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Research Article

The Phenomenon of Fast Efflux of the Vacuolar Content Via Specific Micron-Diameter Holes Intermittently Opening in the Vacuolar Membrane an Attempt to Explain the Biophysics of This Phenomenon

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Abstract

The present paper discusses the phenomenon of fast trans-membrane efflux of the vacuolar content (soluble substances and compounds) via micron-diameter holes (having stiff leaves), which are intermittently opening (and sealing) in the protein shell of the vacuolar membrane. Processes bound up with this phenomenon may be easily observed with the aid of ordinary laser scanning confocal microscopes. In the present article, the consideration and discussion were reduced to isolated plant cell vacuoles. Meanwhile, in principle, the phenomenon discovered potentially applies and may be considered for the membranes of other types of cell organelles. On account of the phenomenon discovered, it becomes obvious that any discussions of efficiency of fast trans-membrane transport processes mediated by nano dimensional pores cannot be considered as having any reliable grounds under them. The problem is that such pores have never been really observed in dynamics because microscopes, which would possess some necessary resolution of such dynamic processes, do not exist.

Keywords: Isolated vacuoles, Natural decay, Efflux of nutrients, Hole in the membrane, Membrane hole sealing, Biophysical characteristics of vacuoles, Laser scanning confocal microscopy

Abbreviations: ABC transporters: ATP Binding Cassette Type Transporters; ANS: 8-Anylino-1-Naphtalensulphonic Acid; CPP: Cell Penetrating Peptide; Cryo-EM: Cryo-Electron Microscopy; DOPC: Dioleoylphosphatidylcholine; DPH: Diphenilhexatrien; FLSCM: Fluorescent Laser Scanning Confocal Microscopy; GABAARs: γ-Aminobutyric Acid A Receptors; GPCR: G Protein Coupled Receptor; GUV: Giant Unilamellar Vesicle; IR: Infrared; KB-Ideology-Knowledge-Based Ideology; LSCM: Laser Scanning Confocal Microscope; LUV: Large Unilamellar Vesicle; NA transporters: Nucleobase-Ascorbate Transporters; NMR: Nuclear Magnetic Resonance; PCV: Plant Cell Vacuole; VLS: Vacuolar Liquid Substance.

Introduction

Appearance of the author's article entitled "The Phenomenon of Fast Empting of an Isolated Vacuole via Holes Forming in Its Membrane, and Perspectives of Application of the Respective Knowledge in Organelle Level Treatment of Heavy Diseases" [1] has provoked a kind of dual reaction of the community of researchers to the author's discovery of the phenomenon of fast empting. Frankly speaking, the author expected that such dual reaction might take place. Some of known researchers considered the author's discovery as very important, and the author's publication as the work giving explanations of many issues earlier unexplained. Some researchers stated that the author's discovery contained a really great driving potential for the further progress expected by them in the research field.

Nevertheless, the author encountered the researchers, who, without doubt, were driven by the heavy feeling of envy. And this was natural. This may be understood. Indeed, how is it possible that an interesting and perspective discovery does not belong to them? These researchers insisted that (i) The holes, which open in membranes of vacuoles, were visible on the snapshots, but not sufficiently well; (ii) Presentation in the form of observations only was insufficient, and, furthermore, immediate theoretical explanations of the observed phenomenon were needed (as if theoretical explanations of many other biological phenomena described with false and absolutely useless "free integrals" were given and really existed); (iii) Explications of statistical data bound up with observations of the processes (bound up with the phenomenon discovered by the author) was needed; etc.

The author has to confess that his publication of "the phenomenon of fast empting" discovered [1] has been intentionally delayed by him for a very long time period (i) In order to make sure in the plausibility of the result, and (ii) Considering the overwhelming pressure of the conception of membrane poration upon the minds of researchers during the recent 30 years.

Furthermore, there were the following three problems. Problem 1. The author's discovery of the holes opening in the membrane has really completely changed the understanding of processes of fast trans membrane transport. Problem 2. In connection with the discovery of the phenomenon of fast empting of vacuoles, the conception of fluidity of membrane lipids (in the aspect of explaining of the processes of sealing membrane pores) may now be recognized as false. Problem 3. Even osmotic phenomena could now be considered only as secondary, because opening of micron-diameter holes was capable of solving any trans-membrane transport problems. So, the author's discovery has inevitably reoriented many well-known approaches to understanding of membrane biology and biophysics. Having taken into account the dishonest negative reaction of some researchers to his discovery, the author has decided to describe the phenomenon discovered by him again, and now in details and on a higher scientific level (with elements of mathematical modelling).

In the present paper, the author has reduced his consideration of the phenomenon discovered to only an isolated Plant Cell Vacuole (PCV) expressed in the form of fast intermittent (stepwise) efflux of substances (soluble and, probably, partially non-soluble content) through the PCV membrane. This phenomenon was for the first time noticed by the author and his team of researchers in 2015, in course of confocal microscopy observations bound up with analysis of peculiar properties of some components in the PCV morphological structure [1]. The phenomenon was specially observed and registered in 2017 [2]. It was later confirmed in numerous experiments from 2017 till 2025.

The phenomenon discovered is principally new. In this connection, there appears the need to consider biophysical characteristics of PCVs, characteristics of PCV membranes, which are bound up with this phenomenon (e.g. biomechanical membrane's stability [3] and other important properties [4,5]), and analyse the opportunities of applying the discovered phenomenon in the aspect of transport of substances from PCVs, which could be useful from the viewpoint of contemporary goals of cytogenetic medicine.

A PCV and its membrane are capable of dynamical morphological reconstructions discussed in publications of *Y Oda, et al.,* (2009), *E Etxeberria, et al.,* (2012), *E Etxeberria, et al.,* (2013), *N Ozolina, et al.,* (2013) and other authors [5-8]. PCV membranes are known to ensure high degree of plasticity. And biochemical diversity of membranes, which are known to be comprised by numerous lipid species, contributes to membrane stability and plasticity.

It is known that principal functions of the central vacuole living inside a plant cell are determined by its participation in processes of redox ionic homeostasis of the cytosol [9,10], storage of primary and secondary metabolites, osmotic regulation, detoxification of xenobiotics, formation of protective responses of the cell under the conditions of biotic and abiotic kinds of stress [9,10], as well as participation in the metabolism of poisons [11] and in the processes of programmed cell death [11]. Furthermore, already in the 2000s, it was shown that vacuoles were bound up with cytoskeletal elements [12]. The role of vacuoles in the stomatal movement [13] was also emphasized [12,13]).

Important investigations were conducted in order to understand functions of the vacuolar membranes. Presently, it is known, for example, that vacuolar membranes form a protective layer against (i) External mechanical, osmotic and toxic forms of stress, while providing for mechanical stability; (ii) Bacterial infecting and even (iii) Viral infecting. Vacuolar membranes are discussed as involved in ongoing signaling, trafficking, and morphogenesis. Furthermore, vacuolar membranes participate in provision of intra-cellular redox homeostasis [3,9,10].

As far as PCV membranes are concerned, from time to time there appear declarations about new insights into the architecture of these membranes [14], and declarations about diversity of specific systems responsible for transport of water and substances through PCV membranes [14]. At the same time, the level of understanding of biological and biophysical properties of PCV membranes remains very low. All the properties of plasma membrane and membranes of organelles are to be understood better.

The author considers it necessary to recollect and briefly describe known concepts of transport via PCV membranes. In diverse investigations conducted in many countries, the researchers postulated and tried to analyse several concepts describing transport of water, solutes and compounds (a) Inside the cytoplasm, (b) Via cellular and organelle membrane transport channels, (c) Via organelle membrane pores into the organelles, etc.

As a result of long-term investigations, the following molecular-level trans-membrane transport mechanisms have been identified and described. These mechanisms are considered to be responsible for transport of soluble substances and compounds from the cellular cytosol into the vacuole: tonoplast-bound ABC transporters [15-18], nucleobase-ascorbate transporters [19-22], tonoplast transporters using the proteomic mechanism [23-25], vacuolar H+-ATPase driven potassium transport channels [26-29], solute/H+ antiporters [27,28] discussed nano disc complexes with small multidrug transporters [30], etc. Noteworthy, the proteomic approach studied for the transport process in the vacuoles isolated from cauliflower buds was represented as providing for a novel (in its time) form of transport [24]).

