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Membraneless physiology of the living cell. The past and the present

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Abstract – Since the 1880s, the concept of compartmentalizing through membranes has taken a firm place in cell physiology and has defined the objects, methods, and goals of physiologists' research for decades. A huge mass of biologists know about the important role of intra-membrane pumps, channels, and lipids, and various hypotheses about the origin of life often begin with explanations about how the lipid membrane occurred, without which it is impossible to imagine the origin of a living cell. Against this background, there was a dissonance of statements that there are membraneless organelles in the cell, the functions of which are rapidly expanding under our eyes. Physically, they are similar to coacervate droplets, which from time to time were used to explain the origin of life, and now the coacervates are being more and more often discussed when describing the physics of the nucleus and cytoplasm of modern cells. However, ideas about the coacervate nature of cytoplasm/protoplasm originated in the first half of the 19th Century, when the contents of cells were likened to jelly, but this approach gradually faded into the shadows. Nevertheless, limited research in this area continued and was completed in the form of a membraneless cell physiology. Now that the focus of attention has turned to membraneless compartmentalization, it's time to remember the past. The sorption properties of proteins are the physical basis of membraneless cell because of water adsorbed by proteins changes the physical state of any biomolecular system, from supramolecular and subcellular structures to the cell as a whole. A thermodynamic aqueous phase is formed because adsorbed water does not mix with ordinary water and, in this cause, is separated from the surrounding solution in the form of a compartment. This article discusses the fundamental physical properties of such a phase – a biophase. As it turned out, the Meyer–Overton rule, which led to the idea of a lipid membrane, also applies to membraneless condensates.

Keywords: Adsorption, Hydrogen bonds, Intrinsically disordered proteins, Membraneless organelles, Meyer–Overton rule, Physical state of water, Physiology

Introduction

The discovery of the fist membraneless organelle, germline P granules [1] is deservedly regarded as a new phase in the development of cell biology [2], but even such an assessment turns out to be insufficient if we recall what place membranes occupied in our ideas about life at the cellular level. The membrane theory of compartmentalization based on lipid membranes, which received universal recognition at the turn of 19/20th Century, became the first theory in physiology that explained the four fundamental physical properties of a living cell ([3], p. 12): (1) semipermeability, (2) ability to selectively accumulate certain solutes and remove others from its internal environment, (3) ability to generate electric potentials, and (4) maintain the osmotic stability. But this success was achieved at the cost of extremely simplifying the nature of a living cell, reducing it to a simple sac surrounded by a lipid membrane and filled with an ordinary solution of organic and inorganic solutes [4].

The key feature of the cytoplasm, according to the membrane theory, is that intracellular water is considered as a free and indifferent enough solvent, whose molecular interactions with proteins and other macromolecules can be neglected. Such ideas about water are still alive to this day and present, for example, in the article by Lafontaine et al. [5]. The peculiarities of the chemical composition of the internal environment of the cell (ion composition, for example) were explained by the special properties of the membrane, the structure of which was revised over time [6], and, in addition, the idea of a lipid bilayer as the mandatory structural basis of the membrane was seriously criticized from time to time [7]. For more than a century, the power of the membrane theory over scientists has led to the idea that the membrane is the only technology of nature used to isolate a cell or its parts (membrane-bound organelles) from the medium. The recognition of the existence of membraneless organelles refuted this age-old belief and raised the question of the existence of other, deeper mechanisms of compartmentalization. Now, in the literature, you can find statements that there are two types of

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organelles – membrane-bound and membraneless ones [8]. However, it is obvious that the organization of these types of compartments from the point of view of physics is fundamentally different. In favor of the idea of a universal physical nature of all cellular organelles, there is the evidence that even such a classical membrane-bound organelle as the Golgi apparatus has the characteristic features of membraneless organelles [9].

Let us review a physical phenomenon that is widespread in nature and plays an important role in it – adsorption (the binding of large quantities of particles by a surface). In relation to the cell, the regulated adsorption of ions and small molecules by proteins due to the action of surface forces is considered. By adsorbing water, proteins produce a new aqueous phase (protein-water condensate), which is a compartment with special conditions inside, essentially a biophase [10]. The phase interface (for example, condensate/medium) is a physically active zone capable to form a variety of structures of different composition, including lipid or protein membranes.

A literature review is not the focus of this article. It offers principles to keep in mind when studying membraneless compartments. With the literature I have cited, it is easy for the reader to expand the range of literature sources.

Physical state of water in the living cell

It was widely believed in the 19th Century that the properties of intracellular water were different from those of bulk water (medium water). However, such an idea has emerged from microscopic observations. This issue was first systematically studied by Afanasy Troshin [11] (who later became the first director of the Institute of Cytology of the Russian Academy of Sciences) on the initiative of Dmitry Nasonov [12], who founded the Institute in 1957.

Troshin investigated the equilibrium distribution of solutes between living cells and the medium [11]. A typical result of his research is shown in Figure 1. At low concentrations of lactose, the intracellular sugar content increases non-linearly (yeast *Saccharomyces ellipsoideus* or *Saccharomyces cerevisiae*, according to the modern classification [13], do not ferment lactose). This is due to the presence of two lactose fractions in the cell, bound and free. As soon as the sugar-binding centers on intracellular structures are saturated, the dependence becomes linear, since from that moment lactose is distributed only between two water fractions: intracellular water and medium water. The equation describing the linear part of the curve in Figure 1:

$$C_c = 62.1\,(\pm 1.8) + 0.57\,(\pm 0.01)\cdot C_s,\quad r = 0.9997.$$

The resulting equation, corresponding to the Langmuir model of nonpolar adsorption describes the data obtained by Troshin. The intercept of the above equation is numerically equal to the Langmuir adsorption limit, that is, the maximum possible amount of solute adsorbed in this system. If we subtract the value 62.1 (the amount of bound lactose) from all points of the linear part of the curve, then we get the concentration of free lactose in intracellular water, and the straight line will fall below line A, which means that the solubility of lactose in cellular water is lower than its solubility in bulk water. Indeed, the distribution coefficient (C_c/C_s) of lactose, according to the equation, turned out to be 0.57 (± 0.01), that is, the concentration of sugar in the water of the cell is only 57% of the corresponding concentration in the medium.

