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Effect of ethylene oxide and propylene oxide block copolymers on the permeability of bilayer lipid membranes to small solutes including doxorubicin

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Abstract

The effects of ethylene oxide and propylene oxide block copolymers (pluronics) on the permeability of several weak acids and bases through bilayer lipid membranes have been studied by the methods of monitoring (1) pH shifts near planar bilayers, (2) doxorubicin fluorescence quenching inside liposomes, and (3) current transients in the presence of hydrophobic anions. It has been shown that pluronics facilitate the permeation of comparatively large molecules (such as 2-*n*-undecylmalonic acid and doxorubicin) across lipid bilayers, while the permeation of small solutes (such as ammonium and acetic acid) remains unaffected. Pluronics also accelerate the translocation of large hydrophobic anions (tetraphenylborate). The effect of pluronics correlates with the content of propylene oxide units: it is enhanced when the portion of polypropylene oxide block in the copolymer is increased. The action of the pluronic on lipid membrane permeability differs from the effect of the conventional detergent Triton X-100, which does not affect doxorubicin transport if added at concentrations similar to those used for pluronics. It has been proposed that pluronics accelerate the processes of solute diffusion within lipid bilayers (in a structure-dependent manner) rather than influencing the rate of solute adsorption/desorption on the membrane surface. We suppose that the effect of pluronics on doxorubicin permeation across lipid bilayers along with the known effect on the multidrug resistance protein determines its influence on the therapeutic activity of anthracycline drugs.

Keywords: Pluronic copolymer; Permeability; Bilayer lipid membrane; Liposome; Doxorubicin

1. Introduction

The crucial role of biological membranes consists in the creation of the permeability barrier for cellular components. For certain medical and research purposes, it is important to find compounds that can break this barrier. These compounds, called permeabilizers, are used e.g. for the release of certain substances and for the study of the metabolism of the

Abbreviations: BLM, bilayer lipid membrane; CMC, critical micellization concentration; PEO, polyethylene oxide; PPO, polypropylene oxide; TPB^-, tetraphenylborate anion; USL, unstirred layer; TTFB, tetrafluorotrichlorobenzimidazole

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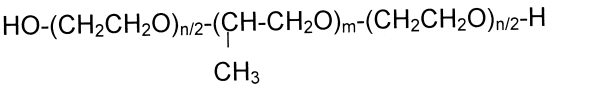
inner cellular components at the level of whole cells [1–3]. Conventional common permeabilizers, such as digitonin and alamethicin, provoke the formation of aqueous pores in membranes inducing their permeability to low molecular weight metabolites [4,5].

Ethylene oxide and propylene oxide block copolymers (pluronics) with the common formula



are widely used in medicine and biotechnology as biocompatible surfactants. Pluronic F-68 has been reported to inhibit thrombosis, decrease whole blood viscosity, and improve perfusion of damaged tissue [6,7]. In a series of research works pluronic copolymers have been tested as emulsifiers for perfluorocarbons used in artificial blood compositions [8–10] and as immunoadjuvants [11,12]. Medical applications of pluronic copolymers are connected mainly with their ability to increase the permeability of biological membranes to different substances. These copolymers promote transdermal delivery of peptides [13,14] and low molecular weight drugs [15,16], facilitate delivery of drugs across the blood–brain barrier [17], and contribute to the loading of cells with fluorescent dyes [18] or ATP [19]. Pluronic copolymers have been reported to potentiate therapeutic activity of antitumor drugs [20,21]. Moreover, pluronic copolymer–doxorubicin compositions have been proved to be effective for the treatment of multidrug-resistant tumors not only in vitro, but also in vivo [21–23].

A number of research works concerned the interaction of pluronics with lipid membranes. Changes in the phase properties of lipid bilayers were observed using calorimetric approaches [24]. It has been shown also that adsorption of pluronics onto liposome surfaces results in an increase of the liposome hydrodynamic size and a reduction of the ζ-potential [25]. The interaction of pluronic F-108 with the surface of liposomes promotes the leakage of radioactive or fluorescent probes incorporated in liposomes [26,27]. Nevertheless, polymer concentrations and experimental conditions in the above mentioned papers are far from those used in biological experiments, and it is still unclear whether the influence of pluronics on cell membrane permeability is mediated by their interaction with a lipid part of the cell membranes. Moreover, there are still no data concerning the mechanism of the pluronic effect on cell membrane permeability.

Two different mechanisms have been proposed to account for the solute permeation across lipid membranes, namely (i) the diffusion through transient pores or defects in the membrane, and (ii) partitioning into the hydrophobic phase of the bilayer followed by diffusion to the opposite side of the membrane [28,29]. The mechanism of the permeation of ions as well as of some small solutes depends on the structure and composition of lipid membranes; in particular in the case of short-chain lipids, it is consistent with the transient pore model [30,31]. On the other hand, the permeation of the majority of solutes across bilayers correlates well with membrane partitioning and can be characterized by the solubility–diffusion mechanism [28,29,32]. Permeation across bilayer membranes is dependent on the size and the shape of a solute molecule [33,34] as well as on membrane viscosity [35].

Planar bilayer lipid membranes (BLMs) and unilamellar liposomes have been used in the present work for the investigation of the effect of pluronics on the permeability of several solutes across lipid membranes. The permeation of weak acids and bases through a planar BLM has been measured by a technique developed in our laboratory which is based on the monitoring of steady-state pH shifts in the unstirred layers (USLs) near the BLM [36–39]. Transport of doxorubicin, the powerful anticancer drug, across membranes of liposomes has been studied by measuring the changes in fluorescence intensity as proposed by Harrigan et al. [40]. It has been found that pluronics facilitate the permeation of solutes across lipid bilayers, the effect being dependent on the chemical nature of the compound.

2. Materials and methods

2.1. Materials

Block copolymers of ethylene oxide and propylene oxide (pluronics P85, L61 and F68) and Triton X-100 were from Serva (Heidelberg, Germany). Doxorubicin hydrochloride (95% purity according to liq-

uid chromatography) was purchased from the Russian Antibiotic Institute. Buffer components were from Sigma (St. Louis, MO, USA). 2-*n*-Undecylmalonic acid (α,α-DC11) was a generous gift of Dr. D. Bondarenko from the A.N. Bakh Institute of Biochemistry, Russian Academy of Science; it was prepared by alkylation of diethyl malonate with *n*-undecylbromide [39]. Synthesized α,α-DC11 contained 98% of the basic compound according to gas chromatography of its dimethyl esters. Ammonium chloride, acetic acid and tetraphenylborate (TPB) were from Sigma (St. Louis, MO, USA). Tetrafluorotrichlorobenzimidazole (TTFB) was a gift of Prof. E.A. Liberman.

