vessels [34]. The growth of ice crystals in microvessels (arterioles and capillaries 10–15 and 4 μm in diameter, respectively [428]) differs from that in large ones. Recent studies of lowering water crystallization temperature [429] and of ice propagation [430] in glass capillaries have revealed a critical radius beyond which the front propagation is considerably retarded or totally arrested. The ultimate supercooling ΔT is written out in the following form, taking into account the front curvature and osmotic effect:

$$\Delta T = \frac{2 v_s \sigma^{ls} T_m \cos\alpha}{Lr} + \frac{v_l \pi_l T_m}{L},$$

where v_s and v_l are the specific volumes of the solid and liquid phases, respectively, π_l is the osmotic pressure of the solution, r is the capillary radius, and α is the angle between the interface and the capillary wall satisfying the Young relation $\sigma^{lb} = \sigma^{sb} + \sigma^{ls}\cos\alpha$ (front curvature $k = 2\cos\alpha/r$).

Experiments with capillaries with a radius from 1.5 to 87.5 μm demonstrated that the critical radius r_0 below which ice propagation is impossible was related with a high accuracy to the minimal wavelength λ_0 in the analysis by Mullins–Sekerka of stability of the flat front: $r_0 \approx \lambda_0/4$. The authors emphasize that caution is needed in extrapolation of their results to cryobiology, the reason being the difference between wetting angles and wall elasticity in glass capillaries and blood vessels.

Despite substantial progress in cryopreservation (Refs [431, 432] report restoration of the viscoelastic properties of human blood vessels), the practical use of cryoconserved tissues (and even more so organs [42]) encounters serious difficulties. First, the presence of different types of cells in a single tissue hinders the choice of optimal freezing and thawing protocols. Second, temperature uniformity is impossible to achieve in a large tissue slab. Hence, there is a thermoelastic stress in the tissue leading to cracks [433] that contribute to its disintegration and pose the risk of devitrification upon heating by stimulating heterogeneous nucleation of ice crystals [127]. The threat of cracking in the course of vitrification is greater than during cryosurgery because the glassy state is more fragile and temperature gradients are rather high. This makes studies of slow tissue cooling in the presence of cryoprotectors as challenging as ever before [434, 435]. Because the coefficient of thermal expansion strongly depends on the presence of a cryoprotector [436], inhomogeneous tissue saturation also contributes to mechanical stress. Tissue strain is a function of both cryoprotector concentration and cooling rate [437]. Analysis of stress patterns in a tissue sample residing in a mixed crystal/amorphous state implies consideration of its viscoelastic behavior [376].

2.2.4 Cryogenic exposure *in vivo*. Cryogenic destruction of cells and tissues in a living organism has more serious consequences than ensue from *in vitro* studies of isolated cells and tissues [438]. A major additional *in vivo* effect is microcirculatory disturbances [35, 439] (the walls of large arterial vessels as a rule recover their properties after thawing).

Freezing-induced injuries of blood vessels were first described in 1877 [366] (to experience this effect, one should take a walk without gloves in the extreme cold). The severity of vessel injuries depends on the thermal history. Cell destruction is governed by the water redistribution mechanism [440]. There are three groups of blood vessel injuries, viz. (1) mechanical deformation of vascular walls by the ice formed in the vascular bed that causes a more than two-fold increase in the diameter of small vessels [441]; (2) the death of endothelium cells or their detachment from vascular walls [439, 442], and (3) failure of circulation itself [438] that show their worth a few hours or days after surgery, depending on the vascular diameter [443].

The difficulty of describing heat exchange in living tissues arises from the necessity of taking into consideration heat transfer with blood; its efficiency is a function of the intricate vascular network [444] that forms fractal structures [445] and of the altered intensity of perfusion in injured tissues [446].

Models of circulatory heat exchange are categorized as continual and vascular [447, 448]. In the former, effective heat conductivity of the medium and/or a spatial heat source are introduced in the energy equation. The most widely used is the Pennes equation [449]

$$\rho c \frac{\partial T}{\partial t} = \nabla \lambda \nabla T + c_b \omega (T_a - T) + \dot{q}_{met} + Q^{ext},$$

where T and λ are the temperature and thermal conductivity of a tissue as a homogeneous medium, ω is the perfusion coefficient having the dimensionality of mass blood flux per unit tissue volume, c_b is the blood thermal capacity, T_a is the arterial blood temperature, and $\dot{q}_{met}$ and Q^{ext} are the heat release in metabolic reactions (which is usually neglected in cryobiology) and energy absorption from an external source. Heat exchange is supposed to occur in capillaries alone, whither blood enters having temperature T_a. In other words, the length over which the thermal equilibrium with the surrounding tissues sets in is assumed to be infinite for all vessels except capillaries, for which it reduces to zero. Tissue temperature in the Pennes model is defined as an average over volume δV:

$$T(\mathbf{r}) = \frac{1}{\delta V} \int_{\delta V} T(\mathbf{r})\, dV,$$

where the linear scale $\sqrt[3]{\delta V}$ is taken to be small compared with the entire region of interest but larger than the diameter of 'thermally important' blood vessels. As shown in Ref. [450] there is no such scale for a well-developed circulatory system. Numerous studies (see, for instance, Refs [451–454]) demonstrated that heat exchange mostly occurs in larger vessels, while capillaries accommodate blood in thermal equilibrium with the surrounding tissues. For all that, the simple Pennes model frequently proves to be more accurate [447, 455] than more sophisticated simulations.