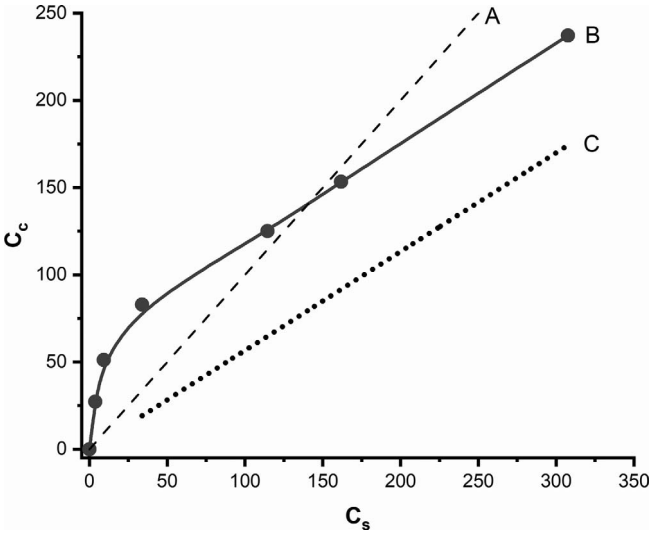

Fig. 1. (A) The theoretical dependence of the equilibrium concentration of a solute in intracellular water on its concentration in the medium, provided that (i) the solvent capacity of water in the cell and the medium is the same, (ii) the solute is not adsorbed on intracellular structures. (B) The dependence of the lactose content (free plus bound) in yeast (C_c, in mM of intracellular water) on its concentration in the medium (C_s, in mM) in equilibrium condition. (C) The dependence of the equilibrium concentration of lactose in intracellular water on the concentration of the sugar in the medium: the bound sugar is subtracted from the linear part of the experimental curve ([11], Fig. 56, redrawn). See text for details.

In addition to yeast, similar data were obtained on other cells [11]: erythrocytes, galactose (Fig. 40); frog muscle fibers: arabinose, galactose, sucrose (Fig. 44); creatinine (Fig. 53); α-alanine (Fig. 65). Thus, the reduced solvent capacity of intracellular water turned out not to be a special case (only seen in yeast), but, apparently, a general pattern for all living cells. Fundamentally important was the fact that the same dependence was obtained also for gelatin-gum arabic coacervates [11]: galactose (Fig. 31) and sucrose (Fig. 32).

Such a similarity between living cells and coacervates leads us to interesting conclusions. Firstly, the results obtained cannot be explained by certain properties of the cell membrane, since coacervates are membraneless compartments. Secondly, the only common component present in both living cells and coacervates is water. Consequently, the physical state of water in the cells and coacervates is the same, but it has changed compared to bulk water. Obviously, this change occurred as a result of the interaction of water with macromolecules (adsorbents), which are part of both living cells and coacervate droplets. Based

on the conducted research, Troshin considered a living cell as a complex system of coacervates. Troshin's conclusion, translated into modern language, is as follows: a living cell consists of membraneless compartments of varying sizes, and adsorbed water with altered properties is the principal determinant of its physical properties.

Native proteins act as the main modifiers of intracellular water because, after their denaturation, the dissolving ability of intracellular water becomes indistinguishable from the dissolving ability of bulk water ([11], Figs. 65, 66). Intracellular coacervates do not mix with each other and impart coacervate properties to the entire cell, which is a polyphase system. As a result of the action of adsorption forces, the interphase boundaries between intracellular coacervates and between the cell and the medium are active zones where various structures may form, such as membranes, among other things. Structures arising at interphase boundaries may play an important role in fine-tuning individual compartments and the whole cell, but they do not ensure the integrity of the cell and membraneless condensates. Integrity is ensured by a protein framework immersed in the phase of adsorbed water formed by it. Generalizing, we can say that the protein, interacting with water, forms a thermodynamic aqueous phase, and if the protein loses the ability to such an interaction (for example, during denaturation or under the action of a regulatory factor), the phase is destroyed, and the compartment disappears. Thus, proteins that adsorb water, changing thereby its physical state, play the role of a vital modifier of the physical state of the system they are part of (macromolecular condensate, membraneless organelle, entire cell). Troshin called coacervates liquid phases, but the reason for the change in the properties of intracellular water remained a mystery to him.

Thermodynamic phases of water in nature and in the living cell

If the water is cooled to 0 °C, then a second aqueous phase will appear, – ice. The two-phase ice/water system in equilibrium state gives a general idea of the conditions under which another phase, a piece of ice, is formed in the water: (1) the strength of hydrogen bonds should increase so much as to reduce the mobility of water molecules; (2) the network of hydrogen bonds between water molecules should acquire a new stable structure. If these conditions are met, the two aqueous phases will coexist without mixing with each other.

The ability of water molecules to form flexible networks of hydrogen bonds of different structures is well known. The study of ice has shown that its structure can change significantly under the influence of external conditions [14], and two phases of liquid water can form in pure water [15]. In a living cell and in phase models of the cell, in coacervates and in Fox's proteinoid microspheres [16], the moderators of water properties are macromolecules and, above among these, proteins, as the most numerous type of polymers. Physicists are well aware that water dramatically changes its structure when interacting with the surface, even if it is not charged [17], but there are always charged functional groups on the molecular surface of proteins, they are represented at least by alternating dipole functional groups of peptide bonds (see below). These changes lead to the restructuring of the network of hydrogen bonds between water molecules and, as a consequence, to the emergence of a new aqueous phase bordering on bulk water (both phases are dynamic, but the differences in dynamics have a functional significance).

Water is a phase-forming agent because the number of its molecules (about 44 M of intracellular water) is many orders of magnitude greater than the number of any other ionic or molecular cell component, therefore, it is the thermodynamic state of water that determines the thermodynamic state of the system as a whole. The physical state of the system determines all processes in it because each physical state corresponds to a certain set of thermodynamic parameters, which at a fundamental level determine the direction of flow of any process. However, this elementary knowledge from physics is not enough to link the protein-water interaction with the fundamental physical properties of a living cell. The solution to this problem was proposed by Ling [3, 18–20], and it explained Troshin's observations in many ways. Let's consider the basic principles of his theory because they are directly related to the physics of membraneless organelles/condensates and can be a good guide for the experimenter.