2.2. Methods

2.2.1. Measurement of fluxes of weak acids and bases across planar lipid membranes

BLMs were formed by a conventional method [41] in a hole, 0.4 mm in diameter, of a diaphragm dividing a PTFE chamber. The membrane forming solution contained 20 mg diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) in 1 ml of *n*-decane (Merck, Darmstadt, Germany). The electrical resistance of the BLM was measured with the help of an OES-2 patch-clamp amplifier (Opus, Moscow, Russia). Gradients on the BLM (ΔpHs) were measured according to Antonenko et al. [36,42] by the method of recording an open-circuit potential in the presence of a protonophore (10 μM TTFB) which was added on both sides of the BLM. The potential had a plus sign on the side of the membrane where the acid was added. The experiments were carried out at room temperature (21–23°C).

J_H^+ were estimated from the following equation [43]

$$J_{H^+} = \frac{D_{buff} B \Delta pH}{2\delta} \quad (1)$$

where D_{buff} is the diffusion coefficient of the buffer molecules, B is the buffer capacity of the solutions, ΔpH is the pH gradient on the BLM, and δ is the thickness of the unstirred layer. We used values for D_{buff} of 5×10^{-6} cm^2/s and for δ of 200 μm following Antonenko et al. [37]. ΔpHs were calculated from the values of protonophore-dependent potentials according to the Nernst equation [36].

pH shifts in the USLs near the BLM were measured directly by means of an antimony pH microelectrode according to Evtodienko et al. [39]. It should be noted that the sum of two pH shifts on the opposite sides of the BLM is equal to the ΔpH gradient on BLM. It has been shown previously that the two methods of pH shift measurement give consistent results [42]. Typically, the electrode tip was 10 μm in our experiments. A smooth approach to the membrane was carried out by means of a hydraulic microdrive.

2.2.2. TPB^- current relaxation experiments

The electric current (I) was recorded under voltage-clamp conditions by application of a step of the potential (voltage jump) [44]. The current was measured by means of a U5-11 amplifier (Moscow, Russia), digitized by DT2814 (Data Translation, Marlboro, MA, USA) and analyzed by a personal computer. Ag/AgCl electrodes were placed directly into the cell, in most experiments a voltage of 100 mV was applied to the BLM.

The conductivity of the unmodified BLM was estimated from the electrical current at a given voltage (50 mV). The water solution in both compartments of the experimental chamber contained 10 mM MES, 10 mM Tris, and 100 mM choline chloride, pH 6.4.

2.2.3. Preparation of liposomes

Unilamellar liposomes were prepared according to Yaroslavov et al. [45]. A phosphatidylcholine ethanol solution (20 mg, Kharkov Bacterial Preparations Company, Ukraine) was carefully evaporated under vacuum. The thin layer of the lipid mixture was dispersed in 0.3 M citrate–Tris buffer, pH 4.0, with a Cole-Parmer 4700 ultrasonic homogenizer. Liposome samples thus obtained were separated from the titanium dust by centrifugation and used during 1 day. The diameter of liposomes measured by quasi-elastic light scattering was in the range of 50–70 nm. To replace the external solution, 1 ml of the liposome suspension was added to the Sepharose CL-4B column equilibrated with 20 mM HEPES–Tris buffer pH 7.0 supplemented with 0.3 M sucrose to avoid osmotic swelling of vesicles.

2.2.4. Kinetics of the influx of doxorubicin into liposomes

The kinetics of the doxorubicin pH-induced uptake by liposomes was measured according to Harrigan et al. [40] and Maurer-Spurej et al. [46]. Doxorubicin (0.1 mM) or its mixture with pluronic was added to liposomes filled with 0.3 M citrate buffer pH 4.0, and changes in fluorescence intensity at 557 nm were monitored with a Hitachi F-3000 spectrofluorometer, using an excitation wavelength of 490 nm. Accumulation of doxorubicin in the liposome interior resulted in a 100–1000-fold increase in its local concentration accompanied by a fast decrease in fluorescence. The rate constant of drug uptake at 30°C was calculated from the kinetics of fluorescence intensity lowering using a first order model. As has been shown by Harrigan et al. [40], the rate constant k in this case depends upon the pH gradient (ΔpH), the liposome surface area (A), the permeability coefficient (P), the external aqueous volume V_o, the apparent membrane–water partition coefficient of the charged form of the drug K^*, the dissociation constant of the drug K_a and the external proton concentration [H^+]:

$$k = \frac{PAK^*K_a}{V_o[H^+]} \quad (2)$$

Acceleration of the doxorubicin influx into liposomes was calculated as the ratio of the rate constant in the presence of pluronic to that measured without pluronic for liposomes prepared in the same series, the liposome concentration was the same for both samples.

3. Results

3.1. Effect of pluronic copolymers on the electric conductance of BLM

One of the advantages of the planar bilayer system consists in the possibility of studying both electrogenic and electrically silent solute fluxes. A series of measurements showed that the addition of pluronics (P85 and L61) alone or in combination with weak acids or bases did not increase the electrical conductance of the BLM which remained within 10 nS/cm^2 under our experimental conditions. These measurements give the upper limit of the electrogenic solute fluxes that can arise in our system. In fact, a conductivity of 10 nS/cm^2 corresponds to a flux of a solute carrying a single charge of 2×10^{-15} mol of charges/cm^2 s [47]:

$$J_{S^+} = \frac{RTt_{S^+}G_m}{z_{S^+}^2F^2} \quad (3)$$

where G_m is the membrane conductance, t_{S^+} is the transference number for ions (1.0 in our estimation), R is the gas constant, T is the absolute temperature, z_{S^+} is the ionic valence and F is the Faraday constant. Since a typical value of a solute flux measured in our system was about 10^{-11} mol/cm^2 s (see below), it can be concluded that the fluxes across the BLM under our experimental conditions were electrically silent.

