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Chapter 35

STUDY OF PHOSPHOLIPIDS MEMBRANES FUSION WITH A PLASMATIC MEMBRANE OF CELLS

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ABSTARCT

The interaction of lipid vesicles formed from neutral lipids with cells of ascetic Ehrlich carcinoma (EAC) has been examined at this investigation.

It is shown that the lipid phase of liposomes may not was bounded with plasma membrane of cells, and occurs the membrane fusion. More accurate distribution of liposomes that are labeled by the fluorescence probe by the calcein on the cell surface is obtained by means of laser confocal microscope.

The process of fusion of phospholipid membranes with cell membranes EAC studied by the method of resonance energy transfer between fume of fluorescence probes, covalently sewn with phosphatidylethanolamine molecules. The fusion with cells initiated by the inductor of fusion - the polyethylene glycol of PEG 300 and change pH of incubating medium of cells.

Downward adjustment pH of incubating medium leads to partial neutralization of negative charge of liposomes on the cell surface, that can contribute to probability increase their collisions and consecutive the membrane fusion. When low values medium pH the cell EAC become more are permeable for ions, the potential on cell membrane changes that initializes the process of membrane fusion.

All changes, happening in cells, logged by means of two methods: fluorescent microscopy, which permits directly observe whole processes, happening in the cell and confocal laser microscopy, allowing of visually to follow these modifications all over cell thickness.

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Keywords: fusion, endocytosis, exocytosis, phospholipid vesicles, ascetic Ehrlich carcinoma, fluorescence probes.

AIM AND BACKGROUND

The process of membrane's fusion is the one of the most fundamental phenomena of life activity of biological cells. It is the necessary component of the important cell processes: endocytosis, exocytosis and intracellular transport processes that occur involving some membranes too. Besides it is necessary make mention of the cell division, fertilizations. The membrane fusion are being used also many viruses when their ingress into host cell and on the contrary, at a point, when new viral particle leaves the infected cell. This is why has not been easing the interest in the job devoted to examining of this phenomenon.

Mission of the present work was interaction study of phospholipid vesicles with membranes of cells of ascetic Ehrlich carcinoma, in which the processes membrane fusions, and the particles endocytosis in cells, are registered by using of indirect observational methods, based on fluorescence analysis of dye molecules are possible.

INTRODUCTION

The fusion of membranes is one of the most fundamental phenomena of the cell viability and it is a necessary component of endo – and esocytosis processes, and endocellular transport processes with the membranes participation. The internal content of cells can get to the cytoplasm as a result of fusion of liposomes to a plasmatic membrane on the cell surface [1-7]. In different works it was supposed that cationic liposomes simply fused with a plasmatic membrane of a cell therefore their contents get to cytoplasm [8]. Further researchers came to conclusion that the most probable way of penetration of various cationic agents in cytoplasm is endocytosis [9]. Experimental researches of membrane fusion *in vitro* processes testify that not only occurs association of membrane material, but the internal content of vesicles as well [1]. It is difficult to observe directly the process of fusion of membranes. More often indirect methods of supervision over the fusion, based on the analysis of fluorescence of the dyes which intensity changes while mixing of lipids or internal vesicles contents [10] or the measurement of sizes of planar membranes electric current [11] are used. Research of fusion of the membranes, based on the usage of fluorescent probes, are rather extended because of high sensitivity of the method and possibility to observe continuously the process. To understand the physical mechanism of fusion of biological objects is extremely difficult and in similar cases modeling systems are used.