Main principles of Ling's theory

All interactions and effects that make up this theory are well described in textbooks of physics. They just need to be used in the right combination and order. The advantage of the theory is that the author explains physiological phenomena on the basis of molecular interactions. In the theory, the author takes us from molecules to the cell.

Purpose of the theory

The purpose of the theory is to establish the physical nature of the living state. The theory explains the physical mechanisms underlying the key phenomenon of life – the distribution of solutes between the cell and its environment and among cell compartments. All other mechanisms important for cell physiology and cell biology depend crucially on our understanding of this phenomenon.

Physical mechanisms that the theory uses

The basic physical mechanism is the regulated adsorption of water and solutes by cell proteins ([3], Ch. 10, 11; [19], Ch. 6; [20], Ch. 4, 5). If some solute accumulates in the cell to a level higher than in the surrounding medium, it means that it is adsorbed by cellular structures. Cell semipermeability, selectivity in accumulating solutes, electrical potentials, and osmotic stability are the result of the adsorptive activity of proteins on water, physiologically

important cations (K^+ and Na^+), and other solutes. Protein, as an adsorbent, differs from mineral adsorbents (for example, clay) in the fact that the dipole moments of its functional groups can vary significantly under the action of ATP (cardinal adsorbate) and other regulatory ligands (Ca^{2+} and hormones, for example) through a change in the distribution of electron density over the molecular surface of the protein (inductive effect) ([3], Ch. 14; [19], Ch. 6; [20], Ch. 6). The theory uses basic physical principles that make it a versatile tool to describe any mechanisms in the functioning of the living cell or in its pathology. The long-range, dynamic structuring of water molecules is due to what Debye called "orientation polarization" [21].

Two-state model of cell function

According to the theory, the functioning of the cell is considered as a reversible transition between two states, the resting state (basic one) and the state of activity ([3], Sect. 14.2; [19], Sect. 6.1.1; [20], Sect. 3.2). The resting state is a stable in time complex of protein-ATP-water-K^+ that has a high energy level compared to the active state (see Section "The structural unit of the living cell"). Since the resting state is stable over time (ATP is not split at the state), it does not require an ongoing flow of energy and matter. The action of an external stimulus or internal signal is to destabilize the resting state (ATP is split, and the whole complex is destroyed) and the cell becomes active. The energy is released and is used to perform biological functions. In the activated cell, metabolic processes start, new ATP molecules are synthesized, and the cell enters the resting state with a reserve of energy for the next cycle of activity. The energy of ATP adsorption on protein molecular surfaces provides energy to a resting cell to use it at transition to an active state. The two-state model can be applied to a whole cell or to every structure in the cell, including single protein molecules. Constant oscillations between these two states are the physical mechanism of the functioning of any cell structure. In this cycle, ATP is consumed, and biological work is performed. When the work is completed, the cell goes into the resting state (like muscle, for example). Different cell structures function in different rhythms, so at any given time, the energy of the high-energy ATP-protein complex is consumed somewhere in the cell.

Which proteins determine the sorption properties of the cell?

In the resting state, fully extended proteins physically adsorb the key components of the cell: ATP, water, and potassium ions. According to modern literature, most of the proteome has extended conformation or extended regions inside molecules, now termed as intrinsically disordered amino acid sequences. Perhaps these proteins (or some of them) belong to the set of fully unfolded proteins considered by Ling's theory as key proteins determining the physical properties of the cell and of intracellular structures.

Physical nature of selective adsorption

The following functional groups of proteins have key significance for the theory: the NH- and CO-groups of peptide bonds, and the carboxyl groups of dicarboxylic amino acid residues. The selectivity of peptide groups oscillations between the two states in respect of (1) affinity for water molecules and (2) affinity for other peptide groups of the same protein, which is needed for the formation of secondary structures. The selectivity of the carboxylic groups oscillates in respect of (1) affinity for potassium ions, and (2) affinity for sodium ions or for cationic groups of a protein (salt bonds). The (1) state of the groups is inherent in the resting state of the cell (or its parts). The (2) state indicates the active state of the protein. Transition (1) → (2) is a phase transition in which energy is released for biological work.

The affinity depends on electron density in the considered functional groups. The low density is characteristic for the resting state, high density for the activated state. The main regulator of the electron density is ATP, which has electron acceptor properties (Ca^{2+}, signal factors, hormones, and chemical modifications of proteins may carry out a more fine-tuning of the protein properties). In the resting state, ATP is bound to a protein and shifts the electron density in the protein molecule in such a way that the functional groups of peptide bonds acquire a greater affinity for water, while the carboxyl groups of the protein acquire an affinity for potassium ions. When ATP is split, the electron density in the functional protein groups increases, and that is why the affinity of the polypeptide chain to water falls, and water becomes free, at the same moment the affinity of the carboxyl groups to sodium ions or to protein cationic groups becomes greater, and potassium ions are released from the bond with the protein ([3], Sect. 14.1; [19], Sect. 6.2, 6.3; [20], Ch. 6).

Adsorption of water

The polypeptide backbone of any completely unfolded protein exhibits a geometrically regular order of positive (NH) and negative (CO) charges at the dipoles (similar to a one-dimensional crystal grid). This geometry is complementary to a space between the water molecules surrounding the completely unfolded protein. The complementarity creates conditions for multilayer adsorption of water on the protein surface. As a result, much of the cellular water (the most massive component of the cell, about 44 M) is transformed into a dynamically ordered structure. Because of its interaction with the backbone dipoles, the dipole moment of the adsorbed water is greater than that of free water (induced increase in dipole moment). With larger dipole moments, water molecules form stronger dipole-dipole interactions in the form of hydrogen bonds. It is more difficult for molecules of a solute to break the stronger interaction between molecules of adsorbed water, so this water is a poor solvent compared to bulk water. Therefore, solutes are displaced from the volume of adsorbed water into the bulk water space under equilibrium conditions. Strongly

adsorbed water is an effective barrier to the diffusion of solutes. It is this water everywhere in the cell (rather than lipids) that explains the property of its semipermeability (Fig. 1 illustrates how the sugar concentration gradient is caused by the different dissolving capacities of intracellular water and medium water, rather than active transport.). During the activation of a resting cell or some cellular structure (e.g., a contraction of a muscle or an action potential), water is released from binding with the extended proteins and the pathway for the diffusion of solutes into the cell becomes open ([3], Ch. 11; [19], Ch. 6; [20], Ch. 5; [22]).