The list of above transport systems is traditionally complemented with peptide-based transporters. These transporters are represented by amphipathic Cell Penetrating Peptides (CPPs) [31,32]. Activity of this type of transporters is known to be mediated also by pore forming peptides and pore forming proteins [33,34]. CPPs, which are known to be capable of inducing dynamical phase separation of phospholipid bilayers, formation and growth of membrane's negative curvatures resulting [35,36] in undulations, and even membrane thinning or thickening, provide for the peptide-based transport. Transport of acid-glutathione conjugate into the vacuole was discussed by *N Ohkama-Ohtsu, et al.,* [37]. Furthermore, the list of above transport systems may also be complemented with SWEET and Semi SWEET transporters [38-40], which provide for (or, probably, facilitate) the functioning of the respective diffusion mechanism. Special attention of well-known researchers was concentrated also on transport processes mediated by protein-bases transporters, which were discussed as mediators providing for transport of nutrients inside and outside a vacuole [41,42,27]. Note, the issues bound up with invaginations in the membrane, which were related to the transport processes, were not forgotten in discussions. It was also stated that some of abovementioned transport systems were needed, for example, in formation of (i) Fe/S clusters, (ii) So called stomatal movements [13]), and probably also (iii) Ion fluxes. Furthermore, the author has to emphasize that (according to the accepted conception) transport of soluble substances from the cytosol into the vacuole may also be mediated by ion channels. The most widely discussed type of such a channel for transport of soluble substances is represented by pores forming in membranes owing to various factors and processes.

In the literature, one will not find any plausible discussions bound up with observations of dynamics of pores in biological membranes of natural cells. Meanwhile, in 2013-2025 discussed were various forms of investigations, which presumed hypothetical formation of pores in such artificial objects as Giant Unilamellar Vesicles (GUVs). Declarations about the applied approaches to formation of membrane pores included:

a) Tension-induced formation of transient pores [43-47];

b) Pore formation under external stress factors [48];

c) Ion-induced transient potential fluctuations provoking pore formation [49];

d) Pore formation owing to membrane electroporation and [50,51];

e) Pore formation owing to mechanical factors (accompanied with lateral sorting of membrane lipids) [52];

f) Pore formation owing to membrane thinning (as the process in course of which peptide-induced pore formation can take place) [53]);

g) Application of bacterial pore-forming toxins (for example, one of the models good for plasma membrane damage assessment implies that the cells are exposed to the bacterial pore-forming toxin Listeriolysin O (LLO), which forms rather large (30-50nm in diameter) protein pores in cholesterol-containing membranes).

h) Furthermore, in [54] analysis of γ-Aminobutyric Acid A Receptors (GABAARs) was conducted. The respective pentameric ion channels were revealed with the aid of Cryo-Electron Microscopy (Cryo-EM).

Unfortunately, the corresponding conclusions of the researchers were bound up mainly with hypothetically assumed possibility of appearance and activity of membrane pores. The conclusion about the hypothetical character of practically all the known conclusions bound up with membrane pores is explained very simply: (1) Practically all the attempts to discuss behaviour of membrane pores have been conducted on GUVs (not, say, on membranes of natural organelles); (2) Membrane pores are very small (1-3nm) and, so, their dynamics (opening, sealing) is absolutely unobservable. Such traditional methods as Cryo-EM, X-ray crystallography, atomic force microscopy, Nuclear Magnetic Resonance (NMR) spectroscopy may

allow one to observe something, which may be qualified as a pore. Meanwhile these methods do not allow researchers consider nano-dimensional pores in dynamics. Observations of dynamic processes are presently available only for the objects of 10^3 larger. Such observations may be conducted only with the aid of Fluorescent Laser Scanning Confocal Microscopy (FLSCM). But, unfortunately, the resolution of FLSCM is very low. FLSCM does not allow one to obtain useful results of observations of any objects smaller than 0.5μm. Furthermore, structures of biological membranes necessitate application of the tools more powerful than FLSCM [55]. Noteworthy, practically all the trans-membrane transport mechanisms mentioned above were discussed mainly in the aspect of transport of water and solutes. Unfortunately, in 99% of cases, the researchers discussed the trans-membrane transport almost exclusively in one way only. As obvious from titles of many articles, this was the way "to the vacuole" (note, not into the vacuole).

A really plausible mechanism responsible for trans-membrane transport of non-soluble substances into the vacuole and in the reverse direction was discussed, for example, in one of the author's articles, discussed was transport via micro-tubes [2]. This is an absolutely realistic transport mechanism observed in hundreds of the author's investigations. This mechanism is responsible for transport of not only solutes but also non-soluble substances and even nutrient protein globules through vacuolar membranes. And this mechanism is responsible for transport (i) Into vacuoles, (ii) From vacuoles outside, and (iii) Inside vacuoles. No wonder that this mechanism was ignored by the community of biologists. This situation is absolutely understandable. Consideration of biological publications bound up with vacuoles published during the recent 25 years gave evidence that the problem was as follows.

The dominating majority of biologists were (and are) involved in (i) Investigations bound up with the trans-membrane transport of water and solutes (this transport being mediated by transporters), and (ii) Experiments bound up with accidental membrane transformations (formation of invaginations, curvatures, pearling in GUVs, formation of membrane tubes on the basis of curvatures, etc.). Noteworthy, the related experiments were conducted mainly on GUVs. Unfortunately, results of such experiments may not be directly applied to membranes of natural organelles.

Meanwhile, new trans-membrane transport mechanisms studied by the author [1,2] have proposed new opportunities. The author has to agree that his new conceptions based on his two discoveries came in contradiction with practically all earlier known hypothetical conceptions of trans-membrane transport. But it is OK, considering the facts that all the mechanisms of trans-membrane transport widely discussed earlier have never been practically observed. These could be only hypothetically simulated, and the results could be represented in the form of very strange computations and hardly ever understandable diagrams. It is strange, but, despite absolutely obvious contradictions, abovementioned transport mechanisms (somehow bound up with invaginations, curvatures, pearling, formation of membrane tubes) have been (in connection with some hardly ever understandable reasons) accepted by the community of biologists as the only possible forms of trans-membrane transport. Meanwhile, the channels of trans-membrane transport discovered by the author were different. It was important that these were completely observable and obvious.

While continuing the discussion, it is important to emphasize also the fact traditionally ignored by practically all the researchers involved in the investigations bound up not with GUVs, but with natural organelles, in particular, with isolated vacuoles. These researchers all conduct (and earlier conducted) their investigations on isolated vacuoles undergoing the process of natural decay. And this is not the same as to work with central vacuoles of living cells. Nevertheless, majority of these researchers construct their investigations as if the vacuoles were not isolated. They ignore (and earlier ignored) to remark about the issue of "isolatedness". Sometimes, the researchers briefly indicate to the fact that they work with "isolated" vacuoles (vacuolar "isolatedness") and, nevertheless, do not (and earlier did not) take into account the facts of:

a) existence of the vacuole observed and studied in the 'saving liquid' (surely, not 'conservation liquid', because isolated vacuoles do not undergo any conservation); furthermore, according to some strange tradition, such liquid is mechanistically qualified as either some 'solution' or some 'buffer

b) existence of the vacuole observed and studied in 'some mass' (which according to another strange tradition has been illogically (and without any shade of doubt) qualified formally, as 'some suspension') represented by other isolated vacuoles (meanwhile, as the author has already come to the conclusion that he deals with some mass of isolated vacuoles specifically interacting inside the saving liquid) (in one of his forthcoming papers, the author is planning to consider the issue of interaction of isolated vacuoles);

c) vacuole undergoing the process of natural decay. Anyway, the researchers inevitably encounter (and earlier encountered): (i) Specific behaviour of the decaying isolated vacuoles (and this looks surely as a variant of collective behaviour, which, considering some circumstances that may not be easily understood (as somebody would like to), has been completely ignored by biologists of the world); (ii) Specific processes bound up with a form of collective behaviour of decaying isolated vacuoles (the issues of collective behaviour and the related processes have been observed by the author, and will be described in the author's forthcoming papers); (iii) Specific biophysical characteristics of isolated vacuoles (which

characterize both the collective behaviour and the processes bound up with these vacuoles); (iv) Specific principles and laws of behaviour typical of membranes of isolated vacuoles, which dominate over primitivism of osmotic laws (noteworthy, almost all the researchers concentrate their attention on osmotic regularities, while completely ignoring any other factors, which surely influence the behaviour of vacuoles and, furthermore, having forgotten that these vacuoles are isolated ones); (v) Specific behaviour of membranes of the vacuoles (undergoing the process of natural decay), which, according to our experience, does not look like a form of accidental behaviour that implies accidental processes bound up with, e.g., liquid character of the lipid layer, which is qualified as its fluidity. The researchers are sure that the property of fluidity forms the basis of the membrane's matrix function, but this is a false idea. It is necessary to recognize that almost all the researchers involved in the discussion of these issues do not (and did not) pay any serious attention to all the serious contradictions mentioned above. Moreover, some of the researchers dare to openly state that "investigation of vacuoles as isolated objects is unimportant".

Development of biological science during the recent 70 years has shown that even experiments conducted by *A. Hodgkin* and *A. Huxley* in the 1950s and bound up with 'functional reconstruction of ion channels' necessitate reconsideration. Today it is possible to state only that functional reconstruction of ion channels has proved that the trans-membrane transport may aid to maintaining the ionic balance on membranes of nerve cells (and, so, forms the conditions for distribution of the nerve impulse) [56]. Understanding of the membrane ion channels is as uncertain as understanding of invisible membrane pores, which presumes elements of fantasy.