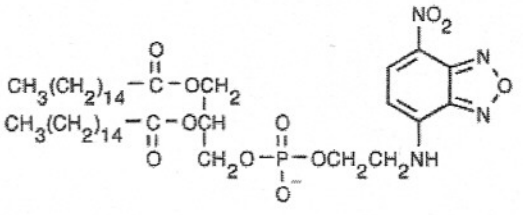
EXPERIMENTAL PART

The large single-layered liposomes, of the size to 1000Å (according to electronic microscopy) were obtained by method of reversed phase with the subsequent extrusion through the polycarbonic membrane filters with diameter of pores 0,1 and 0,2 mm [8] in the

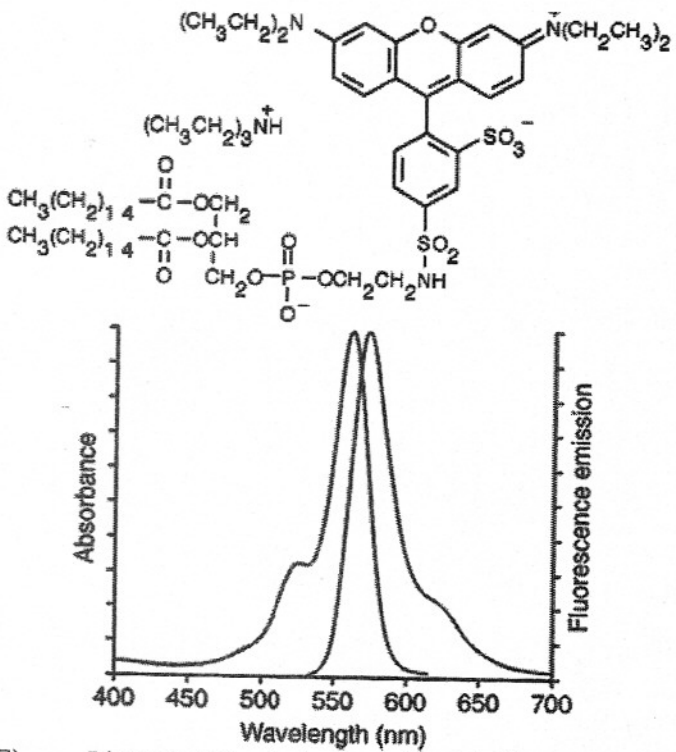
medium of 100 mm NaCI, 10 mm HEPES, pH 7,4. According to molar relation lipid structure of liposomes consisted of palmitoil oleoil phosphatidilcholin, phosphatidilserin and cholesterol in the ratio 2:2:1. The total content of lipids in obtained dispersions was about 10 mM. EAC cells were obtained from cells transfected on mice on the 7 th day. After getting the suspension of cells from abdominal cavity of mice, they were washed out twice by Henks solution containing 20мМ HEPES, pH 7,4. Final concentration of cells was 10^7 cell /ml.

The initiation of the fusion process of liposomes with EAC cells was carried by using of poly (ethylene glycol) 300 (PEG 300) and morpholinoethane sulfonic acid (MES) (pH incubation medium/soup/solution was changed by MES) [12]. The measurement of intensity of fluorescence at interaction of marked liposomes with EAC cells were registered on spectrofluorimetre Perkin-Elmer MPF-44B (USA) at 37 ^{0}C with constant mixing.

The first variant of carrying out of experiments of fusion study of liposoma membranes with EAC cells was held with fluorescent marked liposomes which besides the specified components, also contained N-NBD-PE (N - (7-nitrobenz-2-oxa-1,3-diazol- 4-yl) - 1,2-dihexadecanoyl-sn - glycero-3-phosphoethanolamine, triethylammonium salt) and N-Rh-PE (Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero - 3-phosphoethanolamine, triethylammonium salt (rhodamine DHPE)) in which the fluorescent probes: nitrobenzoxadiazol and a rhodamine B, accordingly, are covalently linkage with polar group of a molecule phosphatidiletanolamin. All reactants were produced by "Molecular probes" company, USA.



Notes: (N-NBD-PE) – N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE).



Notes: (N-Rh-PE) – Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero- 3-phosphoethanolamine, triethylammonium salt (rhodamine DHPE).

Figure 1. Molecule structure and excitation and emission spectrum of the fluorescent marks covalently linkage with phosphatidiletanolamin.