Osmotic stability of the resting cell is provided by fully extended proteins that adsorb most of the intracellular water. In the active state, the proteins lose their ability to bind water, water is desorbed and then the osmotic equilibrium of the cell with the medium could be disrupted. However, along with water, potassium ions also are desorbed and keep osmotic stability. Under normal physiological conditions, the duration of the resting state – active state – resting state cycle does not last long (the duration of the action potential can be taken as a sample) and that is why potassium ions stay close to the protein ([3], p. 102–108; [19], Ch. 13; [20], p. 101–105).

Adsorption of potassium ions

A functionally important part of intracellular K^+ (most of it at any rate) is bound by proteins ([3], p. 67; [19], Ch. 8; [20], Ch. 4). In the bound state, it is physiologically inactive (the resting state). When potassium is desorbed from the binding sites, it becomes free and activates the K^+-activated systems of the cell [23]. Consider this provision of Ling's theory with the example of an action potential ([3], Sec. 15.6; 19, Ch. 14; 20, Ch. 11).

The resting potential is the adsorption (interphase) potential, arising in the microscopically thin surface layer of the axon (ATP is bound to proteins in this layer). When the axon is activated, ATP is split, bound water becomes free, and the carboxyl groups lose their affinity for K^+ and acquire a greater affinity for Na^+ (which is located in the medium) or to fixed cations of the protein (salt linkages appear). Since the water has become free, the barrier for Na^+ has disappeared, and it starts to flow into the upper layer of axon proteins (at this moment Na^+ ions generate a sodium diffusion potential). Then, Na^+ ions displace K^+ ions from their adsorption sites on the proteins. K^+ ions, becoming free, begin to flow out in the medium and generate potassium diffusion potential. These two diffusion potentials shape an action potential. Due to its physical nature, the interfacial adsorption potential can occur at any interface, for example, between a membraneless organelle and the cytoplasm.

Why does a living cell need potassium so much, and not another metal? From the point of view of Ling's theory, this is a natural property of proteins that remains to be explained. However, the sorption properties of proteins are so diverse and poorly understood that the ionic composition of cells can fluctuate significantly in organisms living in extreme conditions or at various stages of evolution in the past.

The structural unit of the living cell

A protein molecule in complex with ATP, water, and potassium ions makes up a minimal structure that retains the fundamental physical properties of the living cell: physiological atom [10, 24] or nano-protoplasm [25]. Functional activity of the cell or a subcellular structure is realized as the oscillated transitions between the two states of the physiological atom (resting and active states):

$$\text{Protein-}(\text{ATP})_m(\text{H}_2\text{O})_n(\text{K}^+)_q \rightleftharpoons \text{Protein} + m \cdot \text{ADP} + m \cdot \text{Pi} + n \cdot \text{H}_2\text{O} + q \cdot \text{K}^+.$$

When the atom is destroyed, energy is released for biological work. Although physiological atoms may have a wide range of compositions, one aspect remain constant: they must be a source of energy for functional activity.

The key points of the theory

Living cells are thermodynamic phases in relation to their medium, and not simply sacks that contain salts and macromolecules in solution.

The phase is formed due to the ability of key cell proteins to adsorb water. Other components important for cell life, such as potassium ions, are also adsorbed by proteins. The adsorption capacity of proteins is regulated by ATP (cardinal adsorbate) and other solutes that play a regulatory role.

The protein-ATP-water-K^+ associate is inherent in a cell or cellular structure at rest and is a high-energy state. The associate is stable enough and there is no need to continuously exhaust energy to maintain it. When this association is destroyed, the system goes into an active state: energy is released to perform biological work. When a cell/condensate transitions from its resting state to its active state, all the four fundamental physical properties change simultaneously. The active state is generally reversible, and the resting state can easily be achieved. In conditions of ATP deficiency, returning to a resting state becomes difficult and, as a result, pathological changes occur in the cell, such as protein aggregation. In the beginning, the damage in the cell is reversible, but later the damage to the cell becomes irreversible, and the cell dies. Physical changes in active and damaged cells result from the same cause – the destruction of the associate.

In the region of interphase boundary act forces that may form structures of different compositions, including lipid membranes. Lipids play an important role in the cell, but compartmentalization in living nature is possible due to only a wide net of physical interactions making up the bulk-phase system – the living cell or membraneless associates inside it.

Phase transitions coordinated in space and time make active transport of solutes in bifacial cell systems possible ([19], Ch. 17).

The significance of Ling's theory for cell biology

Ling's theory is a revolutionary approach to solving the fundamental problems of cell physiology and biology, in spite of all possible disadvantages. By using the theory, we will be able to study both old and new findings in biology and develop a new methodology for analyzing normal cells and cellular pathology. The postulation of membraneless organelles has caused confusion among those who have habitually explained the key physical properties of the cell and membrane-bound organelles using membrane logics. Ling's theory is the suitable basis for the development of the bulk-phase/membraneless physiology of the living cell.

The physical properties of artificial membraneless condensates

There is a large and growing body of evidence showing that coacervates and intracellular membraneless condensates, and coacervates and the cells are highly similar in their physics [26–30]. To coacervates should be added the already forgotten Fox's proteinoid microspheres [16], which, along with coacervates, have been considered for decades as models of protocells, precursors of the first living cells. The similarity of the physical properties of coacervates, microspheres, membraneless condensates, and living cells is too obvious to neglect. For this reason, comparing the physical properties of artificial condensates with natural ones (for example, with living cells) helps us to understand what makes them similar.