A model proposed in paper [452] takes into account the dependence of heat exchange in tissues on blood vessel size by means of the introduction of the 'thermalization length' L_e as the distance over which the difference in temperatures between the blood and the surrounding tissue decreases 'e' times:

$$\rho c\,\frac{\partial T}{\partial t} = \nabla\,\lambda\nabla T + (\rho c)_b\omega^*(T_a^* - T) - (\rho c)_b\bar{u}\,\nabla T + \nabla\,\lambda_p\nabla T + \dot{q}_{met} + Q^{ext}\,.$$

Here, the term $(\rho c)_b\bar{u}\,\nabla T$ describes the contribution of blood motion to heat exchange (the blood being in thermal equilibrium with the tissue), and $\nabla\,\lambda_p\nabla T$ accounts for the effect of additional 'perfusion' heat conductivity

$$\lambda_p = \pi n(\rho c)_b r_b^2\bar{V}\cos^2\gamma\sum_{i=1}^{\infty}\frac{L_e}{L_e^2\beta_i^2+1}\,,$$

where γ is the angle between the direction of blood vessels and the tissue temperature gradient, n is the number of vessels, and r_b is their radius; the parameter β is assumed to be inversely proportional to the vessel length.

Structural elements of the vascular network (in particular, 'artery – vein' pairs) were incorporated into the three-temperature model of Weinbaum at al. [456]. In this model, venous blood temperature is explicitly described. Later on, the model was reduced to a single equation [457]

$$\rho c\,\frac{\partial T}{\partial t} = \nabla\,\lambda_{eff}\,\nabla T + \dot{q}_{met}\,.$$

Effective heat conductivity tensor is defined as

$$\lambda_{eff}^{ij} = k_t\left[\delta_{ij} + \frac{\pi^2 n r_b^2}{4\sigma}\left(\frac{\lambda_{bl}}{\lambda_t}\right)^2 \mathrm{Pe}^2\,l_i\,l_j\right],$$

where n is the geometric parameter specifying blood vessel density, r_b is assumed to be identical for veins and arteries, l_i are the vessels' direction cosines, $\mathrm{Pe} = 2\rho_{bl}c_{bl}r_b u/\lambda_{bl}$ is the Peclet number, and σ is the parameter describing heat exchange between arteries and veins. The authors of Ref. [457] estimated anisotropic corrections to the coefficient of effective heat conductivity as essential when the radius of blood vessels exceeded 100 μm.

Vascular models of blood-mediated heat exchange are numerous and differ in terms of complexity [447]; some of them are based on concrete anatomic data about the circulatory systems. The contribution from a large blood vessel to heat exchange can be considered explicitly by specifying vascular wall temperature [458, 459], or by using the Navier – Stokes equations for incompressible fluids [460], or on the assumption of Poiseuille flow; the rheologic properties of the blood are usually disregarded [19, 461 – 464].

Some approaches make use of a rigorous mathematical apparatus. In Ref. [12], the homogenization technique is applied to a multiscale periodic problem. A hierarchical system of interacting vessels of different levels (sizes) is considered in monograph [465]. The authors show, in particular, that blood-flow velocity nonlocally depends on the tissue temperature distribution.

The contribution of heat transfer by blood to overall heat exchange in cryosurgical situations is important [108], but to a lesser degree than in the analysis of hyperthermia or biophysical tissue reactions to a burn [466]. *In vitro* experiments with artificial circulation (emulation of *in vivo* heat exchange) [467], as well as experimental and numerical investigations of osteonecrosis [468] have demonstrated that this heat exchange mechanism may be essential in thawing. Naturally, a large blood vessel(s) in the vicinity of the cryogenic treatment site is (are) an exception. Consideration of such a heat source is necessary for the correct description of necrotic zones [469].

The cooling rate in cryosurgery [443, 470] is not as critical as in cryopreservation, partly because it changes dramatically at the site of intervention. Slow warming [470] is basically responsible first for the transiently hypotonic milieu in which cells reside (and, as a consequence, gives rise to the delivery of water into cells at the risk of membrane disintegration [34, 40, 312]), and second for intense ice recrystallization, especially in the temperature range from −40 to −15 °C. Refreezing is equally important [55, 123, 124, 471]. By way of example, it allowed the critical temperature to be reduced by 20 °C to perform cryoablation [472].