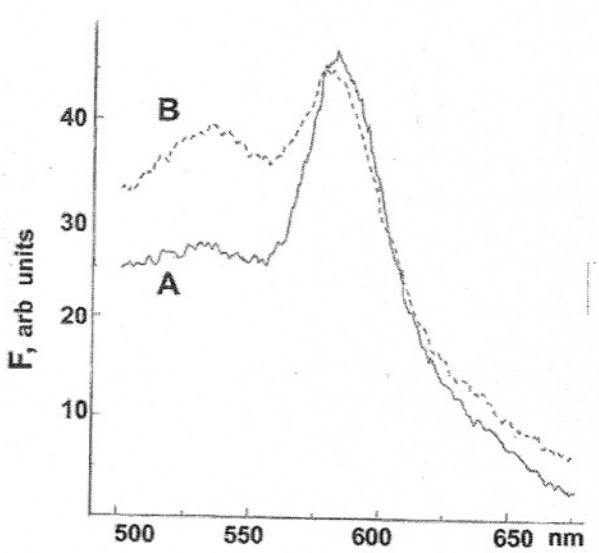


Figure 2. Fluorescent spectrum of N-NBD and N-Rh marks in liposomes (A) and after destruction of liposomes by 0,1% triton X 100 (B). Concentration of fluorescent marked liposomes is 5 μM F – intensity of fluorescence, λ_{ex} = 475 nm.

There were 400-500 molecules fluophror N-NBD-PE and N-Rh-PE (1 % in all lipids) in each liposome. The gel-filtration of lipid dispersions were carried out through columns with sephadex G15, sephadex G25, sepharose 2B and other gels. When both probes are included in the same population of the membranes, originally high efficiency of energy transfer (figure 2,3) is established. The second variant of the experiments of study the of the liposome membranes fusion with EAC cells was carried out with use of fluorescent probe – calcein (2,7 (bis (Carboxymethyl)–amino) methyl) fluorescein). The probe molecules (concentration 100 mm) were included in the liposomes formed by neutral lipids (lecithin soya and cholesterol in the ratio 2:1), during liposomes preparation by method of reversed phase. Concentration of lipids was 4 mM. The unbounded probes were released by means of gel-filtration. It is known that under the high concentration of a probes in liposomes, the luminescence of molecules is quenched by concentration. If the liposome becomes permeable for any reasons, for example, owing to fusion of membranes, dye comes out, and was diluted with the medium, and intensity of fluorescence sharply increases (figure 4).

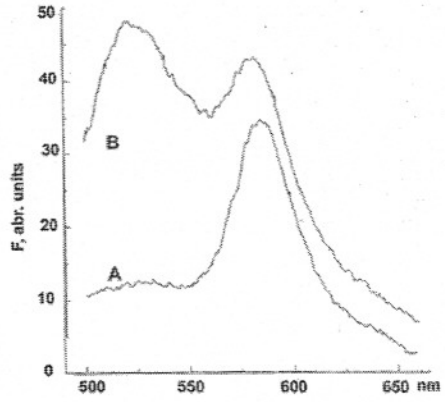


Figure 3. A – fluorescent spectrum of N-NBD and N-Rh liposomes in the sample of EAC cells and after destruction of them by triton X 100 (0,1 %) (B). λ_{ex} = 475 nm. Concentration of liposomes 5 μM. Concentration of EAC=10^7 cells /ml.

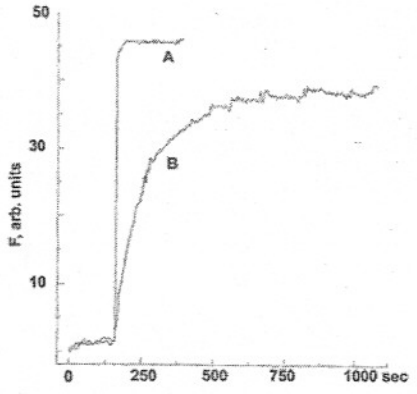


Figure 4. Change of the fluorescent intensity of calcein. A – marked liposomes destructed 0,1% triton X100. B – marked liposomes after the of EAC-cells additions. $\lambda_{ex.}$ = 490 nm, $\lambda_{em.}$ = 530 nm. Concentration of EAC-cells 10^7 cells/ml. Concentration liposomes containing 100mM of calcein - 4μM Liposomes contain lecithin (Soya) and cholesterol in molar ratio 2:1.