It is worth noting that *J. Gao* and *H. Wang* have honestly stated that "Being limited to (current) methods and techniques available, there are still many unanswered questions about cell membranes." "It is not clear how the cell membranes efficiently and precisely accomplish the intricate functions" [55]. And the author of the present article has to agree with these respected researchers regarding the issues noted. Indeed, on the whole, the level of the researches conducted in the direction discussed may not be considered as high. This level may not be considered as "in depth". Instead, the author has to emphasize the following: (1) The majority of researchers prefer to conduct their observations on rather specific objects such as the giant axon of Loligo [57,58] and, nevertheless, the researchers easily apply the conclusions obtained in observations and experiments on these specific objects to the issues of behaviour and functions of cell organelles; (2) Some of the known attempts of numerous researchers to construct formal approaches to investigations of GUVs and construct formal (presently quasi-mathematical) functional models (the results for these models being easily distributed onto organelles), sometimes cause surprises (for example, when constructing mathematical models of processes in the membranes, practically all the researchers compute so called 'free energy', and this is very good; meanwhile, knowledge of this function does not give anything definite to any interested researcher. These and other issues necessitate long discussions. Obviously, there are many issues bound up with the membrane structural details and membrane functions, which are waiting for their detailed investigations and deeper understanding.

In our investigations, the issues related to isolation of vacuoles, which undergo the process of natural decay, were from the very beginning taken into account as very important issues. The present publication discusses the important issue of fast empting of isolated vacuoles, to be exact, the issue of very fast efflux of the soluble vacuolar content from an isolated vacuole. Problem statement. In over than 500 observations, it has been revealed that isolated red beetroot vacuoles can eject their internal content in course of a sequence of events bound up with opening micron-diameter holes in the vacuolar membrane. It is necessary to reconsider and study in detail (i) Processes of fast empting of isolated vacuoles at the expense of these holes, and (ii) Mechanisms of opening and sealing such holes. It is necessary to understand the corresponding regularities.

Materials and Methods

The Materials Used

Our investigation was conducted on isolated vacuoles of Beta vulgaris L. dormant storage red beetroots. The seeds were planted on an experimental field. The beetroots obtained were stored at +4 to 5°C.

The Technique Used for Isolation of Vacuoles

The technique of isolation of vacuoles presumed cutting the beetroot tissues and their placing into some saving liquid (it really saves vacuoles from the fast process natural decay, which (in the norm) comes in some 12h). Practically all inexperienced researchers call saving liquid rather strangely: "suspension", "conservation liquid", "imaging buffer" or else "imaging buffer base". Meanwhile, any saving liquid is not a kind of suspension. The author's long-time investigations have shown that vacuoles in a saving liquid form a mass of objects interacting with each other. A saving liquid is not a kind of conservation liquid because conservation of vacuoles is not implied. Implied is only saving vacuoles for the time of observations. The saving liquid is not a kind of buffer in any sense. Normally, buffer is "an intermediate object or a zone between two objects". In the literature, one can find the following recommendations of insufficiently experienced researchers regarding a "universal imaging buffer base": 10% glucose, 50mM Tris (pH 8.0), 10mM of NaCl; "live-cell imaging buffer base": 10mL DMEM of high glucose without phenol red, 750 lL 1M HEPES (pH adjusted to 8.0), 400lL

50% glucose [59].

The author used the technique of isolation of vacuoles, which has been developed by his team of researchers. This technique was approbated in investigations during the recent 30 years. It presumes cutting the beetroot tissues in the saving liquid containing KCl (as the main component). The mass of isolated vacuoles was placed into the saving liquid containing 300mM KCl, 10mM EDTA, 25mM $NaH2PO_4$ + KOH up to pH 8.0, β-alanine (650 $mOsm \cdot kg^{-1}$ H_2O)). The result of isolation was purified each time. This technique of isolation is preferable (without doubt) in comparison to the approach presuming application of some "buffered sucrose solution". The latter statement may be explained.

KCl is generally used as the main component of the saving liquid for maintaining both the electro-chemical potential (it maintains the charges on both sides of the membrane) and the osmotic potential (KCl represents a good aid to structure water). Meanwhile, sucrose solutions used in the capacity of some forms of the saving liquid are capable of maintaining only the osmotic potential. The long-term author's experience and the experience of his colleagues have shown that the life of isolated vacuoles in the sucrose solution is obviously shorter; potassium goes out of the vacuole according to the law of the concentration gradient. As a result, (i) The electro-chemical potential is violated, (ii) Depolarization of the membrane takes place, and (iii) Very often collapse of the membrane takes place. In this connection, there appeared an idea to add alanine. Addition of alanine presumed the objective to apply a lower size molecule of the osmotically active substance (the size of the molecule participates in defining of the pressure inside the space filled with these molecules). Note, a molecule of sucrose is a large one. A molecule of KCl is an optimally small one. A molecule of alanine has the size intermediate between the sizes of the two abovementioned molecules (sucrose and KCl). Vacuoles placed into the saving liquid containing alanine live longer indeed.

The results of isolation of the medium composition, pH and the temperature of the yield, stability of isolated vacuoles and other biophysical characteristics were analysed. When the micro-method was used, the vacuolar yield was 800-1200 vacuoles per 1sq. cm of the cutting area, and in case of application of the macro-method, it was $4\text{-}6\times10^8$ vacuoles per 1kg of beetroot tissues, what corresponded to 0.8-1.2% of the pigment released from the tissue cut. In all our experiments, the isolated vacuoles were kept in the saving liquid during the time period shorter than 1 hour before the beginning of microscopic observations. Targeted observations of the mass of isolated vacuoles in the process of their natural decay have given the following non-typical results see Figures 1-10.

As obvious from Figure 1, it was possible to observe the vacuoles filled with the vacuolar content (nutrients, possibly, protein globules) inside the mass of fresh isolated vacuoles during the first five minutes after isolation.

The Equipment and the Techniques Applied in Observations of the Processes Bound Up with Transport of Solutes and Compounds Through the Vacuolar Membranes in the Present Investigation

Dynamic localization of the scrutinized objects was routinely followed with the aid of FLSCM and the algorithms oriented to single-object tracking. The locations and trajectories of the isolated vacuoles observed could be traced, mapped. The locations and trajectories were bound up with the respective biophysical characteristics. On this basis, the corresponding physiological and biophysical processes were assessed and analysed (Figure 1).

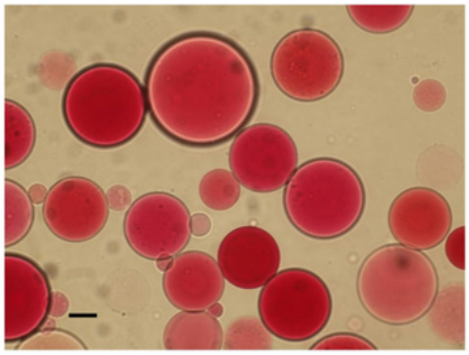

Figure 1: The mass of fresh isolated vacuoles under a light microscope.

In routine observations of the phenomenon to be studied, the author applied a laser scanning confocal microscope (LSCM) LSM710 (Carl Zeiss, Germany) (laser 405nm, objective plan-apochromat 63×/1.40 Oil DIC M27, pinhole 43μm, Ch1: 420-460nm, Ch2: 470-530nm).

In various observations the author and his team used such fluorescence molecular probes as filipin (a well-known sterol-binding antibiotic having an expressed affinity to sterols); laurdan (or 2-(dimethylamino)-6-dodecanoilnaphtaline), a lipophilic probe actively fluorescing in contact with hydrophobic domains; Diphenilhexatrien (DPH) (all Sigma-Aldrich, USA). ANS (8-anylino-1-naphtalensulphonic acid) and bis-ANS known to have affinity mainly to proteins (but surely retaining the affinity to lipids) were used in the cases, when it was necessary to confirm the presence of proteins in the objects observed. The probes mentioned above were added for marking the objects (with the dye) and, so, allowing the researcher identify the vacuoles (0.2% solution in DMSO, the final concentration being 5μM). The size of each confocal microscope's snapshot was 500×500 pixels (1 pixel corresponding to 0.1μm). In each case of observations, the author tried to choose the fluorescent molecular probe providing for (i) Better fluorescence intensity and (ii) A more explicit snapshot.

To the end of binding the probe's molecules and the vacuolar membrane molecules, the probe (diluted in methanol down to the final concentration of 10μM) was added to the mass of isolated vacuoles. The mass of vacuoles was incubated at 20°C±2°C during 10 min and, next, observed via the FLSCM used. The equipment and the techniques used for observations allowed the author observe (a) Activity of the scrutinized isolated vacuoles, (b) Processes in the membranes, (b) Process parameters, and assess some of biophysical characteristics of the vacuoles and the membranes. Observed and assessed were (i) Internal contents of the isolated vacuole under scrutiny (processes of loss of the vacuolar contents by the vacuoles observed): (ii) Structures and behaviour of the vacuolar membranes; (iii) Some relations between isolated vacuoles.