It is noteworthy, however, that the addition of potassium ions in the solutions bathing the membrane resulted in the induction of the pluronic-mediated electrical conductance of the BLM. Since this effect is beyond the scope of the present research, we did not study it in detail. None of the buffer solutions used here contained potassium ions.

3.2. Effect of pluronic copolymers on the transport of α,α-DC11 across planar lipid membranes

The permeation of weak acids across planar membranes was studied using a technique based on measuring the pH shifts in the vicinity of the membrane. This method allows us to measure the fluxes of neutral forms of these substances across the BLM. Permeation of neutral forms of weak bases and acids across lipid bilayers is accompanied by the formation of pH gradients in the vicinity of the membrane due to the dissociation–association processes occurring in the USL near the BLM. It has been shown in our previous works that the transmembrane flux of a neutral form of a solute can be calculated from the pH gradient according to Eq. 1. pH gradients were calculated from the values of the transmembrane potential ($\Delta\phi$) formed on the BLM in the presence of the protonophore TTFB. The Nernst equation gives the linear dependence between the transmembrane potential and the pH gradient with a coefficient of 58 mV at 25°C.

Fig. 1A shows a typical curve of generation of the

transmembrane potential in response to the addition of α,α-DC11 into one of the compartments of the experimental cell. The addition of P85 pluronic copolymer into both compartments led to a six-fold increase in the transmembrane potential corresponding to a six-fold increase in the solute flux across the BLM (from 1.6×10^{-12} to 9.1×10^{-12} mol H^+/cm^2 s). The L61 pluronic effect on the bilayer permeability increased upon raising the copolymer concentration (Fig. 1B).

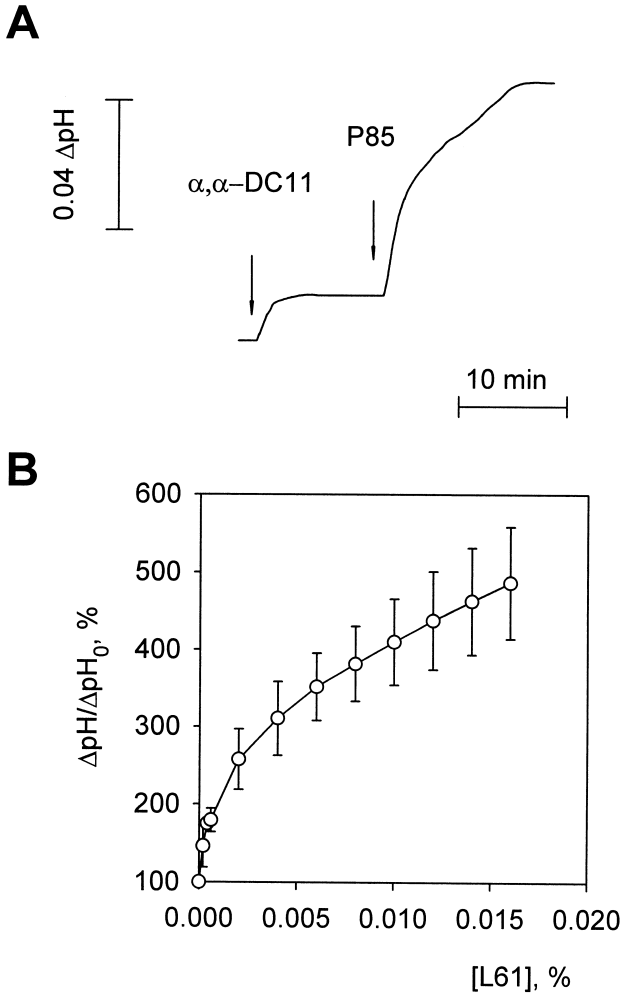


Fig. 1. (A) Effect of P85 pluronic copolymer on the flux of α,α-DC11 across the planar bilayer measured by recording the transmembrane ΔpH. The concentration of α,α-DC11 on the *cis* side is 100 μM, [P85] is 2×10^{-2}% on both sides of the BLM. The solution is 1 mM Tris, 1 mM MES, 100 mM choline chloride, 10 μM TTFB, pH 6.2. (B) Effect of the concentration of L61 pluronic copolymer on the ratio of the α,α-DC11 fluxes in the presence of the pluronic and in the control.

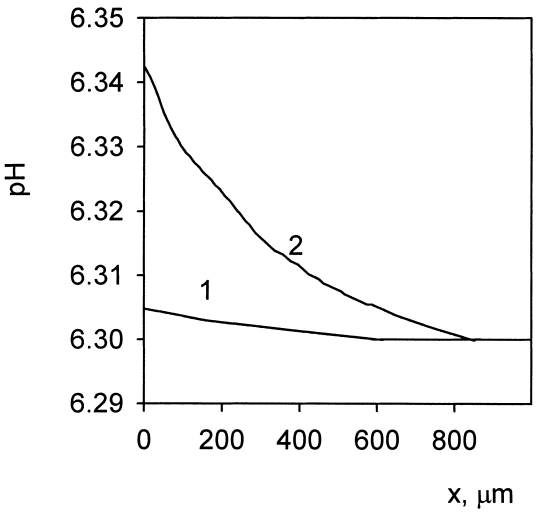


Fig. 2. Effect of P85 pluronic copolymer on the flux of α,α-DC11 across the planar bilayer measured by the recording of pH profiles near the BLM (at $x=0$) by pH microelectrode on the *cis* side of the membrane (where the acid is added). The concentration of α,α-DC11 on the *cis* side is 100 μM, [P85] is 2×10^{-2}% on both sides of the BLM. Other conditions are the same as in Fig. 1. The solution is without TTFB.

These results were confirmed by direct measurements of pH shifts on both sides of the BLM, performed in the absence of TTFB with the help of the pH microelectrode. Fig. 2, curve 1, demonstrates the formation of the pH profile on the *cis* side of the BLM (the side where the acid has been added) after the addition of 100 μM α,α-DC11 under conditions identical to those of the experiment shown in Fig. 1A. The permeation of the acid leads to a pH increase near the *cis* side of the membrane surface reflecting the disappearance of protons due to acid permeation through the membrane in symport with protons. In agreement with the results of the measurements of protonophore-dependent potentials, the addition of the copolymer to both compartments of the cell led to a drastic increase in the pH shift near the membrane surface (Fig. 2, curve 2). Since the addition of pluronics does not affect the membrane conductivity, the proton fluxes induced by weak bases and acids across the BLM in the presence of pluronics are electrically silent and proceed via the symport with the acid.