3. Cryogenic exposure *in silico*

3.1 Microscopic models

The molecular dynamics method *per se* and as a component of more complicated computational procedures (see, for instance, Refs [473 – 476] and references cited therein) provides a major tool for the solution of biophysical problems. However, it is still finding limited application in cryobiology. High labor input requirements of the method account for the use of simplified or hybrid models of macromolecules and/or multiprocessor computer complexes. [7]

[7] Productivity of parallel computations is characterized by acceleration $S_n = T_1/T_n$, or efficiency $E_n = T_1/nT_n$ (T_n is the computation time with a system of n processors) represented by the product of three factors [477]: parallel efficiency, numerical efficiency, and uniformity of processor loading. Parallel efficiency is the ratio of concrete computation time to the overall time of calculations, data exchange, and waiting for the message: $E_n^{par} = T_{comp}/(T_{comp} + T_{comm})$ [time of sending/receiving messages is shortened by the use of an asynchronous regime (simultaneous computation and data exchange]. Numerical efficiency reflects the enhancement of the volume of computations (e.g., the number of iterations) by using parallel algorithms and varies broadly depending on the problem and the method of its solution. The uniformity of processor loading is achieved either statically or dynamically. Ideally, (1) the number of particles or cells 'assigned' to a processor is proportional to its throughput, and (2) the volume and frequency of data exchange between processors are minimal (it is possible to additionally minimize the number of processor pairs in need of data exchange). Obviously, these requirements are contradictory. A single processor must be used to minimize communications. The problem of $T = T_{comp} + T_{comm}$ minimization is formulated with constraints. For a broad class of problems, $T_{comp} = \max_q(N_q T_q)$, where N_q cells or particles are assigned to the qth processor, and T_q is the time of a single step or iteration. For the most common systems (work station clusters connected through Ethernet or Myrinet), one has $T_{comm} = \sum_{pq}[C_{pq}/b + \delta\,(C_{pq})\,L]$, where C_{pq} is the communication matrix, b is the effective bandwidth of the network, and L is its latency [478].

MD simulation with short-range potentials provides an ideal tool for parallelization (see Refs [479 – 481] for the methods of efficacious realization of long-range electrostatic interactions). The efficiency of the NAMD software packet [482] designed to treat biophysical problems is as high as 85 and 70% with systems of 1024 [483] and 2250 [480] processors, respectively. A more challenging task (because of the dynamic nature and heterogeneity of the computing medium) is realization of the method, most of all uniform loading of the processors, based on distributed GRID resources [484]. The architecture of the MDM computation system [485] is designed to make large-scale calculations with the MD method.

A large number of models of the water molecule have been proposed (see, for instance, Refs [153, 486–492] and references cited therein), differing in structure (number and location of charges, nature of chemical bonds, allowance for polarizability of molecules, etc.), adequacy of simulation of experimental dependences, and suitability for high-performance computations. Recent experiments on X-ray Compton scattering [493] showed the necessity of introducing a correlation between the length of the O–H bond and the structure of the H-bond net. References [491, 492] compared models in terms of accuracy of prediction of melting temperature for ice I_h and other parameters of the phase diagram; their comparative applicability to the description of protein and nuclear acid dynamics in solutions was treated in paper [487].

Serious difficulties are encountered in simulating the homogeneous nucleation of water. The very first MD calculations were reported in 2002 [494] (they were highly labor-intensive and required consideration of a time range of 200–300 ns with an integration step of 1 fs). The difficulty arises from the complex nature of the potential energy surface (PES) in the $6N$-dimensional configuration space (N is the number of particles in the computation) that has a large number of local minima, i.e., it accepts numerous potential configurations of the hydrogen-bond net [495]. PES topological properties are related to the thermodynamic and dynamic properties of supercooled water [496]. Results of MD calculations are highly sensitive to the chosen intermolecular interaction potential [152] and may overestimate or underestimate the degree of water structuring [497]. A measure of structuring is a set of order parameters describing the geometric properties of bonds between neighboring molecules; these properties are based on the expansion of the pair correlation function in terms of spherical coordinates (plus the three-particle tetrahedrality parameter). Nucleation is described by the trajectory of the minimal free energy in the order parameter space [498]. Two- and three-particle order parameters were used in work [364] to determine the energy barrier to nucleation in different media, viz. a homogeneous liquid, water in hydrophobic pores, water in a constant electric field, a water–CO_2 mixture, and water plus an antifreeze protein.

A simpler task is presented by simulation of melting and crystallization in spatially confined systems (see references cited in Section 2.1) and crystallization of water in contact with the ice surface [499].

Simulation of crystallization in an NaCl aqueous solution in contact with cubic ice I_c was reported in papers [500, 501]; the unpolarizable SPC/E potential was used for water. Instability of the crystallization front was responsible for the appearance of cavities containing an unfrozen concentrated brine; front propagation was associated with local fluctuations in salt concentration. This problem is equally interesting for geophysicists in that variations in the underice solution density have a dramatic effect on the mass circulation of water in the ocean.