Results

As it was already described and shown in [1], fluorescence microscopy was applied for capturing and tracing of scrutinized red beetroot (*Beta vulgaris L.*) isolated vacuoles as the objects on the snapshots (at 20±2°C). The author obtained video films, some sequences of snapshots from which were chosen below for demonstration of the processes and, so, presentation of the phenomenon postulated by the author.

The observation equipment described above and the fluorescence molecular probes used have given the author an opportunity to observe the processes of gradual (intermittent, stepwise) empting of isolated vacuoles in connection with opening and sealing holes in vacuolar membranes. Targeted observations of individual vacuoles in the mass of isolated vacuoles have confirmed the results earlier described in [1] and made these results more obvious. The phenomenon of fast empting of the vacuoles (i.e. fast efflux of the vacuolar content during first decades of seconds) in the process of their natural decay was first registered by the author's team of researchers in 2015. This phenomenon, which was later periodically observed from 2017 to 2025, necessitated deep understanding and plausible explanation. Preliminary explanations of the phenomenon of opening holes in vacuolar membranes were given in [1].

Consider a sequence of snapshots given in Figure 2. As obvious from Figure 2 (snapshots a i), the internal space of the scrutinized isolated vacuole is gradually blanching in the process of opening and closing (sealing) holes in the vacuolar membrane. It is possible to observe how the vacuole is gradually (and very quickly - during 23sec) losing its internal content. On snapshot i, the vacuole looks as almost empty (Figure 2).

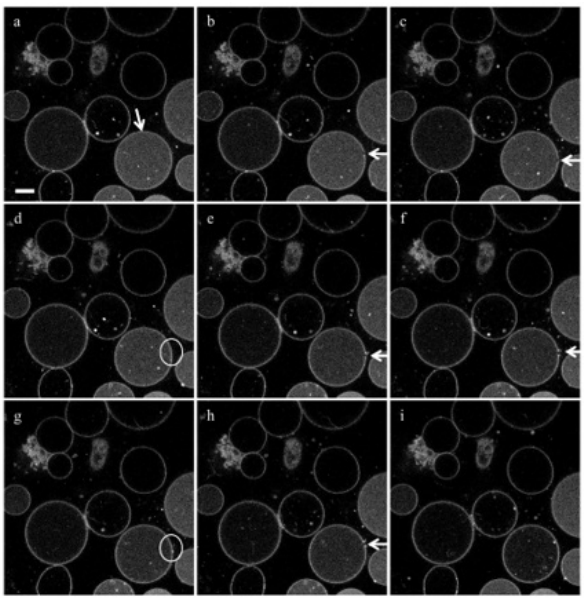

Figure 2: The process of partial loss of the vacuolar nutrients: a-0sec; b-1sec; c-3sec; d-5sec; e-16sec; f-18sec; g-19sec; h-23sec; i-50sec. The fluorescence molecular probe was ANS. Scale bar 10μm.

In course of numerous observations during 2017-2025, the author has made sure that the process of disappearance of the vacuolar content from isolated vacuoles is conditioned by observed (NB!) opening of holes (not nano-dimensional pores, never!) in vacuolar membranes. This process presumes very fast efflux of the vacuolar content. The equipment described above has given the author an opportunity to observe the processes of intermittent formation of absolutely clearly visible (under a LSCM) channels in vacuolar membranes see Figure 2b, c; e, f, h. These channels have the form of observable holes, which form in the stiff protein shell. An average diameter of the holes observed in approximately five hundred observations conducted was assesses to be 0.5-2.2μm. A circumstance, which has attracted the author's attention, is that the sequence of opening and next sealing of the holes proceeds very fast, during units of seconds: see snapshots in Figure 2 see also in Figures 3-10 below. And, furthermore, this process of opening and sealing of holes corresponded to a really fast process of emptying of the scrutinized isolated vacuole via holes intermittently forming in the vacuolar membrane. The process of blanching may be obviously traced by the shade of the vacuolar content, which was blanching.

The processes of opening of holes in the vacuolar membrane looked as intermittent with the processes of sealing. For example, fast opening of a hole in the membrane Figure 2c alternated with its fast sealing in 2sec (the places of sealing are indicated with small rounds Figure 2d, g. The same relates to opening of a hole Figure 2e,f, and its subsequent sealing in one sec Figure 2g. Repeated opening of a hole in 4 sec Figure 2 h slightly above the position of the previous hole and its subsequent sealing Figure 2i indicates to the sustainable character of the processes described. Since the author was told by researchers working in the field that the situation with the holes demonstrated by him on the snapshots in the previous author's publication was not sufficiently obvious, the author decided to elevate the sizes of all the snapshots (and, so, improve visibility of the holes, which appeared in the membrane) by 10 times. As a result, the opportunity to observe the holes with higher quality was obtained see Figures 3-10 below (Figures 3-10).

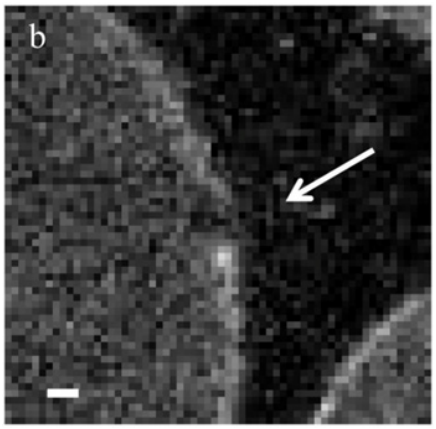

Figure 3: Snapshot b. Opening of a small hole in the membrane during 1sec. The fluorescence molecular probe was ANS. Scale bar 1μm.

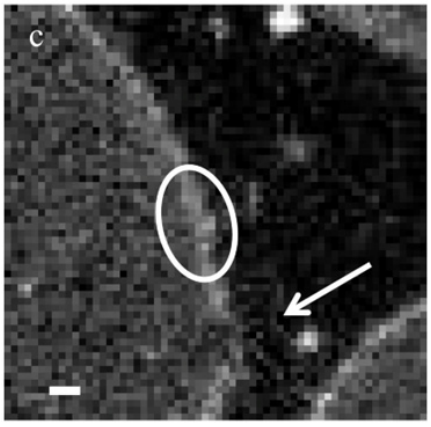

Figure 4: Snapshot c. Sealing of a small hole in the membrane during 2 sec (here and on the snapshots below the place of sealing is indicated by a round). Opening of a very small hole below the position of the previous hole. The fluorescence molecular probe was ANS. Scale bar 1μm.

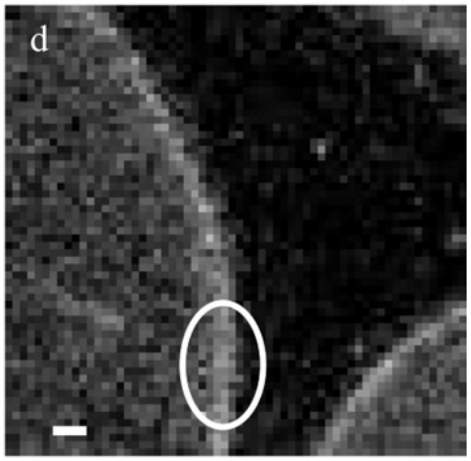

Figure 5: Snapshot d. Sealing of a very small hole in the membrane during 2sec (the place of sealing is indicated by a round). The fluorescence molecular probe was ANS. Scale bar 1μm.

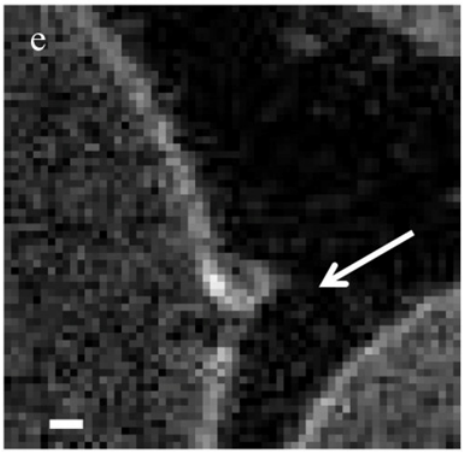

Figure 6: Snapshot e. Opening of a small hole in the membrane in 11sec below the previous hole (the position of the hole is indicated by an asterisk). The fluorescence molecular probe was ANS. Scale bar 1μm.