Semipermeability

The larger the size of the solute molecule, the more poorly it dissolves in intracellular water (the property of semipermeability), the smaller the coefficient of the equilibrium distribution of this solute between the cell water and the water of the medium. Thus, with an increase in molecular mass by 2.3 times, from 150.13 (L-arabinose) to 342.3 (sucrose), the sugar distribution coefficient decreases by 40%, from 0.46 to 0.28 (see [10], Table 1, column for Muscle T). The mass of the D-raffinose molecule is 18.5 times the mass of the methanol molecule, and its distribution coefficient is 91 times less than that of methanol (see the Table 1, column for Muscles P).

In the case of coacervate (gelatin-gum Arabic complex), a 2-fold increase in the mass of the molecule (sucrose/L-galactose) led to a decrease in the distribution coefficient by only 0.02% (from 0.61 to 0.60; see the Table 1). That is, with the obviously low solvent capacity of coacervate water, its sensitivity to the size of the molecule turns out to be extremely weak (this is the only quantitative assessment of this kind in the literature; further research is needed in this area). However, in the case of gelatin gel (18%), an increase in the molecular mass of the solute by 11 times (D-raffinose/methanol) led to a decrease in the distribution coefficient by 34% (see the Table 1). Unfortunately, microspheres were not used to study equilibrium distributions of solutes within microsphere/medium systems.

In living cells and cell models, the reduced dissolving capacity of water is the simplest mechanism for exclusion of free solutes from the systems into the media. In order to accumulate solutes in their internal environment, cells or condensates must have a protein matrix that is capable to adsorb those solutes in physiologically significant quantities.

Selective adsorption

Selective accumulation of solutes by a living cell has always been explained by the role of the membrane. In this light, it was logical to think that the study of the equilibrium distribution of solutes between membraneless condensates and the medium is not of interest to cell physiology. Nevertheless, Troshin cites literature data on the leading role of sorption, and not the membrane, in the selective accumulation by cells of some ions (K^+), and the exclusion of others (Na^+, Cl^-) in equilibrium conditions (see [11], Figs. 90, 104–107, 110, 113, 116, 119). In addition, the bound state of most of the K^+ in muscle cells is shown by a direct, electron microscopic method [31]. Donnan's model cannot be used for a satisfactory explanation of intracellular equilibrium distributions of K^+ since this cation directly interacts with carboxyl groups of proteins [3, 20, 32]. Since the adsorption of solutes makes the participation of membranes unnecessary, so it is expected that membraneless condensates can also accumulate solutes.

K^+ ions are the main cation of the cell and their selective accumulation in the presence of Na^+ is a distinctive feature of living cells. There is, apparently, only one work in the literature that shows that the membraneless Fox's microspheres are able to accumulate K^+ from the medium, and its equilibrium concentration in this membraneless compartment is 1600 times greater than in the medium ([33], Table 1). However, it is important to show that such accumulation of K^+ in microspheres (or in other kinds of membraneless condensates) occurs in the presence of Na^+ in the medium. This would mean that the macromolecular matrix of a membraneless condensate is able to selectively bind K^+. The predominance of K^+ in a living cell indicates the key role of this cation in life processes, so it is possible that its role in intracellular membraneless condensates, which play a diverse role in the functioning of the cell, is no less significant. It should be noted, however, that not only K^+ can accumulate in membraneless condensates. The choice of ions is determined by the adsorption properties of the condensate's protein matrix and by its functional state.

Electrical potentials

Research has revealed that proteinoid microspheres can generate action potentials and demonstrate ion channel activity similar to living cells, despite the fact that they do not have a membrane with specialized proteins integrated into the lipid phase ([34], Figs. 1, 2); the differences

visible in the figures are insignificant and may be due, among other things, to differences in the technique used.

The similarity of the electrical properties of membraneless condensates and living cells has attracted much attention in the context of the problem of the origin of life [35–38]. Indeed, protocells, condensates of the simplest peptides, could not get at their disposal a membrane at least to some extent similar to the axon membrane with complex protein structures (pumps and channels). In spite of this, these same peptides and their associates were able to adsorb on their surface everything necessary for the emergence of life due to their chemical nature. It is clear that the concept of the biological membrane emerged as the result of evolution rather than from lightning striking the primordial soup [34]. Nevertheless, membraneless cell models are able to generate action potentials that indicate a crisis in the membrane theory of electrical potentials: alleged membrane action potentials are in fact interphase ones from the point of view of membraneless physiology. The activity of the "ion channels" of membraneless microspheres also appears in a different light ([34], Fig. 2): it is caused by spontaneous changes in the adsorption potential of the microscopically thin surface layer of microspheres, that is, it is the result of the same physical phenomenon as in living cells, according to Ling's theory [39]. Thus, membraneless protocells, the precursors of living cells, had electrical activity long before the formation of membranes such as the axon membrane.

Therefore, the bulk-phase approach provides a general physical basis for the electrical properties of both cells and cell models – selective adsorption of K^+ in the presence of Na^+ (interphase resting potential) or vice versa, adsorption of Na^+ in the presence of K^+ (interphase depolarization). Other ions can also form electrical potentials, depending on the composition of the phase and its adsorption properties. Using the example of electropotentials, we see that the phase and membrane mechanisms of their generation are fundamentally different: on the one hand, there is a change in the physical state of the ions (as a result of adsorption), on the other hand, the physical state of the ions does not change, only their position relative to the membrane changes (first they are located on one side of the membrane, and then on the other side). From a physical point of view, interphase boundaries are necessarily present in any heterogeneous system. Thus, the peculiarity of the bulk-phase approach is its universality. Membranes are undoubtedly significant interfacial structures that may form at the cell/medium or condensate/medium interfaces, but they are a special case of interfacial interactions whose functionality is often exaggerated or distorted.

Osmotic stability

An idea of the osmotic stability of microspheres is given by data on the effect of the NaCl salt concentration on the diameter of microspheres ([40], Fig. 5). With an increase in the salt concentration (from 0.2 to 10.0%; the physiological concentration of NaCl for humans is 0.9%), the diameter of microspheres increased by 80% (from 1.4 to 2.5 μm with an increase in the salt concentration from 0.2 to 1.3%). With a further increase in the salt concentration, the diameter of the microspheres decreases from 2.5 to 0.4 μm at a salt concentration of 10% (the volume decreased by 6 times), and at higher concentrations, the microspheres dissolve. Thus, microspheres are stable in the 60-fold range of salt concentrations from 0.03 to 1.7 M. The effect of other salts and pH on the size of microspheres was studied by Kokufuta et al. [41]. These data suggest that the strength of the interaction of proteinoids with water is so strong that they are able to provide osmotic stability of microspheres in a wide range of osmotic pressure of the environment: from fresh to seawater, containing about 3–4% salts (mainly NaCl).