So, it may be concluded that pluronic copolymers considerably increase the permeability of the BLM to the neutral form of α,α-DC11. Taking into account

that pluronic copolymers are micelle-forming surfactants, one can suppose that their influence on BLM permeability is caused by the solubilization of the substances in micelles, which in turn fuse with the bilayer. This explanation seems unlikely, because the experimental conditions (21°C, $1–2\times10^{-2}$% of pluronic) are unfavorable for micelle formation. The critical micellization concentration (CMC) has been reported to be about 5% at 20°C (Table 1). It may be proposed that the influence of pluronic copolymers on carboxylic acid permeation across the BLM is due to its interaction with the membrane. The results of the following experiments favor this proposal. Fig. 3 shows the BLM transmembrane potential generation in the presence of α,α-DC11, when pluronic was added initially on the *trans* side of the membrane (panel A) and on the *cis* side (panel B), respectively. In the second case α,α-DC11 and the pluronic can easily interact in the solution, whereas in the first type of experiment this interaction is virtually excluded. It is seen that the addition of pluronic P85 on the *trans* side of BLM resulted in even bigger changes of α,α-DC11 flux than when it was added on the *cis* side. Thus, the solubilization of the solute in micelles of pluronics cannot be responsible for the effect on the transmembrane flux.

Table 1
Effect of different pluronic copolymers on the lipid bilayer permeability, composition, CMC and therapeutic activity

		Copolymer[a]			Triton X-100
		L61	P85	F68	
Number of PEO units (*n*)		4	50	160	10
Number of PPO units (*m*)		30	40	30	–
Molecular weight		2090	4500	8800	642
Effect on the translocation rate constant of TPB^- with respect to control, copolymer concentration 2×10^{-4}%		5.0	4.0	1.8	1.6
Effect on pH-induced doxorubicin uptake into liposomes with respect to control, copolymer concentration 2×10^{-3}%		3.2	1.6	1.05	1.3
CMC (%)[b]	25°C	0.01	5	–	0.016[c]
	37°C	0.0005	0.003		
Optimal therapeutic concentration (g/kg body weight)[d]		0.025	0.1	1.0	–

[a]For formula see Section 1.
[b][23,52].
[c][21].
[d][61].

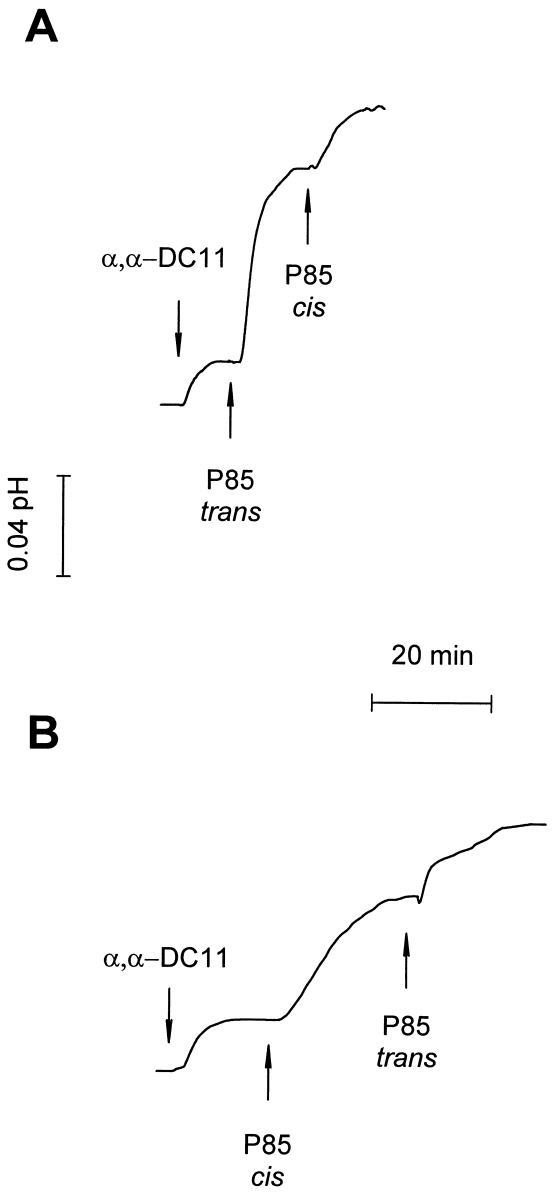


Fig. 3. Effect of P85 pluronic copolymer added on different sides of the membrane on the flux of α,α-DC11 across the planar bilayer measured by the recording of transmembrane ΔpH. The conditions are the same as in Fig. 1.

The transfer of weak acids across planar lipid bilayers can be limited either by diffusion of the neutral form of the solute across the membrane, or by the diffusion of the ionic form across the unstirred layer

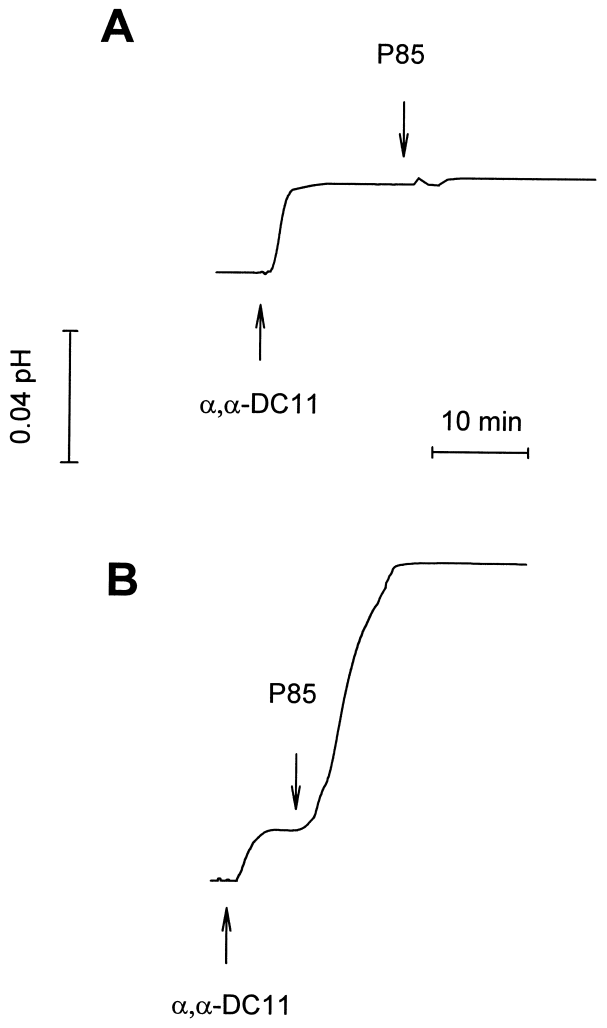


Fig. 4. Effect of P85 pluronic copolymer on the flux of α,α-DC11 across the planar bilayer measured at different pHs of the solutions bathing the membrane by the recording of transmembrane ΔpH. (A) pH 3.8. (B) pH 6.3. The conditions are the same as in Fig. 1.