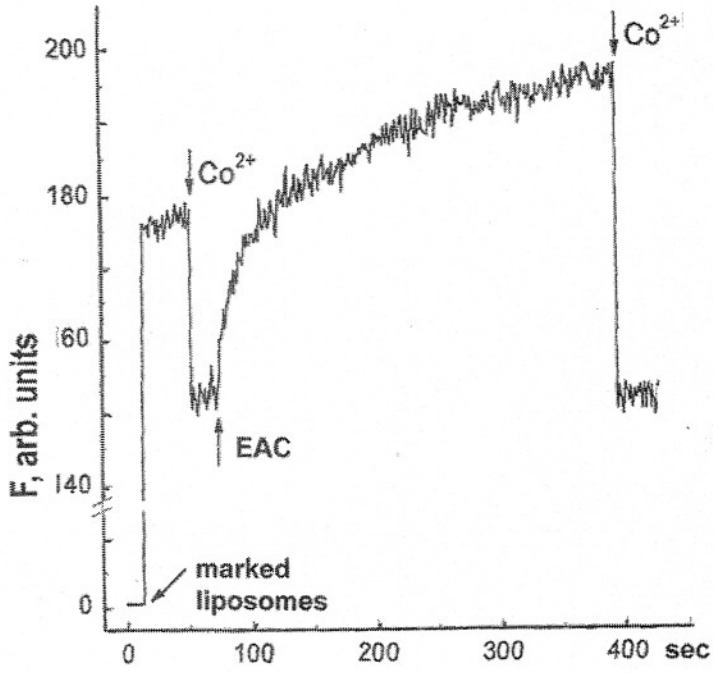


Figure 5. Change of intensity of calcein fluorescent (F) in liposomes. Arrows indicate the points of introducting in the sample of EAC-cells and cobalt ions (40 μM) which put out of the calcein fluorescent. Lipid concentration = 2 μM. Incubation medium: 100 mM KCl, 20 mM HEPES, pH 7,0, EGTA 2 μM. $\lambda_{ex.}$=490 нм, $\lambda_{em.}$ = 520 nm.

After measuring of kinetic of fluorescence of samples of marked lipid vesicles, interacted with EAC cells, these samples were used for registration of a luminescence of cells on laser confocal microscope. Cells washed out twice by centrifuge to remove the unbounded labels from membranes, and the pellet was put on subject glass. A luminescence of cells was observed on laser scanning confocal microscope LSM 510 NLO (Carl Zeiss). As an excitation source was used laser Argon 2 (length of a wave 477нм) and NeNe 1 (length of a wave 543нм). Registration was carried out by three photoelectric multipliers. Processing of images was made by means of program LSM 510 b Lucida Analyse 5.

Results and Discussion

Interaction of lipid vesicles from the neutral lipids loaded with a fluorescent probe calcein, with EAC cells is expressed in the form of fast increase of fluorescence calcein intensity and exit on some plateau (figure 4. B). Fluorescence of the free dye which left liposomes was effectively quenched by cobalt ions. The increase in intensity of fluorescence of a probe in the presence of cells can be connected with partial destruction of liposomes. They become penetrated for a probe that causes an exit of dye and it's diluting (figure 5).

Process of destruction of liposomes as a result of contact interaction with a cellular membrane is called contact lysis. Enclosed in liposome water-soluble substances come out to surface and as, destruction of density of a liposome at such way of interaction occurs near to a cellular surface these substances can be gathered in near membrane layer of cells.