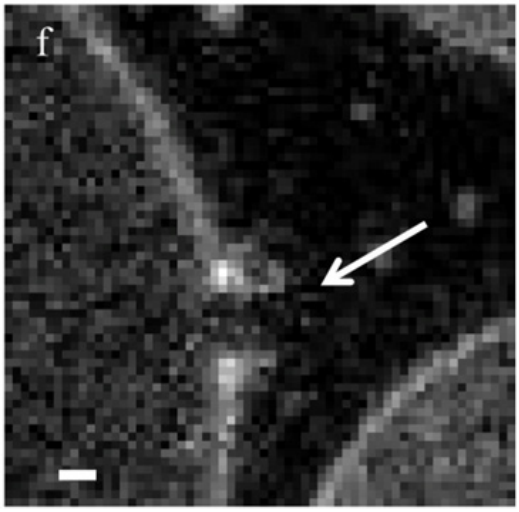

Figure 7: Snapshot f. Extension of the hole earlier opened (see snapshot e) during 2 seconds. The fluorescence molecular probe was ANS. Scale bar 1μm.

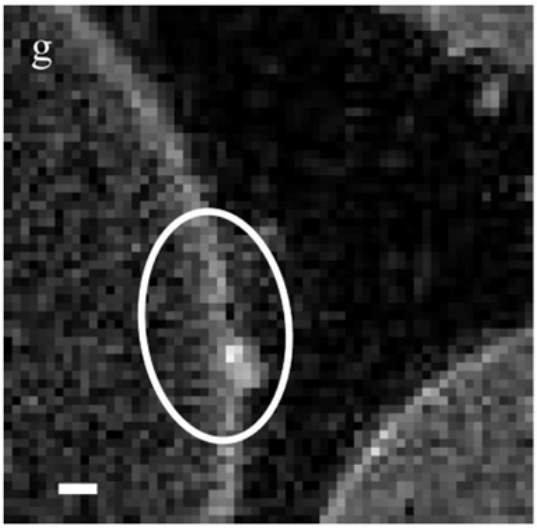

Figure 8: Snapshot g. Fast sealing of the hole during 1 second. The fluorescence molecular probe was ANS. Scale bar 1μm.

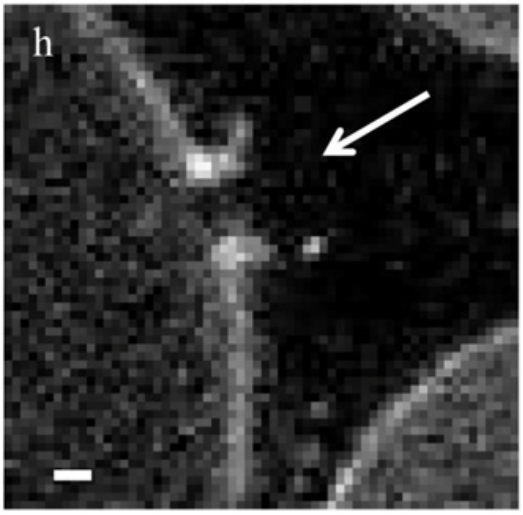

Figure 9: Snapshot h. Opening of a pretty large hole in the membrane in 4seconds at the place slightly above the position of the previous hole (the place of this hole is indicated by an asterisk). The fluorescence molecular probe was ANS. Scale bar 1μm.

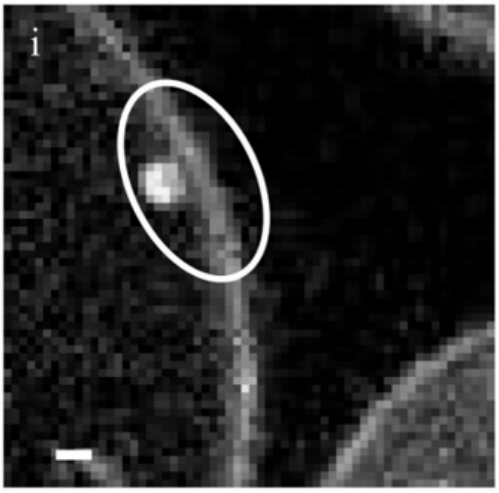

Figure 10: Snapshot i. Complete sealing of the hole after 27sec. The fluorescence molecular probe was ANS. Scale bar 1μm.

When analysing the results of this investigation, it is possible to state that the author observed the process of efflux of the vacuolar content from the vacuole. When considering the time scale, one can make sure how fast the process of efflux really is and how quickly (intensively) the process of empting proceeds.

Principal Results Obtained by the Author with the Use of Enlarged Snapshots

a) The phenomenon discussed represents a complex issue, which presumes (i) Opening of a hole, when the membrane tears at some place, and obviously stiff leaves of this hole, which forms around the opening in the membrane, deflects into sides (to get open), while forming a channel. (ii) Any opening of a hole is intermittent with closing of the hole. On the 2D snapshot, this channel looks as having the form of a "double door opening from a room". Meanwhile, in reality, on the 3D image, any such hole represents a round. This explains why the leaves of the openings look illegible on snapshots with membrane openings Figures 4,7,9. Anyway, the leaves of the channels formed in the membrane are stiff. Otherwise, it is not possible to explain the processes of (i) Fast opening of the holes, and (ii) Fast closing (sealing) of the holes.

b) One can look at Figures 2 to 10 and make sure that the process of empting of the vacuolar content is obvious, and it proceeds very fast (look at the process of blanching of the vacuolar content from one snapshot to another).

c) The micron diameter holes opening in the vacuolar membrane are really larger than it has been expected: 0.5-2.2μm.

d) In principle, the process of empting is going on until the isolated vacuole turns out to be optimally emptied (in connection with (i) Pressures inside and outside the vacuole and, probably, (ii) The process of vacuolar decay of isolated vacuoles). The process of empting of a vacuole turns out to be gradual and intermittent. And this is obvious. Nevertheless, it is obvious from Figure 2i and Figure 10 that the process of empting of the scrutinized vacuole is incomplete even after 50 seconds. Presently, on account of the present level of knowledge, it is not possible to rigorously judge about the completeness of the process of vacuolar empting observed.

e) It is obvious from Figures 7 and 9 that pretty large (micron-diameter) holes (having stiff leaves) are intermittently opening (and sealing) in the protein shell of the vacuolar membrane (see snapshot f and snapshot h). On a flat snapshot (2D projection), such holes look, in each case, like a "double door" opening outwards from the room. In reality, on 3D projections, any such hole is a round, and this explains why the leaves of the opening look illegible. When there are no conditions for opening of a hole (and later, for sealing of the hole), the leaves of such an opening look as being closed.

f) Opening of a hole may take place in any part of the membrane. It depends on the processes, which take place with the dynamics of pressures inside and outside the vacuole at a given time interval.

g) It is absolutely obvious that a course of the two processes (opening of the hole and sealing of the hole) cannot be explained by the well-known conceptions of membrane organization and functioning (e.g., portion; membrane fluidity; etc.). (i) The conception of membrane formed by some lipid bilayer cannot explain the process of formation of a hole with the opening (and closing) stiff leaves. (ii) The conception of fluidity of membrane lipids is not capable of explaining the process of opening and closing of rigid (not fluid) leaves during 1-2 sec. At this point of reasoning, the author really understood why he earlier experienced very heavy pressure of reviewers of two popular biophysical journals. In connection with the two author's discoveries discussed in [1] and above, do we all (researchers of the world) have to reconsider the two abovementioned basic conceptions of contemporary biology and biophysics?

h) There is no slightest doubt (and this fact has been confirmed by reactions of the molecular probes used in the author's observations) that stiff leaves of the hole are represented by proteins (more exactly, by pieces of the stiff membrane's protein shell, which are ready to form a sort of a "double door" (seen in the 2D projection) at any place of the membrane).

i) Processes of opening and closure of membrane holes are bound up with fast splitting of an obviously stiff membrane shell, and next tilting (rotation during 1-2 seconds) of small pieces of this shell (with forming of a hole) outwards, furthermore, opening of the hole on the two sides. These pieces of the membrane's shell are obviously represented not by soft lipids. Noteworthy, process of tilting, invaginations and similar reactions of pieces of membranes were earlier discussed for GUVs. But in case of opening holes in membranes of natural vacuoles, the reaction of membranes was specific. It was a reaction of the stiff protein part of the membrane, So, it is absolutely obvious that (i) The structure of the vacuolar membrane is represented by not only two lipid layers (Note, there are no specialists, who dared to explain why there are two lipid bilayers).

j) After all, the author insists on the conception, which presumes existence of a stiff protein layer between two lipid layers of the vacuolar membranes (and, probably, any other membranes). And this is a stiff layer, which builds (fixes) the two lipid layers on its two sides. This rigid protein layer and the mechanisms of opening holes and sealing of the holes are to be specially studied.