near the membrane [39]. The relative contribution of these processes depends on the ratio of ionic and nonionic forms of the solute determined by its pK_a and pH of the solution. In order to rule out the possibility that the effect of pluronic corresponds to the diffusion of the anionic form of α,α-DC11 across the unstirred layers, we studied the pH dependence of the flux of the acid. Provided that pluronic mainly affects the permeation of the neutral form of the solute across the BLM and does not influence its diffusion through the USL, the effect should be observed only under alkaline conditions. Fig. 4 shows the effect of pluronic P85 on the value of the BLM potential generated upon the addition of α,α-DC11 under acidic (panel A) and more alkaline (panel B) conditions. Pluronic had no effect on carboxylic acid transport at acidic pH, showing that the copolymer affects the permeation of the neutral form of carboxylic acid across the lipid bilayer.

3.3. *Effect of pluronic copolymers on the transport of small weak acids and bases across BLM*

Fig. 5 shows the effect of L61 pluronic copolymer on the protonophore-dependent BLM potential generated as a result of the permeation of acetic acid (panel A) and ammonia (panel B) across the membrane. The experiments were carried out under conditions where the transport was limited by the diffusion across the lipid phase and the contribution of the diffusion across the USLs was negligible. L61 pluronic had only a minor effect on the acetic acid and ammonia fluxes across the BLM. This finding shows that the effect of pluronic copolymers on the permeation of solutes is very sensitive to the nature of the solute. On the other hand, these experiments confirm that the effect of the copolymers on membrane permeability is not mediated by the formation of pores in the lipid membrane, since small molecules of acetic acid (or ammonia) would penetrate through such pores more readily than large molecules of α,α-DC11.

In the experiments with ammonia and acetate the values of the potentials were close to those measured with α,α-DC11 (about 2 mV). This means that the transmembrane fluxes of the neutral forms of these substances were close to each other since the experiments were carried out under conditions where the transport was limited by the diffusion across the BLM and the contribution of the diffusion across the USLs was negligible. This was achieved by adjusting the pH of the solution to substantially higher (or lower for ammonia) values than the pK_a so that the pH decrease led to a concomitant decrease of the value of protonophore-dependent potentials. However, the values of membrane permeabilities for small solutes and α,α-DC11 differ considerably. In view of this, the selectivity of the action of pluronic copolymers may be accounted for by the differences in permeability. It may be proposed that the effect of co-

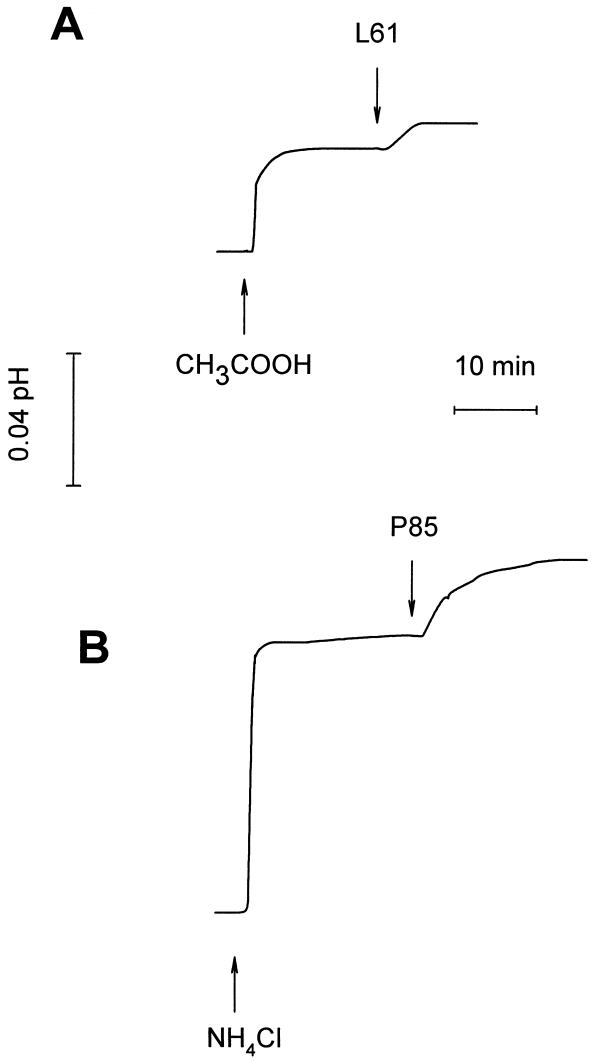


Fig. 5. (A) Effect of L61 pluronic copolymer (1×10^{-2}%) on the flux of acetic acid (50 μM on the *cis* side of the BLM) across the planar bilayer measured by the recording of transmembrane ΔpH (acidification on the *trans* side). The conditions are the same as in Fig. 1, pH 6.3. (B) Effect of P85 pluronic copolymer (2×10^{-2}%) on the flux of ammonia (100 μM on the *cis* side of the BLM) across the planar bilayer measured by the recording of transmembrane ΔpH (acidification on the *cis* side). The conditions are the same as in Fig. 1, pH 6.5.

polymers is more pronounced for substances which have a lower membrane permeability. In particular, as described in Section 3.4, pluronics considerably accelerate doxorubicin permeation through membranes of liposomes. The values of the protonophore-dependent potentials detected in the presence of doxorubicin are negligible (data not shown), therefore the membrane permeability of doxorubicin is obviously considerably lower than that of acetate.