Osmotic transport in cylindrical pores in the absence of molecule–wall interactions was examined in Ref. [502], and water transport across the lipid bilayer in Ref. [503]. The characteristic time for water molecule transport being large (on the order of 100 ps), the authors considered direct MD simulation inefficient and proposed using this method to obtain coefficients necessary for the macroscopic description of the transfer process (based on the computed water-molecule-depth distribution function).

The use of lattice models of macromolecules [286] relaxes the requirements imposed on computational resources due to a drastic decrease in the number of possible conformations. This, however, *a priori* implies the introduction of space anisotropy [504]. Therefore, it accounts for the increasingly higher popularity of off-lattice models. Water molecules and constituent elements of macromolecules are regarded as spheres of different diameters; interactions between elements i and j of the molecule that are not close neighbors are described by a short-range pair potential, most often by the truncated Lennard-Jones potential

$$V_{ij}(r) = \begin{cases} \epsilon\left[\left(\frac{\sigma_{ij}}{r}\right)^{12} - 2\left(\frac{\sigma_{ij}}{r}\right)^{6} + v_{ij}\right], & r \leqslant R_{ij}\,, \\ 0, & r > R_{ij}\,. \end{cases}$$

Here, the shift v_{ij} ensures potential continuity at $r = R_{ij}$, and the cutoff radius is chosen depending on the properties of elements i and j. Particle positions are monitored using the 'random lattice' algorithm [505].

Major approaches to the construction of hybrid models include: (1) the substitution of a spatial element (e.g., phosphate or methyl group) of a macromolecule by one or two particles having certain effective properties; likewise, a group of water molecules can also be represented by an 'effective' atom [506], and (2) a combination of the discrete (atomic) consideration of macromolecules and the continual description of solvents (in this case, water) effects [507]. Methods of such an 'implicit' description of water in application to simulation of properties and functions of biological membranes have been recently reviewed at length in paper [508].

In the HP model of protein [509] and its off-lattice generalization (AB-model) [510, 511], amino acid residues are regarded as pointlike objects, either polar (P, represented by a charge or a dipole) or nonpolar (N) [282]. Hydrophobicity is described as a trend toward the reduction of the surface of contact with water and implies the ordering of its nearest-neighbor molecules into an energetically more advantageous structure like ice. Formally, this effect can be represented as an attractive interaction between nonpolar amino acid residues to eliminate the degrees of freedom associated with water. However, the above authors considered water explicitly as located at lattice sites unoccupied by protein elements. The ability of water to form structures is taken into account by the introduction of q states in one of which water (or a group of water molecules, to be precise) occurs at a lattice site. The authors used the bimodal model with $q = 2$. The energy of each amino acid residue depends on the water state at the neighboring sites. The explicit description of water is necessary for the correct simulation of protein cold denaturation [512].

A simple model of hydrophobic interaction does not reproduce the pressure effect on the cold denaturation of proteins. The description of this process at elevated pressures requires that account be taken of water density fluctuations [513]. This work considers a nonpolar homopolymer whose interaction with water (partial organization of water molecules around protein) is defined by the Hamiltonian

$$H_p = J_r\, n_{HB}\left(n_{max} - \sum_{i,j} n_i n_j\right),$$

where the parameter J_r specifies the repulsive interaction force, $n_{HB} \equiv N_{HB}/N_W$ is the numerical density of hydrogen bonds enlarging the system's volume, N_W is the number of

water molecules, $n_{\max}$ is the maximum number of contacts between amino acid residues of the protein, $n_i = 1$ if the ith site of the lattice is occupied by a residue, and $n = 0$ if it remains unoccupied. The expression in parentheses defines a measure of protein compactness. The Hamiltonian of interaction between water molecules is written out as

$$H_{\mathrm{HB}} = -J \sum_{i,j} \delta_{\sigma_i,\sigma_j} ,$$

where the variable σ_i defines water molecule orientation, the formation of a hydrogen bond between neighboring molecules i and j being feasible if $\sigma_i = \sigma_j$ [513]. Enthalpy for a system of 'protein in a water-bath' is given by $W = H_{\mathrm{p}} + H_{\mathrm{HB}} + PV$. The authors calculated the two-parametric (the numbers of H-bonds and contacts between amino acid residues) density of states using the multicanonical algorithm of the Monte Carlo method [514]; thereafter, they found the dependence of the mean number $\bar{N}_{\mathrm{c}}$ of contacts on temperature and pressure; the denaturation condition was formulated as $\bar{N}_{\mathrm{c}}/n_{\max} \geqslant \alpha_{\mathrm{d}}$ (on the assumption that $\alpha_{\mathrm{d}} = 0.96$ for definiteness).

Other approaches to the explicit consideration of water molecules are employed, too, for example, the model of a phantom solvent in which water molecules interact with macromolecules but not with one another [504].