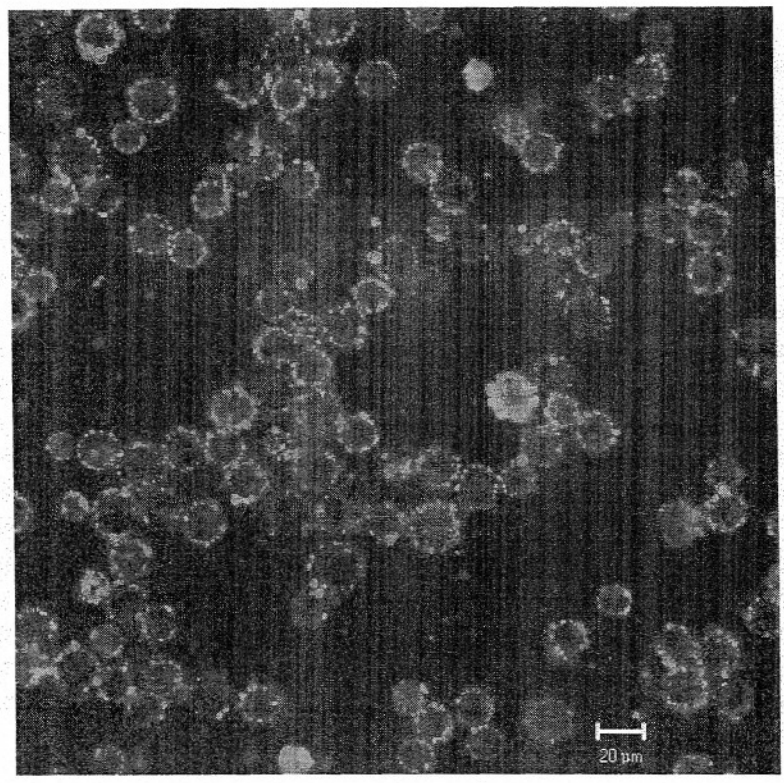


Figure 6. Laser confocal microscopic of EAC-cells after contact with liposomes contained calcein molecules. Cells incubation medium – 100 mM KCl, 20 mM HEPES, pH 7,4. $\lambda_{ex.}$ = 488 нм, $\lambda_{em.}$ =500-550 нм. Mark 20µ.

Wherefrom depending on permeability of the cell and properties of the substances which left liposome they can get into cytoplasm. As fluorescence calcein, left liposomes, is completely quenched by cobalt ions, it is possible to assume that dye avoids penetrating into cytoplasm of cells, and remains outside and is accessible to a quencher. The plasmatic membrane of cells is a barrier to penetration into cytoplasm of water-soluble molecules.

The lipid phase of liposomes at such version of interaction can and can not bounded by cell. More accurate distribution of marked calcein liposomes on a surface of cells was gained by means of laser confocal a microscope (figure 6).

At the microphotography it is seen that liposomes are adsorbed by a cellular surface.

Whether there is such a fusion of liposomes into a membrane of cells? Such type of interaction at which liposomes the membrane ceases to be closed is meant fused of liposomes to cells and entirely is included in a plasmatic membrane. Liposome contents thus come out onto the surface . Depending on the type of fusion it can appear in outer environment or get to cytoplasm.

In order to study processes of membranes fusion the method of resonance energy transfer between of fluorescent probes, covalent bounded with phosphatdileetanolamin was used.

Diluting of lipid molecules, marked by pair of probes NBD and a rhodamine B, as a result of membranes fusion leads to reduction of efficiency resonance energy transfer on an acceptor, and intensity of fluorescence of the donor increases (figure 7). As lipid vesicles contained negatively charged phosphatidilserin, the process of fusion to cells was initiated,

creating the certain conditions. In one case polyetilenglycol PEG 300 was used as fusion indicator, and in other - the acid environment of incubation medium for cells.

The curve of intensity of fluorescence change (figure 7. B) actually reflects two various processes: aggregation of cells (reaction of the second order, figure 7. D) and process of fusion which is the reaction of the first order (figure 7. C).

On the figure 8. the curve of intensity of fluorescence change of dyes during interaction of marked liposomes with EAC cells are presented. The fusion was induced by change pH environment of cell's incubation medium by addition to the sample the buffer from solution of Morpholinoethane sulfonic acid (MES, Serva) 1,0 M.