3.4. *Effect of pluronics on the transfer of tetraphenylborate across lipid bilayers*

As shown earlier, the application of a voltage jump to the BLM in the presence of permeating anions (TPB^-) results in a current relaxation kinetics with a steady-state current substantially less than the initial current [44,48]. This kinetics can be accounted for by the model assuming that the rate constant of the translocation of the permeating anions across the BLM substantially exceeds those of their association with or dissociation from the BLM. Therefore, the initial transmembrane current vanishes after the depletion of TPB^- anions on one side of the membrane due to translocation of the major part of the anions to the opposite side [44]. The number of coulombs moving through the bilayer during a single current transient (Q) corresponds to the total number of TPB^- anions transferred through the BLM. The characteristic time (τ) of TPB^- translocation was

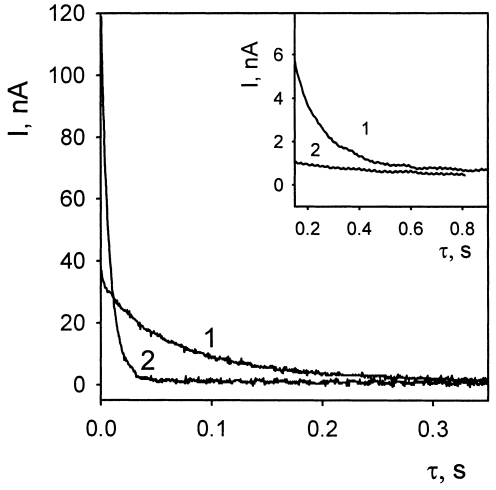


Fig. 6. Effect of P85 pluronic copolymer (5×10^{-4}%) on the TPB-induced current transient after the application of a voltage jump (100 mV) to the BLM (curve 2). Curve 1 is a control without P85, it is well fitted by a single exponential with $I_0 = 30$ nA and $\tau = 83$ ms. Curve 2 is fitted by a single exponential with $I_0 = 120$ nA and $\tau = 7.5$ ms. The solution is 10 mM Tris, 10 mM MES, 100 mM choline chloride, pH 6.4. TPB^- concentration is 1 μM. (Inset) Filtered traces of curves 1 and 2 at the end of the recordings.

83 ms in our experiments (Fig. 6, curve 1). The addition of 5×10^{-4}% of P85 copolymer resulted in an 11-fold decrease in the value of τ and a four-fold increase in the initial current (Fig. 6, curve 2). The corresponding first order constant ($k=1/\tau$) increased from 12 s^{-1} to 133 s^{-1}. The number of coulombs transferred across the BLM, calculated as an integral,

$$Q=\int_0^\infty I(t)\mathrm{d}t,$$

also displayed a 2.2-fold decrease indicating that pluronic adsorption on the BLM reduced the amount of membrane-bound TPB^-.

The relaxation of the current resulted in a low but definite steady-state level determined presumably by the rates of TPB^- adsorption/desorption kinetics (inset in Fig. 6). It is seen that the addition of pluronic L61 did not alter the steady-state current showing that the pluronic selectively affects the stage of TPB^- translocation across the membrane. Fig. 7 shows the concentration dependence of the copolymer's effect on the TPB^- translocation rate constant (panel A) and the amount of transferred TPB^- (electrical charges in nC) upon the voltage jump (panel B). The translocation rate increases with the copolymer concentration indicating that the effect is mediated by polymer adsorption on the BLM.

It is worth noting that pluronic copolymers also increased the valinomycin-mediated K^+ electrical conductance (by about 30% at a BLM conductance similar to that mentioned above in the experiments with TPB^-). Therefore, the effect of pluronic copolymers is not accounted for by the change in the dipole potential of the membrane, since the increase in the dipole potential should accelerate the translocation of anions but decrease the translocation rate of cations [49,50].

We compared the effect of different pluronic copolymers and the polyethylene oxide (PEO)-containing detergent Triton X-100 (*p*-iso-octylphenyl monoether of PEO ($n=10$)) at a fixed copolymer concentration (Table 1). It may be concluded that the increase in copolymer hydrophobicity and the portion of the propylene oxide (PPO) units in the pluronic molecule enhances the effect on TPB translocation. At the same time, the duration of the PEO block mainly determines the copolymer effect on the

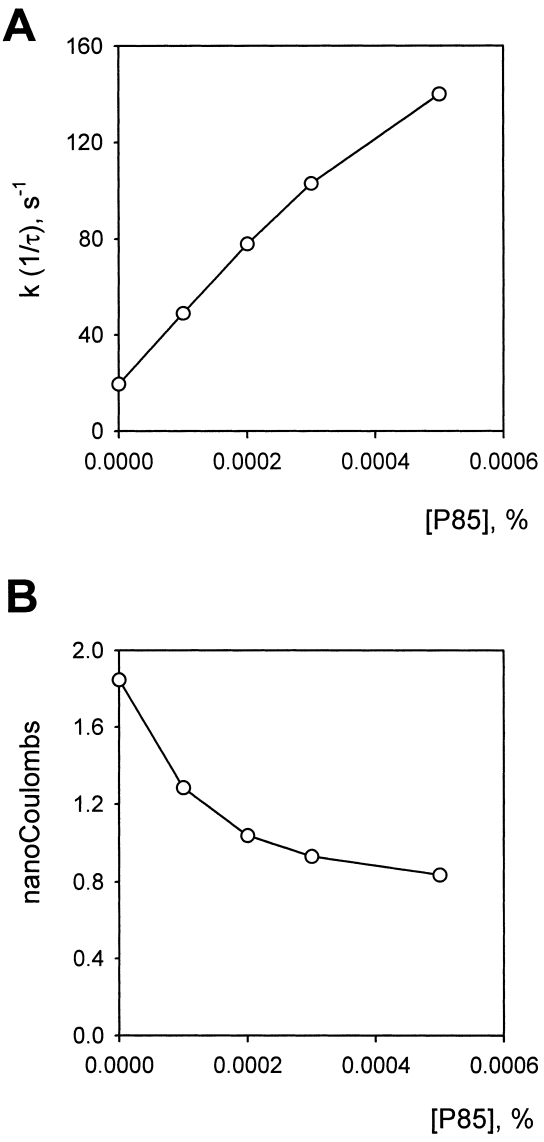


Fig. 7. The dependence of the translocation rate constant of TPB^- (A) and the magnitude of the transferred charges (B) on the concentration of P85 pluronic copolymer. TPB^- concentration is 1 μM. Other conditions are the same as in Fig. 6.