The MD method was applied to analyze the state of an antifreeze protein isolated from the flatfish *Pseudopleuronectes americanus* in a vacuum, in aqueous solution, and adsorbed on the ice surface [515]. Such an analysis revealed the most preferable crystallographic planes of ice for the inhibiting action of the protein, the comparative role of hydrogen bonds and van der Waals forces, and the effects of possible protein mutations (removal of a peptide group). The same approach and minimization of energy in an ice – antifreeze protein system in a vacuum and water were used in papers [362, 516, 517] to elucidate what peptide groups are responsible for inhibition of the growth of ice crystals. It turned out, in contrast to the widely accepted opinion, that hydrophobic amino acid residues play an important role in the binding of antifreeze proteins to ice. Solvent effects in a water – antifreeze protein system were considered in Ref. [518].

Comparative analysis of three disaccharides with a view to their possible use as cryoprotectors was carried out by the MD method in Ref. [519]. Trehalose (α – D-glucopyranosyl – α-D-glucopyranoside) was found to contribute to the binding of more water molecules and to a higher degree of homogeneity of the solution, thus reducing the probability of nucleation. The ability of trehalose to effectively destroy the tetrahedral water structure and prevent ice crystallization was confirmed by the results of a study of thermophysical properties of several disaccharide solutions using the DSC technique [520].

The properties of lipid membranes depend on physical and chemical processes on atomic to mesoscopic (microns) scales in a wide range of characteristic times. The available models are designed for different spatial and temporal scales; ideally, they must be complementary (multiscale simulation) which implies analysis of the two-way information flow in the transition from one level of description to another. A review of studies on MD simulation of bilipid membranes at normal temperature, conducted prior to 2000, can be found in paper [521]; recent models are reviewed in Refs [272, 504, 522]. Model lipid systems (monolayer at the water – air interface known as Langmuir monolayer, planar bilayers, bilayer assemblages in the lamellar phase) [301] are designed not only as tools to study the general properties of biological membranes but also as ideal (due to their relative simplicity) objects for the development and verification of simulation techniques. A recent work [523] compared oligomer chains residing in free state and incorporated in a membrane structure. Calculations of the order parameter for C – C and C – H bonds with respect to the principal axis of the macromolecule's inertia tensor showed that the inherent properties of the chain determined by its structure manifest themselves in the membrane, too. Simulation of the behavior of a membrane protein was reported in Ref. [199]. To date, most attention has been given to simulation of ion channels [403, 524, 525]. Considerable interest is being shown in the employment of hydrocarbon tubes for physical simulation of biological transport processes [526] and collective dynamics of ion channels [527].

MD simulation was invoked to study interactions between several cryoprotectors (trehalose, glucose, methanol, DMSO) and model membranes differing in the degree of lipid saturation at constant temperature [522, 528]. It was shown, in particular, that small organic molecules reduce membrane thickness and increase surface area per lipid molecule and membrane permeability. The protective effect of sugars, such as saccharose and trehalose, is related to the replacement of water molecules [203] in hydrogen bonds with lipid heads (which are provided by water molecules in the absence of cryoprotectors) and stabilization of the lipid bilayer structure (an appreciable shift of the main phase transition temperature). This effect is quantitatively described by the order parameter

$$S_{\mathrm{CD}} = -\left(\frac{2}{3} S_{xx} + \frac{1}{3} S_{yy}\right),$$

where $S_{\alpha\beta} = \langle 3 \cos \Theta_\alpha \cos \Theta_\beta - \delta_{\alpha\beta} \rangle$ $(\alpha, \beta = x, y, z)$; $\cos \Theta_\alpha = \mathbf{e}_\alpha \mathbf{e}_\beta$ and $\mathbf{e}_z$ is the unit vector in z direction in the laboratory frame of reference, while $\mathbf{e}_\alpha$ is the unit vector in the local system constituted by three sequential carbon atoms $\mathrm{C}_{i-1}, \mathrm{C}_i, \mathrm{C}_{i+1}$ in the tail of a lipid molecule, and $\mathbf{e} = \mathbf{r}_{i+1} - \mathbf{r}_{i-1}$. This parameter, available for experimental determination by NMR method, characterizes orientation of hydrocarbon tails relative to the normal to the lipid bilayer.

Tail ordering increases at low temperatures, which promotes phase transition of the layer to the gel. It is worth mentioning that the problem of interaction between low-molecular organic compounds and lipid membranes is encountered in wine-making. The interaction of alcohol molecules with yeast cell membranes alters the conformation of transmembrane proteins and interferes with their functions (arrests fermentation) [528].

The above models describe macromolecules as flexible chains. Simpler (so-called spin) models considering the orientation of amphiphilic molecules as that of a single object are applicable in many cases [504]. Finally, the continual description of amphiphilic systems is feasible by the free energy functional dependent on a few macroscopic parameters, e.g., local concentrations (models of the Ginzburg – Landau type).

3.2 Imitation models

Propagation of intracellular ice. Ice propagation in a close-packed cluster of four concentric spherical cell layers was assumed to be instantaneous in Ref. [529]. However, the

assumption was soon described as incorrect, since it disregarded the finiteness of the propagation rate of intracellular ice [530].