It is apparent from figure 8. A, that the decrease of pH of medium of the sample with EAC cells and marked liposomes initiates the increase of intensity of fluorescence of probe NBD which is interpreted as fusion of membranes. Decrease of pH of incubation medium can lead to partial neutralization of negative charge of liposomes and at the surface of the cells that can promote increase in probability of their collisions and the subsequent fusion of membranes. At low values of pH environments of EAC cells become more penetrated for ions, the potential on cell membrane changes that initiates process of membrane fusion.

There are some mechanisms of interaction of liposomes with cells: fusion, endocytosis, stable adsorption, increase in permeability of cells under the influence of liposomes, contact lysis of liposomes, an exchange lipid molecules between a liposome and a membrane, is possible also inclusion of liposomes in a cellular membrane as a result of fusion (figure 9) [13].

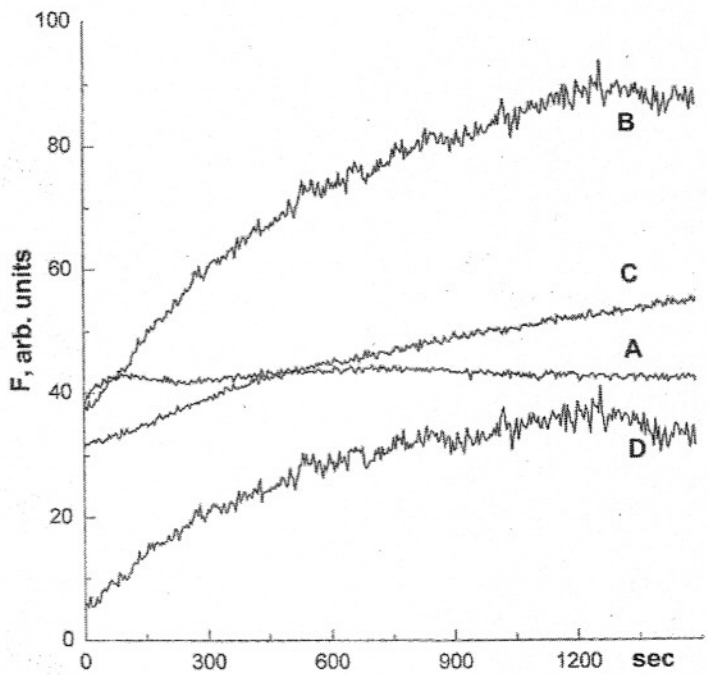


Figure 7. Change of fluorescent intensity of N-NBD probe. A – control: EAC-cells + marked liposomes; B – experiment: EAC-cells + marked liposomes + 15% PEG; C – difference from curves (B - D);D - light scattering by EAC-cells suspension in 15% PEG presence; cells + unmarked liposomes+ 15% PEG. $\lambda_{ex.}$ – 475 nm, $\lambda_{em.}$ – 530 nm. EAC cells concentration 10^7 cells/ml. Concentration of the liposomes marked by N-NBD and rodamin B, 6 μM .