Q value, probably via the influence on the membrane area covered with PEO chains.

3.5. *Effect of pluronics on the doxorubicin influx into liposomes*

Following Harrigan et al. [40] and Maurer-Spurej et al. [46] our experimental model of doxorubicin transport is based on the accumulation of doxorubicin in response to the pH gradient formed on lipo-

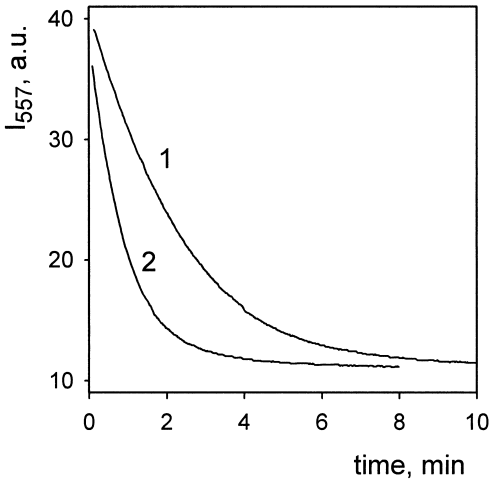


Fig. 8. Effect of the pluronic copolymer on the kinetics of the pH gradient-induced uptake of doxorubicin into liposomes. Curve 1 is a control, and curve 2 is 3.6×10^{-3}% L61. Internal buffer contains 0.3 M citrate, pH 4.0, external buffer contains 20 mM HEPES, 5 mM Tris, 0.27 M sucrose, pH 7.0.

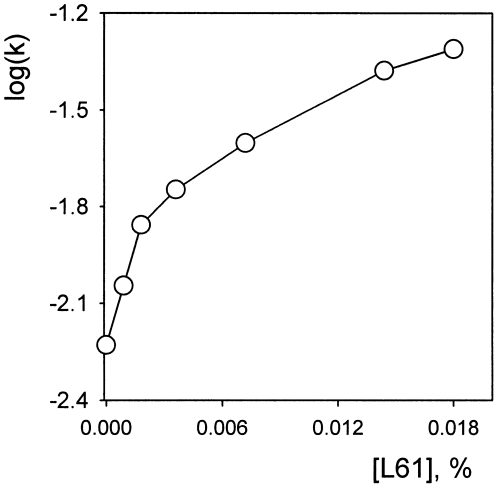


Fig. 9. The dependence of the first order rate constant of doxorubicin uptake into liposomes on pluronic L61 concentration. The conditions are the same as in Fig. 8.

some membranes (pH = 4 inside and 7 outside). Doxorubicin is a weak base which binds proton under acidic conditions; it is unable to permeate through the membrane in the charged form. The influx of doxorubicin into liposomes leads to an increase in its local concentration inside liposomes, which results in a decrease in the drug's fluorescence due to self-quenching [40].

Fig. 8 shows the kinetics of the doxorubicin uptake into liposomes in the absence (curve 1) and in the presence of 3.6×10^{-3}% L61 pluronic (curve 2). Both curves are single exponential, so the simple first order model describes well the uptake kinetics. The addition of L61 pluronic resulted in an about three-fold acceleration of doxorubicin transport across the liposome membrane. The increase in the L61 pluronic concentration resulted in a gradual increase in doxorubicin uptake (Fig. 9). At the high pluronic concentration the rate constant becomes eight times higher than that in the control.

The pH dependence of the kinetic constants of drug uptake into liposomes gives information on the mechanism of the action of the pluronic on doxorubicin transport. If the permeation of doxorubicin is limited by the transfer of the neutral form across the bilayer, a reduction of the external buffer pH should result in a decrease of influx rate due to a lowering of the neutral form content in the external buffer. This dependence is observed in the experiment shown in Fig. 10 (curve 1). The same pH dependence was observed in the presence of pluronic L61, showing that the pluronic facilitates the uptake of the neutral form of doxorubicin (Fig. 10, curve 2).

Variation of the duration of PEO and PPO blocks in the pluronic molecule results in drastic alterations of its properties [51–53]. An increase of the PPO/PEO ratio in the copolymer molecule leads to a decrease of its CMC (Table 1), water solubility and a change of micellar structure. It is reasonable to sug-

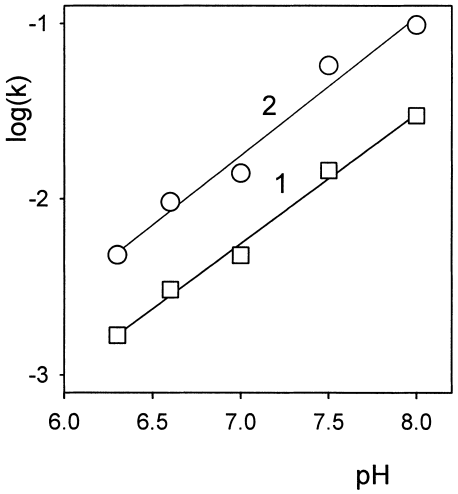


Fig. 10. pH dependence of the first order rate constant of doxorubicin uptake into liposomes in the presence (curve 2) and in the absence of pluronic copolymers. The conditions are the same as in Fig. 8.

gest that more hydrophobic pluronics containing more PPO units will cause stronger effects on the membrane structure than hydrophilic ones. A comparison of the therapeutic effects of different pluronics in combination with doxorubicin [21] has shown that more hydrophobic pluronics (L61, P85) are substantially more effective than hydrophilic copolymers (F68, Table 1). In our experiments with doxorubicin influx into liposomes, the more hydrophobic pluronics L61 and P85 caused more pronounced changes in flux than the hydrophilic copolymer F68. The same correlation was observed for the transport of TPB^- (Table 1). The correlation of optimal therapeutic concentrations of the pluronics and the effect on doxorubicin influx indicates that the effect of pluronic copolymers on the permeability of the lipid bilayer is important for their biological activity.