A pair of isolated cells is considered in paper [404] as a system with a finite number of states: (0) — ice is absent in both cells; (1) — ice is present in one of the cells, and (2) — ice is present in both cells. The state of an ensemble of cell pairs is described by two parameters, for instance, the probabilities of unfrozen, $P_0 = N_0/N$, and 'singlet', $P_1 = N_1/N$, states ($P_2 = 1 - P_1 - P_0$). Evolution of the ensemble is governed by two independent stochastic processes — spontaneous nucleation J_s, and ice propagation J_p across an intercellular junction. Evidently, the following equations are valid:

$$\frac{dN_0}{dt} = -2J_s N_0 \quad \text{and} \quad \frac{dN_2}{dt} = (J_s + J_p)\, N_1 .$$

The introduction of dimensionless time $\tau = \int_0^t J_s\, dt$ and interaction parameter $\alpha = J_p/J_s$ gives

$$\frac{d}{d\tau}\begin{bmatrix} P_0 \\ P_1 \end{bmatrix} = \begin{bmatrix} -2 & 0 \\ 2 & -(1+\alpha) \end{bmatrix} \begin{bmatrix} P_0 \\ P_1 \end{bmatrix} .$$

Clearly, $P_0(\tau) = \exp(-2\tau)$. The analytical solution for P_1 at constant α has the form [404]

$$P_1(\tau) = \begin{cases} \dfrac{2}{1-\alpha} \exp\left[-(1+\alpha)\,\tau - \exp(-2\tau)\right], & \alpha \neq 1 , \\ 2\tau \exp(-2\tau), & \alpha = 1 . \end{cases}$$

The highest probability of the singlet state, viz.

$$P_1^{\star} = \left(\frac{1+\alpha}{2}\right)^{(1+\alpha)/(1-\alpha)} ,$$

is achieved at

$$\tau^{\star} = \frac{1}{1-\alpha} \ln \frac{2}{1+\alpha} .$$

Ice formation and propagation in cell aggregates was considered on the assumption that the rate of nucleation in a given cell is defined as $J = J_s + mJ_p$, where m is the number of neighboring cells [531]. The total number of states in a system of N cells equals $M = 2^{N-1} + 2^{(N-1)-2}$. Evolution of vector $\mathbf{P}$ of probabilities P_ξ for each state ($\xi = 0, \ldots, M-1$) in an ensemble of identical cell aggregates was described as

$$\frac{d\mathbf{P}}{d\tau} = \mathbf{Q}(\alpha)\, \mathbf{P} ,$$

where the matrix $\mathbf{Q}$ contains all transition rates between states. Reference [531] proposed an analytical solution for $N = 4$. At large N, intracellular ice formation was simulated by the Monte Carlo method. A one-dimensional cluster of 100 cells was considered in paper [531], and two-dimensional problems for the number of neighbors $m = 6$ and $m = 4$ in Refs [532, 533]. For the region containing both pathologic ('t') and healthy ('h') tissues, the nucleation rate was written out as [532]

$$J = J_s + m^{t} J_p^{t} + m^{h} J_p^{h} ,$$

with the emerging new parameter $\beta = J_p^{t}/J_p^{h}$ characterizing the ratio of ice propagation velocities. Calculations in a wide range of ice propagation rates in healthy tissues revealed three growth regimes differing, in particular, in terms of dependence of the probability of eliminating pathologic tissue on parameter β [532]. Modification of the aforementioned Johnson–Mehl–Avrami–Kolmogorov model was also proposed in paper [533].

3.3 Macroscopic models

3.3.1 The cell in solution. The two-dimensional problems of interaction between an individual cell and the ice front have been considered by the arbitrary Euler–Lagrange method on regular and unstructured triangular meshes [534, 535] and by interface matching [536] on fixed Cartesian meshes [537]. In the former case, the cell was trapped by the moving crystallization front. In the latter case [536], the interest was focused on the behavior of the cylindrical crystallization front coaxial to the centrally positioned cell (resolution of the evolution of the front shape required meshes containing up to 250,000 unit cells). In either case, variations in cell volume due to dehydration were taken into account, but the shape of the cell (cylindrical in the two-dimensional design) was deemed invariant.

The calculation of the freezing of biological suspensions based on the Osher–Sethian 'level set' approach has been proposed in Ref. [538].

3.3.2 Numerical heat exchange studies. Methods for the calculation of heat exchange accompanied by crystallization were described in many publications (see, for instance, Ref. [539]). Typical values of the thermophysical parameters of biological tissues are reported in Ref. [540].

Exact solutions of the one-dimensional equation for heat exchange in tissues for a multiblock region with constant thermophysical properties have been obtained in papers [541] (Cartesian and spherical coordinates) and [542] (cylindrical coordinates). Nonstationary sources and boundary conditions were considered in Ref. [543], and sinusoidal time-varying heat flow for a semiplane in Ref. [544]. A solution in the form of series for a two-dimensional problem on a pair of vessels in a confined medium was written out in paper [545].