The semipermeable membrane has been proposed to explain osmotic properties of living cells for decades, but the membrane approach has been flawed even with regard to living cells [11, 22], and the osmotic properties of the membraneless microspheres prove the need to study the interaction of water with the macromolecular matrix of cells/condensates as the root cause of their osmotic stability.

Of course, these data are too few in comparison with what we would like to know about membraneless condensates, but they give an idea of a promising direction of research.

Is there bound water in membraneless condensates?

Changing the structure of water under the influence of any surface with which it interacts is a fundamental physical phenomenon [17]. Cytologists came to the phenomenological conclusion that intracellular water differs in its properties from bulk water almost 200 years ago, but, even more surprisingly, they believed that the altered state of water in the cell allows it to be considered as a membraneless compartment [4]. A significant obstacle to the development of these ideas was the lack of knowledge about the physics of the interaction of water with cellular structures.

Ling [42, 43] drew attention to the fact that the chain of peptide bonds of any protein is a geometrically correct alternation of positive (NH) and negative (CO) dipoles. It is clear that the electrostatic interaction of a dipole water molecule with these dipoles will lead to an increase in its dipole moment, that is, to polarization. Polarized water molecules form stronger hydrogen bonds among themselves compared to bulk water (see [10] for refs). Since the mentioned dipoles of the peptide backbone have different signs of electric charge and are staggered along the polypeptide chain, the water molecules interacting with them will also have antiparallel dipole moments, that is, the first layer of water molecules will also represent an alternation of positive and negative poles in a staggered order. Stronger hydrogen bonds between the water molecules of the first layer will make it a matrix for the adsorption of the second layer of water, and the second layer will become a matrix for the adsorption of the third layer, etc. Under crowding

conditions, an adsorption phase of water with a thickness of 5–9 layers will be enough to bind all or most of the intracellular water. Stronger hydrogen bonds between the water molecules of the adsorption phase make it difficult to embed solutes into it, which is the reason for the reduced dissolving power of such water (Fig. 1), and are a key physical factor explaining many other biologically significant phenomena [3, 10].

The mechanism of formation of "biological" water proposed by Ling is equally applicable not only to proteins but also to any macromolecules: artificial polymers, polysaccharides, RNA, DNA, as well as to mineral (as mica) and artificial surfaces (as graphene). The secret of such universality lies in the flexible nature of the water molecule itself and the networks of hydrogen bonds formed by it. However, bound water may become a factor controlling the physical properties of a cell or membraneless condensates only if its physical state is able to constantly change in order to regulate different cellular processes. The only class of proteins meeting such requirements are intrinsically disordered proteins with their dynamic (labile) conformation [44]. In the context of Ling's theory, disordered proteins are converters of the physical state of the system between two ones, resting state (water is adsorbed) and active ones (water is desorbed).

Disordered proteins or disordered regions of complex proteins (for convenience, let us replace these two terms with a single one, intrinsically disordered peptide chains, IDPCs, which can both be separate molecules and be a part of complex proteins) are of interest to Ling's theory primarily because they have an expanded conformation, that is, a conformation in which all or most of the NH- and CO-groups of peptide bonds participate in hydrogen bonds with water. Indeed, the molecular surface area accessible to water is significantly larger in disordered proteins than in ordered ones [45]. Thus, IDPCs are the key adsorbents of water, and due to their conformational variability, water adsorption can be cyclically replaced by its desorption. A change in the physical state of water determines a change in the physical state of a cell or condensate: the phase is formed – the phase disappears (just as ice is formed – a two-phase ice/water system arises; ice melts – the system becomes single-phase one). By changing its conformation, IDPCs plays the role of a converter of the physical state of the system, therefore, factors affecting the conformational transitions of IDPCs control the physical properties of the system. These factors can be ions, organic compounds (for example, ATP), proteins, other biopolymers, for example, RNA and DNA. Interestingly, membraneless condensates are recognized as phases recently [46], and only disordered proteins are recognized as phrase-forming proteins [47–54]. These trends of recent years are in good agreement with the logic of Ling's theory, but the role of water in the formation of membraneless condensates is underestimated in these articles. Studies such as those conducted by Troshin (Fig. 1) would make it possible to establish whether membraneless condensates have semipermeability. A positive answer to this question would be proof of the key role of water in the formation of biophases. In general, we have to state the following: there is no evidence yet that membraneless condensates possess at least one of the fundamental physical properties. There are but isolated observations in the literature that agree with Ling's theory. Consider them.

According to Ling's theory, ATP, binding to a peptide, enhances its ability to adsorb water. The observation that the binding of ATP to polylysine leads to the formation of a membraneless condensate [55] looks like experimental proof of the validity of Ling's approach. Indeed, polylysine, in complex with ATP, changes the physical state of such a large amount of water that it becomes possible to have an aqueous phase that is immiscible with bulk water. Membraneless condensates are also formed by the interaction of ATP with a number of short peptides [8], with actin [56], immunoglobulin [57]. ATP tends to bind to the most flexible and hydrated regions of the polypeptide chain, and this interaction of ATP with proteins is physically universal [58]. The result of this interaction is also probably universal: in most cases, membraneless condensates (organelles, coacervate droplets) are formed. ADP appeared after ATP splitting does not cause the formation of coacervate droplets [59]. It is understandable because, secondary structures are formed in the protein after ATP splitting [60], decreasing the contact area of the polypeptide backbone with water, and decreasing the amount of adsorbed water, leading to the disappearance of membraneless condensates. Thus, controlling the ATP/ADP ratio is one of the ways of regulating phase formation.