Taking into account that the PEO/PPO ratio correlates with the ability of the copolymer to form micelles in a water solution (Table 1), one may suppose that the effect of pluronics on lipid membrane permeability is somehow related to their surface activity. In order to examine this assumption, we studied the effect of Triton X-100 containing the alkyl radical in its hydrophobic block. It turned out that Triton X-100 has a negligible effect on doxorubicin transport across liposome membrane, when added at a concentration of $4\times10^{-3}\%$ (Table 1). The addition of Triton X-100 at higher concentrations led to leakage of buffer components from a portion of the liposomes. At the same time the doxorubicin influx rate measured by fluorescence intensity was affected only slightly. Thus, the surface activity of pluronic copolymers is not the only factor that determines its influence on bilayer permeability. Detergents with hydrocarbons in their hydrophobic blocks exhibit quite different behavior.

4. Discussion

In a series of papers [17–19,54] pluronic copolymers have been reported to promote permeation of water-soluble drugs across cell membranes. This effect was attributed to the pluronic copolymer-mediated inhibition of the multidrug resistance protein [21–23]. The present results show that pluronic copolymers can accelerate the permeation of different solutes across the lipid part of biological membranes.

The process of permeation across lipid membranes includes the adsorption of a solute on the membrane, the translocation through the hydrophobic region of the lipid bilayer and the desorption from the membrane. The total permeability value P_M depends on the rate constants of these processes as proposed, for example, by Miller [55]:

$$P_M = \frac{k_{WL}k_T}{(k_{LW} + 2k_T)} \quad (4)$$

where k_{WL} is the rate constant of solute translocation from the water phase to the lipid bilayer, k_{LW} is the rate constant of its translocation from the lipid bilayer to the water solution and k_T is the rate constant of the diffusion of the solute across the membrane. Since pluronic copolymers are widely used to facilitate the incorporation of hydrophobic compounds into membranes (see, for example, [56]) the increase in membrane permeability caused by pluronics may be attributed to the influence on the k_{WL} and k_{LW} constants. However, the results of the experiments with TPB^- show that pluronics increase the k_T value of TPB^-. Moreover, steady-state current measurements in the presence of TPB^- indicate that pluronics do not alter the k_{LW} and k_{WL} values. These results support the idea that the action of pluronic copolymers on membrane permeability proceeds via the effect on inner membrane diffusion.

Therefore, the selectivity of pluronic action on the transport of different substances is probably caused by the different mechanisms of solute permeability in the membrane interior. In fact, ammonia and acetic acid are comparatively small molecules and their permeation can proceed at least partially via membrane microdefects. On the other hand, the permeation of α,α-DC11 or doxorubicin can hardly proceed via the above mentioned process and apparently goes via the solubility–diffusion mechanism.

Another possible mechanism of the selective effect of pluronics may be associated with the dependence of the process of the transmembrane diffusion of solutes on the size and/or the form of the molecules [34,57,58]. It was shown that the membrane interior can hardly be regarded as a classical liquid [59] and that permeant diffusion is strongly dependent on its shape and size. Thus, the selective action of pluronics

may be accounted for by their disturbance of bilayer lipid packing which mediates the process of solute permeation in the case of large molecules. This conclusion is in line with the recent finding that pluronics can lower the membrane microviscosity of plasma membranes of tumor cells [60].

As discussed above, it seems unlikely that pluronics form channels or hydrated pores in the BLM. If so, pluronics should increase the planar bilayer electrical conductivity. Besides, we observed that the permeation of small hydrophilic acids (CH_3COOH) and bases (NH_3) is not affected by pluronic, while the flux of large molecules (α,α-DC11 and doxorubicin) is strongly increased in the presence of pluronic copolymers. These findings also contradict the channel mechanism of the effect of pluronic copolymers. Thus, the action of pluronics differs from that of commonly used permeabilizers [1–5].

Importantly, the effect of pluronic copolymers on membrane permeability is quite different from that of conventional detergents such as Triton X-100 that also contain PEO groups. The addition of Triton X-100 at low concentrations (similar to that of pluronics) has no effect on the doxorubicin pH-induced uptake into liposomes, while the increase in the detergent concentration results in leakage of liposomes and complete inhibition of the pH-induced drug uptake. We suppose that peculiarities of the action of pluronics in comparison with traditional PEO-containing detergents are caused by the structure of the hydrophobic PPO block that contains polar oxygen groups. Since the overall hydrophobicity of the PPO block is rather high, it binds to the apolar part of the lipid bilayer [25]. However, oxygen atoms placed in the hydrophobic environment can disturb it. Apparently, this disturbance mainly determines the action of the copolymer on the membrane permeability.

We suppose that the effect of the pluronic on doxorubicin permeation across lipid bilayers provides the basis for its influence on the therapeutic activity of anthracycline drugs. Measurements of the pharmacokinetics of pluronics in mice have shown that their concentration in the bloodstream remains at a level of 5×10^{-3}–5×10^{-4}% for several hours after injection of therapeutic doses of the copolymer [21]. Our results demonstrate that such copolymer concentrations can considerably accelerate drug permeation across the lipid part of the plasma membrane of cells.

In a series of recent publications [21–23] pluronic copolymers have been shown to increase the sensitivity of multidrug-resistant (MDR) cells with respect to various chemotherapeutic agents, such as anthracyclines, *Vinca* alkaloids, and others. In the presence of pluronics P85 and L61, the cytotoxic activity of the drugs against MDR cells increased 100–1000 times, whereas only marginal changes in the drugs' cytotoxicity were observed in the case of sensitive cells [21–23]. It was shown that pluronic copolymers facilitate accumulation of doxorubicin in the resistant cells, the efficacy of their action being dependent on the expression of the MDR phenotype. It was suggested that pluronic copolymers stimulate drug accumulation in the cell cytoplasm by inhibiting the P-glycoprotein-mediated drug efflux from the resistant cells. The results of the present paper suggest that along with the above proposed mechanism an additional effect of pluronic copolymers can take place. In fact, copolymer adsorption on the cell plasma membrane accelerates doxorubicin translocation across the lipid parts of the membrane and should facilitate its accumulation in the cytoplasm. According to the findings presented above, this will allow the drug to bypass P-glycoprotein located in the plasma membrane of resistant cells. The reduced effect of pluronic copolymers on sensitive cells may be accounted for by the dependence of the effect on the lipid composition of cell membranes. We suppose that the study of the influence of pluronics on the transport of different substances across lipid bilayers will be important for the elaboration of pluronic-based drug formulations.

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