To our knowledge, the finite difference method for the calculation of a two-dimensional temperature field near a cryoprobe was first applied in work [546]. Paper [547] (see also references cited therein) described the boundary element method applied to the calculation of the temperature field in two- and three-probe systems. Reference [548] reported the finite element method for two-dimensional calculation of heat exchange in cryosurgery. The finite volume method on unstructured triangular meshes [549, 550] was used in Refs [551, 552]. The axially symmetric problem of ice ball formation around a lone cryoprobe was resolved in work [553] by the finite element method in the three-dimensional formulation in the Cartesian coordinates. Anisotropic adaptation of the unstructured mesh had been proposed by the authors earlier [554, 555]. Linearized equations were solved by the GMRES method from the PETSC library with preconditioning using the incomplete factorization method (ILU).

The accuracy of heat exchange computation may be improved by monitoring the ice front boundary [556–560]; in this case, the boundary element method is applicable [561, 562]. Reconstitution of the shape of a region exposed to a cryogenic impact from experimental data poses a separate problem [563]. The solution to this ill-defined problem in the cryosurgical context is simplified by the use of *a priori* information on the character and evolution of the frozen

region [561]. However, the majority of this information, for example, the assumption of front bulging, applies only to single-probe systems.

Heat exchange in a multiprobe system. The success of cryosurgery depends on the adequate choice of the cooling/thawing rate and temperature and on the precise localization of the applied cold, excluding injury to the surrounding healthy tissues. It is rather a difficult task to overcome. To begin with, a freezing protocol is as a rule based on general recommendations virtually disregarding the size and the geometric shape of the tumor, the degree of development of the capillary network, properties of the surrounding tissues, proximity to the walls of hollow organs, etc. Second, it is difficult to optimally place a large number of cryoprobes based on the surgeon's experience alone. Finally, intraoperative monitoring by a variety of methods (ultrasound [3], thermometry [564], matrix thermovision [564], X-ray computer tomography (CT) [565, 566], magnetic resonance tomography [567, 568], optical coherence tomography [569], electrical impedance tomography [5]) does not provide all necessary information about the three-dimensional temperature field. Temperature distribution inside an ice ball can be deduced either by extrapolation based on mathematical models [570] or from the empirical temperature dependence of measurement results [in Hounsfield units (HU) normalized to the water – air contrast] [566] if X-ray CT is the method of choice. In this case, the accuracy of conversion is limited by noise sensitivity and the nonmonotonic temperature dependence of HU. It is relatively easy to determine localization of the ice front but not the boundary of the direct cryonecrosis region, the volume of which is normally one fourth of the ice ball volume [571].

Simulation usefulness depends on the precision of predictions. The sources of errors in cryosurgical practice are summarized in Ref. [572], one of the most important being the vague thermophysical properties of tissues at low temperatures.

Analysis of the choice of solution based on the temperature dependence of effective heat conductivity of frozen tissues is presented in Refs [551, 552]. (The problem was analytically considered earlier for a one-dimensional case [573].) Consideration was focused on the stationary problem, i.e., the exposition stage of cryosurgery. In this case, the temperature distribution and the location of the phase transition region were determined by tissue heat conductivity alone and did not depend on specific thermal capacity or latent crystallization temperature. At the same time, stationary two-dimensional computation is the 'worst possible scenario' from the standpoint of protection of the surrounding healthy tissues [574].

It was assumed for the purpose of the above calculations that heat conductivity of a frozen tissue depends on temperature alone (and as a rule equals that of ice). The error introduced by this assumption can hardly be assessed *a priori*. Two options were considered: (1) constant heat conductivity (it should be noted that its value does not enter the formulation of the stationary problem), and (2) heat conductivity obtained by joining the temperature dependences for water and ice.

Calculations for systems with up to 18 cryoprobes and a cryoheater showed that the uncertainty in the thermophysical properties of tissues measured at a fixed point may be dozens of degrees or a few centimeters in the zone of cryonecrosis, the length of which was estimated from the 'lethal' isotherm [551].

The development of an adequate model of heat conduction in a frozen biological tissue considered as a heterogeneous medium including the intercellular matrix and vitrified or bound water besides ice [196, 575, 576] is an independent problem. An intermediate solution may be obtained by traditional methods for the assessment of the effective properties of multiphase composite materials [577]. This, however, does not eliminate all difficulties because the fraction of uncrystallized water and the state of the intercellular matrix vary during cryogenic exposure. As known, the first 'freezing – exposure – thawing' cycle enhances effective heat conductivity in the tissue [470] and thereby significantly (by dozens of percent) increases the ice ball volume [441]. The putative mechanisms include injury to cell membranes and blood vessels [578]. Therefore, an adequate description of heat exchange during cryogenic exposure must combine the description of macroscopic heat transfer with the analysis of structural changes in the medium at the mesoscopic level, in particular, with the analysis of cell dehydration and intracellular ice formation effects determined, first and foremost, by the (macroscopic) local cooling rate of the tissues [305, 578].