An Attempt to Explain the Biophysics of the Phenomenon of Fast Efflux of the Vacuolar Content Via Holes in Membranes

The pressure generated in a vacuole p_{inside} and exerted from the inside upon the vacuolar membrane includes $p_{inside} = p_{1\,hyd} + p_{ex}$, where p_{ex} is the Laplace pressure, i.e. some excessive pressure exerted upon the curved (spherical in our case) surface. This pressure exerted upon the vacuolar membrane is 2 times larger than in the case, when the surface is flat. So, this excessive pressure p_{ex} is some additional pressure experienced by the liquid substance, which is placed under the spherical surface, $p_{ex} = 2\sigma / r = 4\sigma / d$, where σ is the liquid substance density of the external saving liquid [N/m]; r and d are, respectively, the radius and the diameter of the vacuole. So, we have

$$p_{inside} = F / S + p_{ex} = m_1 g / S_1 + p_{ex} = \rho_1 V_1 g / S_1 + p_{ex} = \rho_1 g h_1 + 4\sigma / d$$

where *ρ1* is the density of the vacuolar content; *h1* is the vacuolar radius; σ is the liquid substance density of the external saving liquid [N/m].

According to the existing conception of hydrostatics, when there is more than one covering spherical surface around the vacuolar liquid substance (solute) inside the vacuole, then the liquid substance inside the vacuole experiences another additional pressure. This hydrostatic effect was earlier noticed and taken into account, for example, in investigations bound up with of a soap bubble. So, when the vacuolar membrane is considered as a bilayer (having 2 layers), one may speak about double pressure exerted upon the liquid (solute) inside the vacuole, i.e. $p_{ex\,bilayer} = 4\sigma / r = 8\sigma / d$. This circumstance changes the situation, and we have

$$p_{inside} = F / S + p_{ex\,bilayer} = m_1 g / S_1 + p_{ex\,bilayer} = \rho_1 V_1 g / S_1 + p_{ex\,bilayer} = \rho_1 g h_1 + 8\sigma / d$$

The pressure equilibrium for the vacuole (and hence its integrity) maintains, while the pressure ρ_{inside}, which is exerted upon the vacuolar membrane from the inside, remains in balance with the pressure $p_{outside}$, which is exerted upon the membrane (and, so, upon the liquid substance concentrated in the vacuole) from the outside. The pressure from the outside is represented by the following 2 components: $p_{outside} = p_{atm} + p_{2hyd}$, where p_{atm} atmospheric pressure (normal p_{atm} is 101.7 kPa); $p_{2\,hyd} = F / S_2 = m_2 g / S_2 = \rho_2 g h_2$ hydrostatic pressure, where ρ_2 is the density of the saving liquid; h_2 is the level of the saving liquid over the vacuole (which is very low because the vacuole is studied *in vitro* under a LSCM).

In connection with the above reasoning, it is possible to draw the following conclusions.

a) The pressure equilibrium for the vacuolar membrane maintains (i.e. the Vacuolar Liquid Substance (VLS) stays in the vacuole), when p_{inside} (internal pressure generated by this VLS) is equal to $p_{outside}$, i.e. $\rho_1 g h_1 + 8\sigma / d = p_{atm} + \rho_2 g h_2$.

b) When the internal pressure p_{inside} generated by the VLS is larger than pressure $p_{outside}$, i.e. $p_{inside} > p_{outside}$, i.e. $\rho_1 g h_1 + 8\sigma / d > p_{atm} + \rho_2 g h_2$, then an impulse directed from the centre of the vacuole is generated, and this impulse provokes either (i) Opening of a hole in the vacuolar membrane or, probably, a sequence of holes, which are later sealed (as discovered and described in the present publication), in order to relieve the pressure exerted upon the membrane; or, probably, (ii) Vacuolar collapse in extraordinary cases.

Meanwhile, there arises a problem bound up with the recent author's discovery discussed above. If (according to the existing conception of hydrostatics) there are two covering spherical surfaces around the internal liquid substance (and this has been earlier noticed and taken into account, for example, for a soap bubble), then the excessive pressure experienced by this liquid substance from the outside is 2 times larger. It is known from the biological literature that any vacuole is surrounded with a membrane, which is considered as a lipid bilayer (many specialists in biology and biophysics are sure that there are 2 layers in the vacuolar membrane), so, $p_{ex\,bilayer} = 4\sigma / r = 8\sigma / d$. Meanwhile, according to the recent author's discovery bound up with the membrane's protein shell, really there are 3 layers in the vacuolar membrane (one of the layers being represented by a stiff protein shell). In this connection, it is possible to state that really the excessive pressure p_{ex} 3-layer is larger, i.e. $p_{ex\,3-layer} = 6\sigma / r = 12\sigma / d$.

So, we have that the condition of pressure equilibrium for the vacuolar membrane writes: $\rho_1 g h_1 + 12\sigma / d = p_{atm} + \rho_2 g h_2$.

Discussion

It is worth noting that processes of the type observed in the author's experiments have never been earlier discussed in the world biological and biophysical literature. The details of biophysics of the observed efflux (and, so, loss) of the vacuolar content via opening holes are also quite specific. Specificity of biology and biophysics

bound up with membranes is planned to be discussed in detail in the author's forthcoming articles.

Many researchers consider the PCV as a temporary storage compartment. Probably, in this connection, they reduce their investigations to mechanisms of transport of solutes, mainly into the vacuole. And they represent these mechanisms within the frames of one-aspect understanding, which is grounded on the related investigations described in the literature published in the world during the recent 4 decades. For example, formation of pores in organelle membranes has for long time been considered as the main "candidate mechanism" of trans-membrane transport of substances. Unfortunately, having forgotten that this mechanism was understood as only candidate one, 99% of researchers immediately transformed it into the "main mechanism", and later into the "only existing mechanism". Numerous discussions of this inadequately studied mechanism were complemented with (i) False data about observations of membrane poration in dynamics (unfortunately, even now, no microscopic techniques good to observe poration in dynamics really exist); (ii) Data about techniques of artificial membrane poration discussed not simply as somehow studied in laboratories, but as the techniques already actively employed in practical medicine (and this is not so); (iii) Application of amphipathic peptides internalized; etc. The situation with obtaining really useful (and, so, practically applicable) biomedical knowledge was aggravated in connection with the fact that majority of experiments were conducted by researchers of the world on artificial membranes. And results of such experiments were without any attempts of verification applied to natural membranes in the capacity of ready conclusions.

Brief Discussion of Known Concepts Bound up with Types and Forms of Membranes (In Connection with Formation of the Protein Pattern in the Membranes)

While trying to adapt to possible variants of the concepts of constructing membranes, some researchers (see, e.g., *Lie Wu,* and *Xiue Jiang*) oriented themselves to investigations of structures of membranes of artificial objects (such as GUVs) [60]. These researchers stated that there were 3 forms of membranes, but, in their opinion, these were, nevertheless, all represented as biomimetic lipid membranes (hybrid lipid bilayer membrane, lipid-tethered bilayer lipid membrane, and protein-tethered bilayer lipid membrane) [60].

Lipid-tethered bilayer lipid membranes were considered to be constructed on the nanostructured SEIRAS enhancing substrate to incorporate gA [60]. This substrate consists of a mixed self-assembled monolayer of synthetic lipid-thiols like (cholesterylpolyethylenoxy) thiol (CPEO3) (with "dilution" of small thiols such as 6-Mercaptohexanol (6MH)), which is used to create a sub-membrane reservoir attached to the surface, and a lipid layer on the top of the layer formed by fused unilamellar vesicles. The process of formation of such a bilayer membrane (considered to be represented by lipids) by vesicle spreading and fusion on the mixed monolayer was monitored by ATR-SEIRAS through organizing a spectrum of the mixed monolayer. It was assumed that the positive and negative bands represented contributions of the species binding to and removing from the substrate surface, respectively [60].

Protein-tethered bilayer lipid membranes deserve special and detailed consideration. Such membranes have never obtained any due consideration earlier. Such consideration cannot be found also, say, in [60] and in some other publications. The appropriate consideration of even only this problem with membranes necessitates deeper understanding of the form of presence of proteins (proteins in various membranes). Meanwhile, when any young researcher addresses to the literature published in this research field, he obtains an impression that almost all the issues bound up with membranes are studied, understood and well-known. Planning to be published in the journals without any problems, many researchers have long ago given up any attempts to express doubts and find anything really new and really useful. This may not be acceptable. And there are other researchers, really honest researchers. For example, in Chapter 2 of the monograph "Membrane Biophysics: New Insights and Methods", outstanding researchers *J. Gao* and *H. Wang* have honestly stated: "We must realize that the membrane structure is far from being completely understood. Biological details are generally more complicated than the resolving power of a simple model, which describes generalized, uniform behavior of molecules in the membrane…" The author has to agree with the ideas of these honest authors. These authors also stated: "Even the cell membrane structure needs to be refined continually…" [55].

J Gao and *H Wang* are sure that "the most important issues bound up with membrane proteins are still unknown". These unknown issues include the issue of "localization of proteins in the membrane". Even in 2023-2025, in 100% of the corresponding publications, the pictures demonstrating strange and ugly inclusions of proteins into membranes (which are demonstrated as distributed locally, at least in vacuolar membranes [62] were still drawn on a list of paper with the aid a pencil [61]. No wonder that J. Gao and H. Wang honestly stated that "relationships between membrane proteins", "relationships between membrane proteins and membrane lipids" also remained unknown. Furthermore, "the mechanism that underlies the formation of the protein pattern in the membranes" is also completely unknown [55].