A new phenomenon for Ling's theory is that the formation of phases induces not only ATP but also nucleic acids, DNA, and RNA [61]. This similarity is obviously explained by the fact that both ATP and the nucleotides that compose DNA and RNA belong to the same type of chemical compounds – the phosphoric ethers of nucleosides. Therefore, polynucleotides interacting with proteins increase affinity of their polypeptide backbone for water, like ATP, which leads to the formation of an adsorption aqueous phase.

Interestingly, the relationship between the physical state of water and ATP level is observed on the scale of the whole cell. With a cyclic change in the intensity of yeast cell metabolism, expressed in fluctuations in ATP content, the dynamics of intracellular water and its dipole moment changed synchronously with the amount of ATP [62–68]. If the level of intracellular ATP decreased below the critical level, the fluctuations of the dipole moments of water stopped. The authors note changes in water properties such as viscosity, dielectric permittivity, and density occur not only at the level of the whole cell but also at the subcellular level. The authors believe that the data they obtained are best explained on the basis of Ling's theory. Another example of the usefulness of Ling's theory can be given: nuclear pores. It is known that there are many disordered proteins in the lumen of nuclear pores [69], which regulate their permeability to various molecules. If the disordered proteins of the nuclear pore adsorb more water, then the lumen of the pore will narrow and the diffusion of solutes through the pore will slow down. Thus, the

physical state of water is the regulator of the size of the lumen of nuclear pores.

A great difficulty for those who do not take into account the contribution of water to the physics of membraneless condensates is the explanation of the nature of condensates that contain subcondensates [5, 70]: why do subphases in the condensate not merge, why they coexist separately? If we accept, in accordance with Ling's theory, that water is the component that determines the phase nature of the cell and membraneless condensates, then this possibility opens up. For example, it turns out that adsorbed water is more hydrophobic than bulk water [10], that is, from a physical point of view, a condensate can be likened to a drop of oil in an aqueous environment. In addition, water is capable, as already mentioned, of creating a wide variety of structures depending on the nature of the surface with which it interacts, so if the polyphase condensates have water phases with different hydrophobicity, with different hydrogen bond net structures, with different thermodynamic parameters, then such phases will be structurally incompatible with each other and will coexist as separate condensates in a hierarchical system. However, as soon as, due to changes in the properties of the adsorbent, the aqueous phases converge in their thermodynamic parameters, they will merge into one, and vice versa, if sorbents with different properties appear in the condensate, the condensate will split into two or more phases. In this context, the idea that it would be useful to replace the "from structure to function" paradigm with the "from physical state to function" paradigm is of interest, and replace the usual "key–lock" specificity with the idea of physical specificity, when the specificity of interactions is determined by a unique combination of physical parameters of interacting systems [71]. Perhaps it is in physical specificity that it is necessary to look for the specificity of interactions (albeit weak) of disordered peptide chains with each other and with other macromolecules.

From coacervates to atomistic cell physiology

The studies of Nasonov [12], Troshin [11], and Ling [3, 22] laid the foundation for the membraneless physiology of the cell. Structural changes in the cytoplasm caused by various external stress factors were considered by the Nasonov School as resulted in the loss by proteins of their native sorption properties: water bound by intracellular structures became free, and the structures themselves turned from hydrophilic to hydrophobic state. However, with a more thorough study, it was possible to observe a barely detectable paradoxical phase in the cell response, when structural changes were of the opposite nature to changes in the excited/damaged cell ([24], Fig. 1). A clear example of two-phase cell response to hydrostatic pressure is shown in Figure 2. At moderate pressure (100–200 atm), the cell, under conditions of diffusion equilibrium, adsorbs less vital dyes than control muscles. Since the dyes used are highly hydrophobic substances [72], the experimental curves indicate a change in the hydrophobicity of cellular structures

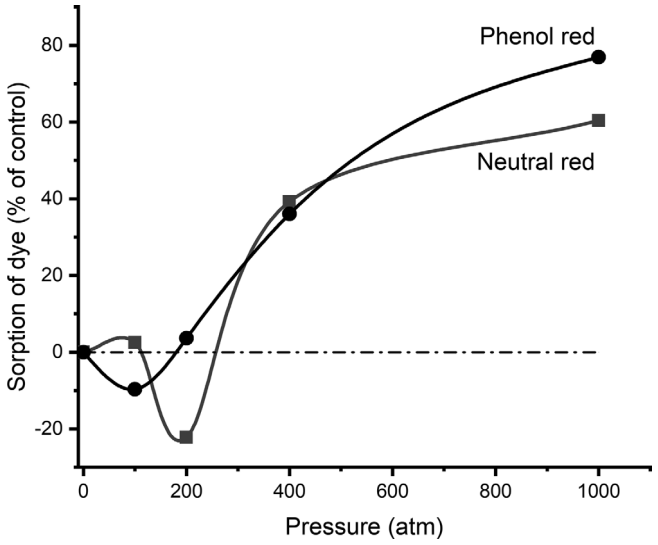

Fig. 2. Binding of the vital dyes by sartorius frog muscle during the action of hydrostatic pressure ([12], Figs. 20, 21, redrawn).

depending on the pressure. It is obvious that under the action of moderate pressure, the cell structures become more hydrophilic, that is, they bind more water. This phase of the cell response is characterized by a decrease in the viscosity of the cytoplasm, and as a result of the disaggregation of protein complexes, the cytoplasm becomes more transparent [12]. From the point of view of Ling's theory, these changes can be explained by an adaptive increase in ATP synthesis in a stressed cell that then interacts with target proteins, increasing their ability to adsorb water (the theory does not exclude other reasons either). With further increasing pressure, changes characteristic of the excited state of the cytoplasm begin to occur, such as action potential, synaptic activity, muscle contraction, the increased secretory activity of the cell, and the like; similar physical changes also occur with inflammation and cell damage [12].

Since Nasonov and Troshin considered the cell as a system of complex coacervates, this first phase of the cell's reaction to pressure indicates the appearance of new hydrophilic coacervates in the cell, which increase the volume of the bound water fraction in the cell. In modern terms, under unfavorable conditions, stress-coacervates, stress-bodies, stress-condensates begin to form in the cell (this is the adaptation phase of the cell). With a further increase in the effect of the stress factor, hydrophilic stress coacervates are destroyed, and after them, those coacervates that are present normally in the resting cell begin to collapse. With a further increase in the stress factor, cell structures are damaged, first reversibly, then irreversibly.