Optimization of cryogenic exposure. Formulation of the optimization problem implies defining optimization parameters $\mathbf{p} = (p_1, p_2, \ldots, p_n)$ and the objective function $F(\mathbf{p})$. In cryosurgery, such parameters are self-evident, namely, temperature (to be precise, the law of its variation in time), the number of cryorpobes and their localization [579 – 581]. Sometimes (and in all cases of cryopreservation), they include parameters determining administration/withdrawal of a cryoprotector (usually a tuple of three parameters: concentration, temperature, and duration, for each step of the process [39, 582]). Stepwise cooling by prolonged exposure of the specimen to an intermediate temperature [583, 584] is used in cryopreservation to achieve crystallization of extracellular ice and cell dehydration followed by rapid cooling [44, 585]. Traditional cooling (heating) regimes with piecewise constant temperature variations are considered to be nonoptimal because of essentially nonlinear tissue response [586].

The objective function is not so easy to choose. Radiotherapy deals with the *irradiation dose* and the *probability of eliminating* (*saving*) pathologic (healthy) tissue based on the approximation of experimental data [587]; the resulting objective functions are referred to as physical or biological, respectively. The use of *dose – volume* histograms is also possible bearing in mind that the reaction of a tissue depends on the dose delivered to its vicinity [89].

Hypertermic exposure is frequently planned taking into account a quadratic function $F(\mathbf{p}) = \int_\Omega (T - T_\mathrm{o})^2 \, \mathrm{d}V$ that describes the mean deviation of the theoretical temperature field T from the desired (optimal) T_o. Sometimes, one or a few reference points in the tumor region are considered [588, 589].

It is possible to identify T_d, i.e., the minimal destruction temperature, without setting an upper bound on pathologic tissue temperature. The maximum temperature of healthy tissues, T_lim, is given additionally. When solving the optimization problem $\min F(\mathbf{p})$, where

$$F(\mathbf{p}) = \int_{\Omega_\mathrm{tumor},\, T<T_\mathrm{d}} (T - T_\mathrm{d})^2 \, \mathrm{d}V,$$

the temperature limits $T \leqslant T_{\rm lim}$ in the healthy tissue region $\Omega \setminus \Omega_{\rm tumor}$ are used explicitly or introduced by the penalty method in the objective function [13]

$$F(\mathbf{p}) = \int_{\Omega_{\rm tumor},\, T<T_{\rm d}} (T - T_{\rm d})^2 \, {\rm d}V + w \int_{\Omega\setminus\Omega_{\rm tumor},\, T \geqslant T_{\rm lim}} (T - T_{\rm lim})^2 \, {\rm d}V .$$

Similar approaches are employed in cryosurgery [581, 590]. The simplest way to define the objective function is to assign the critical ('lethal') temperature ensuring cryodestruction or to use the following representation

$$F(\mathbf{p}) = \int_{\Omega} w(T) \, {\rm d}V ,$$

where the weight function $w(T)$ equals 0 or 1 in the appropriate temperature intervals and geometric regions [580, 591]. In Ref. [548], the 'freezing exposure index' was introduced, which is equal to the product of time for which tissues remain at a temperature below the given one and the mean temperature during this time interval:

$$I = \int_{T_1}^{T_2} T(t) \, {\rm d}t .$$

Unfortunately, these criteria are not universal. One objective of optimization may be to reduce the duration of surgery [124].

4. Conclusion

Analysis of processes in biological objects undergoing freezing with regard for the different behavior of cells in aqueous solutions and tissues exposed to cryogenic effects *in vitro* and *in vivo* permits distinguishing several levels (scales) of physical problems, listed in the table below.

One of the most challenging tasks is the development of a model of heat conduction in a frozen biological tissue as a heterogeneous medium with time-dependent structural variations, including an intercellular matrix and vitrified or bound water, besides ice. A hybrid model combining the macroscopic description of heat transfer and the analysis of structural changes in the medium at the cellular level is needed to resolve this problem. Other topical problems include: (1) microscopic simulation of ice formation in cytoplasm via homogeneous and heterogeneous nucleation or propagation through membrane pores, and of interactions of crystals with plasma membranes and cytoplasmic structures (in the presence or absence of cryoprotectors), and (2) identification of an adequate objective function for the solution of optimization problems.

Table. Major physical problems in cryobiology.

Biological object	Problem
Subcellular objects	Interactions of cryoprotector molecules with cell membrane Evolution of membrane and cytoskeleton Membrane rupture Ice propagation through membrane pores
Isolated cell	Heat and mass exchange with the environment Interaction with the ice front
Homogeneous tissue	'Effective' thermophysical properties Heat exchange Ice front propagation Ice recrystallization Microcirculatory effects
'Tumor + surrounding tissues + cryoprobes + cryoheaters' system	Global heat exchange in the system Ice front interactions Mechanical stress Optimization of cryoexposure protocols

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