It is a great pity, but almost 50% of researchers consider proteins in plasma membranes and organelle membranes as heterogeneities of these membranes [59]. And the lipid bilayer (traditionally considered as the basis, at least in the plasma membrane) has for several decades become a traditional model in

any membrane-related studies.

Brief Discussion of the Concepts Bound Up with the Known Studies of the Forms, In Which Proteins Are Present in Various Types of Membranes

The well-known membrane structure conception states that all plasma membranes, organelle membranes and, surely, vacuolar membranes contain channels (these are ion channels), which are represented by numerous channel-forming membrane insertions represented by proteins. It has been stated that such numerous protein insertions form protein channels, which gate the flow of liquid substances (ions) through the membrane [60]. Non-liquid substances were not discussed. In order to study membrane protein channels, the researchers working in the field applied various methods: magnetic force microscopy [62], atomic force microscopy [63], electrostatic force microscopy [64], single-molecule force spectroscopy [65], infrared spectroscopy [60], etc. In one of the known cases, super-resolution imaging was used to observe proteins in the plasma membrane [59].

Despite several attempts undertaken by talented scientists to attract attention of researchers of the world to the problems, which seemed completely obvious, it so happened that practically almost all the researchers in the world were not ready to accept any novel scientific truths. And one of the very important truths (directly stated by *H. Chu* and his co-authors) was that "membrane lipids are assembled around membrane proteins" [66]. These authors have emphasized that, as a result of such assembly, "a protein-tethered bilayer lipid membrane" is formed [60].

Owing to the non-perturbing and "molecular fingerprint" features, Infrared (IR) spectroscopy was widely applied in plasma membrane-related investigations [61]. IR spectroscopy might serve not only as a characterization tool good to determine physicochemical characteristics of membranes (such as phase transition, hydration state, membrane fluidity, etc.), but also to provide detailed information about the processes of molecular conformation and orientation. In this connection, the researchers realized the opportunity of application of traditional IR techniques (IRRAS, ATR-FTIR) in characterization of various membranes. As noted above, several strategies are known to be successfully employed in constructing artificial biomimetic membranes on nanostructured Aufilm, including, the lipid-tethered bilayer lipid membrane, the protein-tethered bilayer lipid membrane and the hybrid lipid bilayer membrane. In publications of *L Wu* and *X Jiang* unilamellar vesicles were used as the lipid source in all the strategies of constructing membranes [60].

The publication of *L Wu* and *X Jiang* described an attempt of the authors bound up with characterization of physicochemical properties of membranes. But this did not mean that characterization of some natural lipid membrane was implied. These talented researchers honestly considered the characterization of a lipid membrane model only. And only a model has formed the basis of plasma membrane investigations conducted by *L Wu* and *X Jiang* Furthermore, the impact of bioactive substrates upon the membrane was considered as evaluated by the changes of the membrane's physicochemical properties. These talented authors have briefly summarized the application of IR spectroscopy techniques in physicochemical characterization of membranes [60].

Nevertheless, in the chapter published by *Lie Wu* and *Xiue Jiang* they described the following four important results bound up with description of: (1) Conception of the protein-tethered lipid containing membrane; (2) Membrane protein-reconstituted phospholipid vesicles; (3) Process of restoration of proteins in the membrane, which was considered as a lipid bilayer membrane, and, moreover, (4) Process of assembly of lipids around the protein core (and, in this connection, formation of the protein-tethered lipid membrane) see Figure 11.14d in [60].

The author of the present publication is ready to assume that there may be exist a specific viewpoint of the community of researchers, who work in the field, with respect to the honest statements and results of the outstanding researchers mentioned above. The author is ready to understand that all these important statements may be considered by the majority of researchers as "emotional statements only". Meanwhile, the author cannot ignore the phenomena he has discovered. These are (i) The phenomenon of fast empting [1] and (ii) The phenomenon of opening of a hole (in the membrane), which on a 2D projection has the form a "double door". The conception of membrane formed by some purely lipid bilayer cannot explain the process of formation of the rigid leaves forming a sort of an opening like a "double door" in the membrane, which opens and seals in 1-4 seconds. In this connection, the author has to repeat his arguments. The second author's discovery, which is discussed in the present publication, is bound up with fast opening of a sequence of pretty large (micron-diameter) holes in vacuolar membranes. In case of 2D observations, each hole looks like a "double door opening outwards from the room". The leaves of such a hole open outwards from the vacuole for 1-2 seconds (and are closed (sealed) in 1-2 seconds after the efflux of some part of the vacuolar content). There is no slightest doubt (and this effect is confirmed by reactions of the molecular probes having affinity to proteins) that the leaves, which, in case of 2D observation, look (each hole) like a kind of "double door", are represented by proteins (more exactly, by inseparable (from the membrane) pieces of the membrane's stiff protein shell). These author's discoveries have proved that membranes of vacuoles only by inertia are qualified by majority of researchers as lipid bilayers.

In this connection, the author is ready to repeat above honest statements of respected scientists *Lie Wu* and *Xiue Jiang*, *J. Gao* and *H. Wang*, *Huiying Chu*, *Yuebin Zhang*, *Yan Li* and *Guohui Li*, and

state that lipids of any membrane are only secondary elements constructed on the protein skeleton. It seems to the author that this truth was known at least a century and a half ago. In order to achieve real progress in science, all possible forms of incorrectness must be excluded. Lipids as biological substances are not capable of forming any stable biological structures, which may be characterized as morphologically stiff and, so, reliable in this connection.

Discussion, Which Presumes Practical Confirmation of the Author's Statements About Protein Shells, Which Really Form Membranes

Any researcher surely understands that above shocking statements about stiff protein shells, are to be confirmed. Meanwhile, the observed facts of opening of holes in membranes are absolutely obvious and do not need any confirmation. It was observed. It does not need any additional confirmation. And it represents a substantially more complex problem than it may seem. The leaves of an opened hole are really stiff. When there are no conditions for opening of a hole (and later, for closing of the hole), the leaves of such an opening are either do not open or are quickly closed. It is important to emphasize that the author has encountered the process of "closing of the holes" (closing, not simply uncertain sealing or healing of the holes), Any declarations are to be confirmed by some practical proofs. Below, these proofs will be represented only by visual demonstrations of real protein shells of membranes, which have been registered by the author in observations of extraordinary situations bound up with membranes (in this case, with membranes of vacuoles). Necessary theoretical explanations are planned to be discussed in the forthcoming author's publications.

For the first time in history of publications bound up with membranes, the author is ready to demonstrate the protein shell of the vacuolar membrane. This shell has become visible owing to the process of natural decay of an isolated vacuole observed in course the author's investigations see Figures 11 and 12 below. Figure 11 may be found in the author's publication in Advances in Biological Chemistry [2]. It shows one of the results of natural decay of the vacuole, and decomposition of phospholipids of its membrane into vesicles (Figures 11,12).

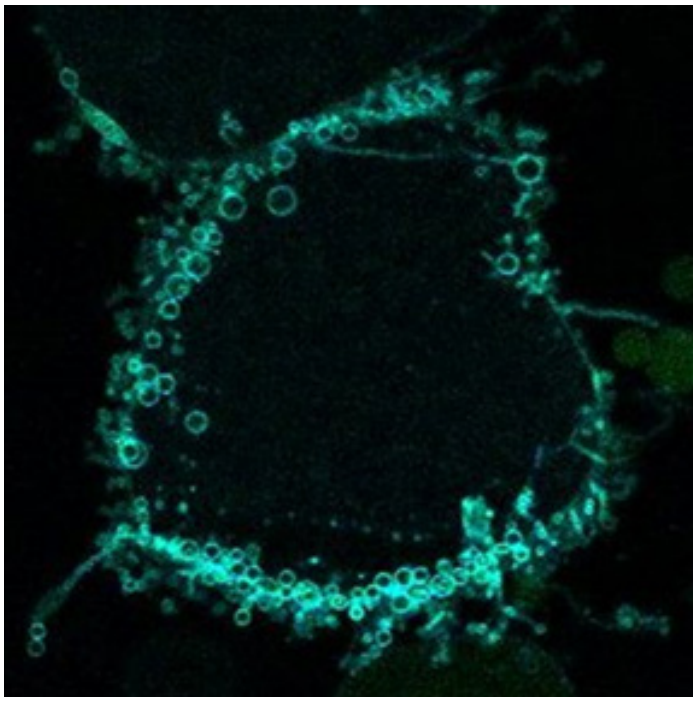

Figure 11: Appearance of traces of the membrane's protein shell in the process of vesiculation of membrane lipids of an isolated vacuole (as a result of the process of natural decay). The fluorescence molecular probe was ANS (having affinity to proteins); optical section at the distance of 5μm from the substrate. Scale bar 10μm. Remark: The author has made sure that the influence of hyperosmotic stress upon the isolated vacuole fastens the process of membrane's decay, and from the outside the picture of vacuolar decay looks similarly.