Ling's theory allows us to understand the phenomena under consideration at the molecular level. In its light, our attention is switched to disordered proteins whose conformation is extremely sensitive to environmental conditions and to interactions with regulatory ligands. It is the IDPCs that are the best candidates for the role of converters of the physical state of membraneless organelles,

coacervates, and condensates, and all membraneless compartments together determine the physical state of the entire cell. Ling's idea of the complex protein-$(ATP)_m(H_2O)_n(K^+)_q$ seems to be a good basis for understanding the physics of membraneless condensates. Since this complex is a minimal structure that exhibits the fundamental physical properties of the whole cell, it can be called a physiological atom [10, 24] or nano-protoplasm [25].

If we assume that the physiological atom was indeed the basis for the formation of the first living cell [10], then the further evolution of the cell not only expanded this basis, increasing the diversity of IDPCs, but also improved the control of their properties. An important stage in the evolutionary development of IDPCs was their inclusion in the composition of more complex proteins containing ordered regions with their diverse enzymatic activity and high specificity of interactions. In addition, ordered regions were able to influence the properties of disordered regions and vice versa, which expanded the functionality of both IDPCs and complex proteins [73, 74].

Due to the fact that water has a wide polymorphism (see above), each physiological atom formed on the basis of IDPCs has a unique set of thermodynamic parameters, that is, it has physical specificity. Due to this specificity, physiological molecules (condensates) are formed with certain properties necessary for a particular cell function. It is possible that physiological atoms will also be able to be combined into a periodic table since in physics appeared the opportunity to systematize nanostructures according to principles resembling Mendeleev's periodic table [75].

The principles of membraneless physiology outlined here need further experimental confirmation. And to do this, they need to be known at least as a working hypothesis when studying membraneless condensates. If in the future it is shown that membraneless condensates possess all four fundamental physical properties of a living cell, discussed above, it will mean that the membrane physiology can no longer claim the influence on the minds of scientists it has exerted in the past.

Membraneless organelles must obey the Meyer–Overton rule

At the end of the 19th Century, membranes were recognized as a structure critical to compartmentalization, and then the question of their chemical makeup arose. The discovery of the Meyer–Overton rule (around 1900) helped to answer this question, according to which the better the anesthetic dissolved in olive oil, the faster it accumulated in the cell and the less it was required to achieve anesthesia. As was logical for that time, the conclusion was made that the cell membrane consists of lipids. Recognition of the lipid nature of the membrane for decades predetermined its study as the primary site of application of anesthetics, however, about 40 years ago it was discovered that proteins in fact are the primary site of action of anesthetics [76].

Interestingly, long before the recognition of the protein theory of anesthesia, it was proved that hydrophobic regions in the cell are formed not only by lipids, but also by hydrophobic nuclei of globular proteins (containing residues of hydrophobic amino acids), and the latter can change in size, disappear and reappear again depending on conformation [77]. In addition, it turned out that lipid and protein hydrophobic phases are indistinguishable from each other by their thermodynamic properties [78, 79]. This means that the Meyer–Overton rule proves not the lipid nature of membranes, as was believed at the beginning of the 20th Century, but only the hydrophobic nature of the interaction of anesthetics (and other low-polar substances) with the cell [76]. In other words, if the cells were surrounded by protein membranes, then the Meyer–Overton rule would also apply.

The level of hydrophobicity of a cell is one of the key indicators of its physiological state [24]. The number of binding centers for hydrophobic molecules in a cell varies widely: (1) under the action of hydrostatic pressure on muscle tissue (Fig. 2), on brain tissue ([12], Fig. 23), on the pancreas ([12], Fig. 24), on the submandibular gland ([12], Fig. 25), on the parotid gland ([12], Fig. 26); (2) under the action of an audible sound on the *sartorius* frog muscle ([12], Fig. 13); (3) with irritation of the crab nerve ([12], Fig. 50) and the frog sciatic nerve ([12], Fig. 51); (4) with denervation of rat muscle ([12], Fig. 52); (5) with electrical stimulation of the cat's ganglia ([12], Fig. 56).

The study of changes in the hydrophobicity of the cell under various influences did not spread, since the cause of these changes was unclear. Indeed, if we accept that the hydrophobicity of the cell is due to lipids, then their amount could not change significantly in isolated tissues in minutes or hours of experiments (as in Fig. 2, for example). Now, when the hydrophobicity of proteins and protein complexes and their variability have been well studied, when the nature of such significant fluctuations in the size of their hydrophobic phase has become clear, the way opens up for studying the contribution of changes in hydrophobicity to physiological processes [73].

Since proteins are a mandatory component of membraneless organelles, it will not be surprising that the Meyer–Overton rule will be applicable to them as well. Membraneless condensates of macromolecules demonstrate high dynamism: they appear, disappear, and rearrange during functioning, and the level of their hydrophobicity will be another physical parameter that will give valuable information about the structure of these formations and their dynamics. In the study of membraneless condensates, it is not necessary to use only anesthetics, since the Meyer–Overton rule is also satisfied by substances that do not have anesthetic activity [76].

Functional changes in the hydrophobicity of proteins and protein complexes during the transition from resting to active state will inevitably cause a redistribution of hydrophobic substances between different cellular structures and membraneless condensates. Examples of such substances include lipids, hydrophobic amino acids, nucleotides, and many other hydrophobic metabolites [80]. By its hydrophobicity, ATP comes close to the hydrophobicity of chloroform [72], therefore, the hydrophobic regions arising

in the active cell may act as temporary depots for this amphiphilic compound. Thus, the sorption properties of proteins retain their functional significance not only at rest, but also in the active state, in the language of Ling's theory.

After a long period of history, the Meyer–Overton rule has proven to be an effective tool for studying physiological phenomena. Attempts to understand the underlying physics of the rule have led to significant discoveries and generalizations, so there is no reason to believe that it would be ineffective when applied to membraneless organelles.

Conflict of interest

The author declares no conflict of interest.

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