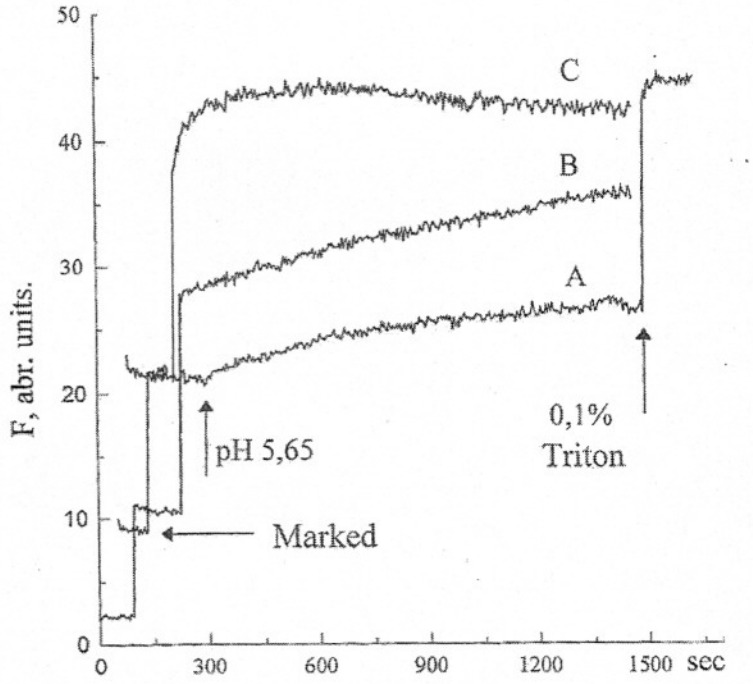


Figure 8. pH-caused change of the fluorescent intensity of N-NBD mark in liposomes under the interaction with EAC cells. A – solution pH was changed from 7,4 till 5,65 (marked by arrow) by addition of 1,0 M MES after additions of EAC-cell and marked liposomes. B – solution pH was changed from 7,4 till 5,65 before addition EAC-cell and marked liposomes in the incubation medium. C – control sample. Incubation medium: Henks, 10 mM HEPES, pH 7,4, 37°C. Cells concentration 10^7 cellsl/ml. pH of medium was changed by addition of corresponding suffer volume of original 1,0 M MES solution. $\lambda_{ex.}$ – 475 nm, λ_{em} – 530 nm.

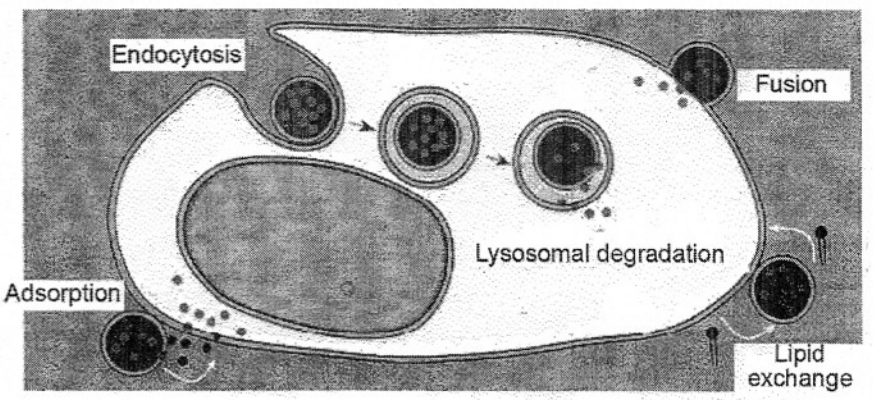


Figure 9. The scheme of possible ways of liposomes and cell interactions.

In the case of exchange of lipids, thanks to small solubility of liposomes in water, they can exchange with cellular membranes lipid molecules while adsorption in the case with smaller molecules, than lipid molecules, to a less degree depends on a membrane condition. Probably, vesicles and cellular membrane are fused by means of intermediate formation of the inverted micelle (figure 9). Such process, however, occurs seldom.