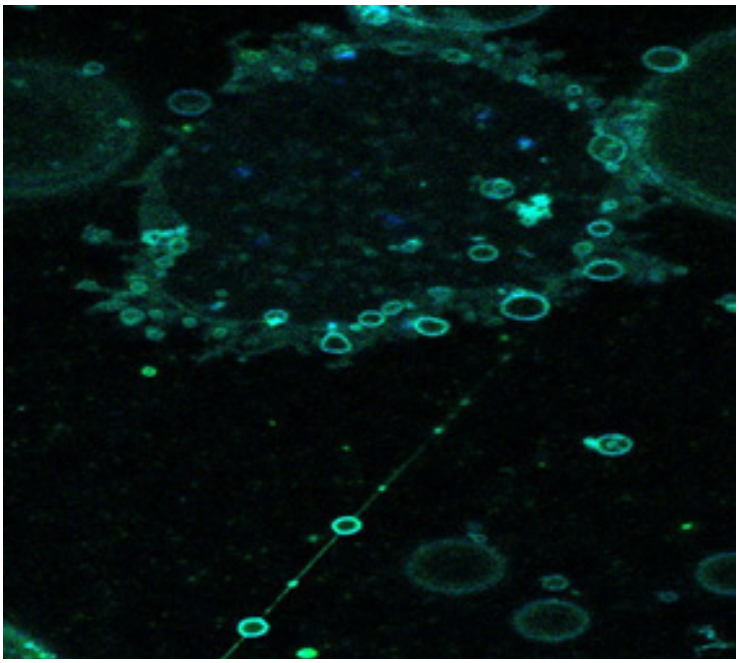

Figure 12: Appearance of traces of the membrane's protein shell in the process of vesiculation of membrane lipids of an isolated vacuole (as a result of the process of natural decay). The fluorescence molecular probe was ANS (having affinity to proteins); optical section at the distance of 5μm from the substrate. Scale bar 10μm.

In Figures 11 and 12, one can observe the process of vesiculation of membrane lipids of an isolated vacuole (*Beta vulgaris L.*) in course of the process of natural decay. As it has been noted in [61], the membrane's bilayer part transforms into protein-reconstituted phospholipid vesicles. In the processes of decay and decomposition of phospholipids into vesicles, the protein shell (freed from the two parts of the lipid bilayer) becomes visible. It is natural that the protein shell becomes distorted (not spherical) or even torn into pieces in the process of natural decay. Nevertheless, the protein shell remains stiff during some short time, even when the vesicles leave their places.

So, while discussing the results of the present investigation, the author would like to state that membranes of cell vacuoles, which during 5 decades were considered exclusively as "lipid bilayers", may now be reconsidered as not simply "the protein-tethered lipid containing membranes", but as membranes, in which two phospholipid layers are fixed on stiff protein skeletons. The author is completely sure that 99% of researchers will insist that either the author is completely not right or his arguments are incomplete. Furthermore, numerous researchers will insist that the author has no right to make such direct and categorical statements about membrane proteins. Having no slightest understanding that the era of membrane pores, portion and other funny things has gone forever, these researchers will insist on the habitual (traditional) conceptions of membranes. Nevertheless, it would have been a lie if these researchers would state that they had never registered traces of stiff protein shells (like those shown in Figures 11 and 12 in their observations of decaying vacuolar membranes. In his forthcoming articles, the author plans to give additional arguments confirming not simply the presence of protein shells in membranes. The author plans to approach to explaining theoretical grounds of the two phenomena he discovered.

Conclusion

There is no doubt that knowledge and application of new methods of biophysics, biochemistry, cell and organelle biology, microbiology and contemporary microscopy techniques may bring the researchers closer to new discoveries. E. Etxeberria and his co-authors [7,8] placed their hopes upon architectural remodelling of the tonoplast during fluid-phase endocytosis. Many researchers lay their hopes upon the known approaches and, in this connection, to use the traditional understanding of definite patterns of intracel-

lular transport systems, and, particularly, systems bound up with transport of fluid substances across organelle membranes.

The present publication has described the material, which has allowed the author to make a step forward to discoveries. These discoveries are in several respects bound up with the phenomenon of fast empting of isolated vacuoles (fast efflux of the vacuolar content). The set of phenomena discovered, observed and studied includes, first of all, opening of a hole, when the membrane ruptures at some place. In this case, the leaves of the formed opening (these are obviously stiff), which rise around the opening in the membrane, deflect into sides (to form the opening), while forming a channel. On a 2D snapshot, this channel looks as having the form of a "double door opening from a room". Meanwhile, in reality, on a 3D image, any such hole represents a round. This explains why the leaves of the holes look slightly illegible on the snapshots showing openings (Figures 4,7,9). Anyway, the leaves of the channels, which form in the membrane, are stiff (these cannot be non-stiff, otherwise, these will not close), furthermore, otherwise, it is not possible to explain the processes of (i) fast opening of the holes, and later (ii) fast closing (not sealing in the well-known sense) of the holes. It is important to emphasize that the author encountered the process of "closing of the holes" (not simply some uncertain "sealing or healing the holes").

The great thing was that the phenomenon described in the previous author's publication, which was bound up with the phenomenon of fast efflux of the vacuolar content via specific micron-size holes of micron diameter (0.5-2.2μm) [1], gave preliminary explanations of this process of efflux of the vacuolar content. Explanations of the related processes were not given in [1]. Meanwhile, it is necessary to emphasize that actually several related phenomena and the related mechanisms have been discovered. The mechanisms discovered were: (i) An unexpected mechanism of opening of micron-diameter holes in membranes, and also (ii) A non-typical (earlier unimaginable) mechanism of closing of such holes. Noteworthy, both the holes and the mechanisms did not correspond to any earlier conceptions and understandings.

In reality, the phenomenon bound up with sealing of the holes formed in membranes represents a separate and absolutely new discovery, which presently may not be reasonably explained on the basis of all the knowledge available in the contemporary biology. When observing the processes of opening and closing of the holes, the author was thinking about the presence of the "Hand of God Himself". (iii) In this connection, all the conceptions earlier postulated in connection with trans-membrane transport (formation of pores, sealing of pores, etc.) unexpectedly appeared to be funny. And the funny character of the portion mechanism also became an unexpected discovery for the author. Furthermore, as it followed from the results obtained by the author, the conception of membrane's fluidity also appeared to be not completely good for explaining of the closing process for any openings in membranes. From the moment of the phenomenon observed by the author, it was possible to state that membrane's fluidity does not have any slightest relationship to opening and sealing of the holes. This fact has become especially obvious on account of results of the author's observations.

Still what is presented and discussed by the author in the present paper is a combination of four real discoveries: (1) Discovery bound up with the stiff protein shell, which forms a core of the vacuolar membrane; (2) Discovery bound up with the phenomenon of fast empting of isolated vacuoles (efflux of the vacuolar content) via micron diameter holes opening in the membrane; (3) Discovery bound up with intermittent opening of micron diameter holes in the vacuolar membrane, each hole having the form of an opening with stiff leaves of this opening temporarily (for 1-2sec) moving aside, while forming a kind of a door; (4) Discovery bound with the mechanism of closing of such micron diameter holes (not in accordance with the conception of fluidity, which has existed until present).

The phenomena related to the holes, which open in membranes of isolated vacuoles, were probably bound up with the processes of natural decay, which these vacuoles underwent. This explanation is surely general and superficial. And plausibility of this explanation is to be proved. Meanwhile, when studying the corresponding biomedical literature, the author has come to the conclusion that many researchers (despite the fact that they often work with isolated vacuoles as with the material of their investigations) consider special investigations of isolated vacuoles as uninteresting and, so, unnecessary. Anyway, no special investigations of isolated vacuoles oriented to at least superficial understanding of mechanisms of their natural decay have been conducted by numerous representatives of the biological community. The author knows for sure that there were no respective investigations (experiments) conducted to the end of understanding of the behaviour and biophysical characteristics of isolated vacuoles. Such investigations (complemented with mathematical modelling approaches) have been conducted by the author. The results of these investigations will appear in author's forthcoming publications.

It is also expedient to note that, in the present case, the author in his work has relied not upon new methods themselves, but upon the KB-ideology [67]. The author has also relied upon the factor of the necessity of due completeness of the programs of investigations (observations and experiments), which may not be reduced only to some well-known or "universally accepted" (by the biomedical community) programs and also to the conceptions and theories bound up with such programs. Only an extended research platform can bring the researchers to a desired result useful for the humankind.

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Data availability

Data sharing is not available.

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