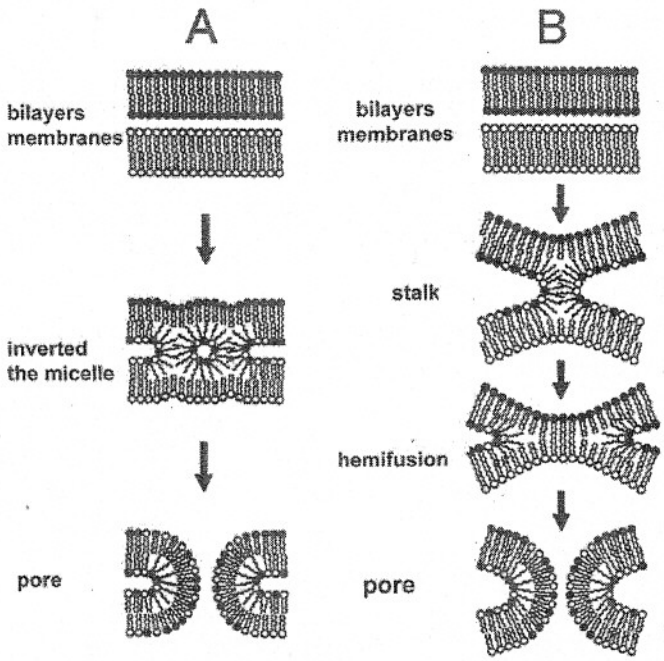


Figure 10. Possible mechanisms of the membrane fusion [14] A –the inverted micelle formed between two neighboring membranes, what provokes the fusion. B – the intermediate of fusion is the intermembrane stalk, then integration of adjacent monolayers occurs (hemi fusion), after that membranes merged and form the pore.

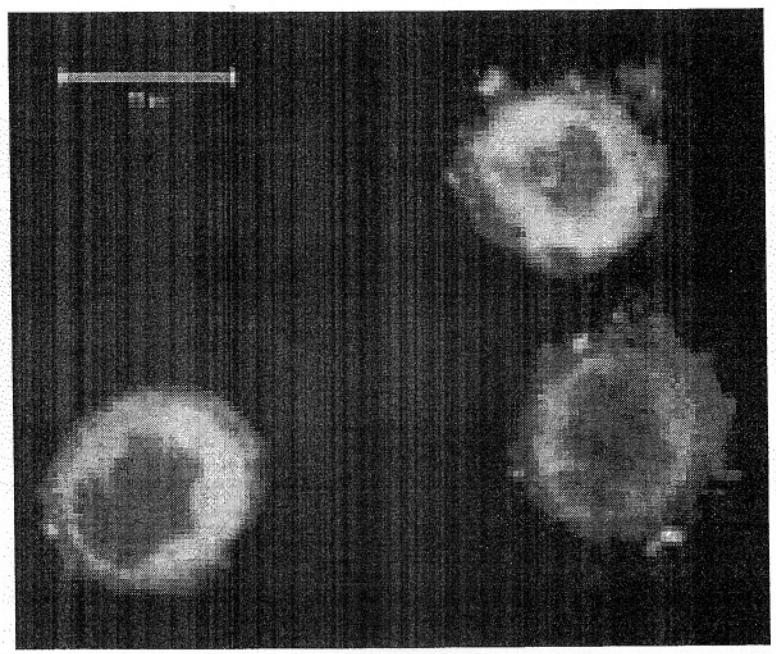


Figure 11. *Confocal laser microscopy of EAC cells treated by fluorescent marked rodamin B and NBD liposomes in the presence of 15% PEG 300.* $\lambda_{ex.}$ – 488 nm, $\lambda_{em.}$ of NBD – 500-550 nm, of rodamin B = 560 nm. Mark = 10 μ.

The most important kind of interaction is endocytosis at which the cell includes the adsorbed or connected molecules in vacuoles (enology). After fusion with lysosomals, bearing the lytic agents, occurred the digestion of liposomes, and the transferable agent also, probably,.Thus, all changes occurring in cells, we registered by means of two methods: fluorescent microscopy which allows to observe directly all processes occurring in a cell and laser confocal microscopy, allowing visually to trace these changes through all thickness of a cell. On figure 11. there are pictures of distribution of probes in EAC cell, was registered on laser confocal microscope, in samples which were used for registration of change of intensity of fluorescence on spectrofluorimeter (figure 7) after entering PEG 300 are presented.

It is seen at the photo that marked liposomes in most cases were endocytated into the cell. Also owing to small solubility of liposomes in water, they can exchange lipid molecules with cellular membranes which also were painted.

CONCLUSION

The results of our experimental studies of the interaction of lipid vesicles from neutral lipids with cells of ascetic Ehrlich carcinoma (EAC) indicated that the liposomes in most case are endocytated inside cells. Also owing to low solubility of liposome in water, they can exchange lipid molecules with cell membranes.

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