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PERMEABILITY OF BILAYER LIPID MEMBRANES FOR SUPEROXIDE (O_2^-) RADICALS

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Egg yolk phosphatidylcholine monolamellar liposomes (1000 Å in diameter) loaded with cytochrome *c* were placed into an external solution, in which superoxide radicals, O_2^-, were generated by a xanthine-xanthine oxidase system. The penetration of the superoxide radicals across the liposomal membrane was detected by cytochrome *c* reduction in the inner liposome compartment. The effects of modifiers and temperature on this process were studied. The permeability of liposomal membrane for O_2^- ($P'_{O_2^-} = (7.6 \pm 0.3) \cdot 10^{-8}$ cm/s), or $HO_2^\cdot$ ($P'_{HO_2^\cdot} = 4.9 \cdot 10^{-4}$ cm/s) were determined. The effect of the transmembrane electric potential (K^+ concentration gradient, valinomycin) on the permeability of liposomal membranes for O_2^- were investigated. It was found that O_2^- can penetrate across liposomal membrane in an uncharged form. The feasibility of penetration of superoxide radicals through liposomal membrane, predominantly via anionic channels, was demonstrated by the use of an intramolecular cholesterol-amphotericin B complex.

Introduction

It is common knowledge that the toxic action of O_2 on biological systems is mediated by activated O_2 species: superoxide radical (O_2^-), singlet oxygen ($^1O_2$) and hydroxyl radical ($\dot{O}H$) [1]. These O_2 species possessing a much higher reactivity than the molecular oxygen can interact with various compounds, as well as with the molecular components of biological membranes, causing their damage or even destruction [2,3]. However, the damaging action of activated O_2 species is not exclusively due to their different reactivity toward biological molecules or structures, but to the differences in their diffusion capacity as well. For example, O_2^- possessing a weak reactivity and, correspondingly, the longest lifetime (several milliseconds in aqueous media [4] as compared to 2 μs for $^1O_2$ [5] and parts of microseconds for OH [6]) can penetrate deep inside the cell and be transformed there into a highly reactive species, OH, which exerts an appreciable damaging action. Presumably, O_2^- can penetrate through the anionic channels of erythrocyte membranes [7] or across the lipid bilayer of biological membranes [8,9]. The aim of the present work was a quantitative estimation of the O_2^- ability to penetrate across the bilayer lipid membranes as well as an investigation of exogenous factors and membrane modifiers on this process.

Materials and Methods

Egg phosphatidylcholine was prepared by precipitation from a total phospholipid extract of egg yolk as described previously [10]. The purity of the preparation was controlled by thin-layer chromatography; its fatty acid composition was deter-

mined by gas-liquid chromatography [11]. Other reagents used were: sodium deoxycholate (Merck), cytochrome *c* (Sigma), cholesterol (Serva), α-tocopherol (Serva), xanthine (Sigma), valinomycin (Serva) and safranin (Spofa, CSSR). Xanthine oxidase was kindly supplied by Dr. L.S. Vartanyan (Institute of Chemical Physics, U.S.S.R. Academy of Sciences, Moscow); superoxide dismutase was a gift from Dr. R.M. Nalbandyan (Institute of Biochemistry, Armenian U.S.S.R. Academy of Sciences, Yerevan).

The loaded liposomes (approx. 1000 Å in diameter) were prepared as follows. The cytochrome *c* (final concentration $1 \cdot 10^{-4}$ M), the superoxide dismutase (in some experiments, (final concentration is $1 \cdot 10^{-5}$M) and the sodium deoxycholate (final concentration 2-times less than that of phosphatidylcholine) were added to a suspension of small monolamellar liposomes (250–300 Å in diameter) in Tris-acetate buffer (pH 8.5)/100 mM NaCl. A 5–10 min incubation at 25 °C resulted in spontaneous formation of loaded 1000 Å liposomes [12]. To remove excess protein and detergent, the liposomes (30–40 mg lipid) were applied on a Sephadex G-100 column (45 × 2.5 cm).

Small liposomes (250–300 Å) were prepared by sonication of the multilayer liposome suspension in an ultrasonic disintegrator UZDN-2 ($I = 0.5$ A, $f = 22$ kHz) and a subsequent gel filtration of sonicated liposomes (60–80 mg lipid) on a Sepharose 4B column (35 × 2.5 cm) to remove the multilayer liposomes and phosphatidylcholine degradation products. The concentration of phosphatidylcholine was determined by phosphorus content [13].

For liposome modification, the cholesterol (15–20 mol%) or the α-tocopherol (10 mol%) was added to the phosphatidylcholine solution in chloroform. The effect of cholesterol on the state of membranes was studied, using the spin-probe 6-doxylpalmitic acid. The 6-doxylpalmitic acid EPR spectra were recorded on a Varian E-104 spectrometer, using the order parameter, *S*, as a measure of the structure-sensitive parameter of lipid bilayer [14]. In studies with oxidative degradation of α-tocopherol, α-[^{3}H]tocopherol (Amersham) was used [15].

O_2^- was generated in an enzymatic reaction of xanthine ($5 \cdot 10^{-5}$ M) oxidation by xanthine oxidase (approx. $1 \cdot 10^{-8}$ M) and was followed by cytochrome *c* (approx. $1 \cdot 10^{-5}$ M) reduction by measuring the increment in absorbance at 550 nm on a Hitachi 556 spectrophotometer operating in a dual-wave regime (bearing wavelength $\lambda_2 = 600$ nm).

To create the transmembrane electric field on the liposomal membrane, a transmembrane K^+ gradient was used in the presence of valinomycin. The field was controlled with a potential-sensitive dye, safranin, by the decrease in absorbance at 520 nm [17].

Results and Discussion

To study the permeability of lipid bilayer for O_2^-, we employed the method in which the acceptor for O_2^- and the O_2^--generated system were divided by the liposome membrane. In our opinion, the most suitable acceptor for this purpose is ferricytochrome *c*, a highly specific reagent for O_2^- [18], which has a relatively high value of molar extinction coefficient [19] and a high value of the rate constants for the interaction with O_2^- [20]. Besides, this acceptor does not penetrate across the lipid membrane and, at concentrations used, is not adsorbed on the phosphatidylcholine liposome surface [21]. The xanthine-xanthine oxidase system commonly used in many studies [22] served as a source of O_2^-. Monolamellar liposomes of large diameter were selected as a membrane model, which, in contrast to small-sized liposomes (250–300 Å) can be loaded by large protein molecules.

The content of cytochrome *c* in the inner volume of the liposomes was determined spectrophotometrically after addition of Triton X-100 (0.2%, v/v) and sodium ascorbate (approx. $1 \cdot 10^{-4}$ M) to the liposomes, until a complete reduction of the cytochrome was reached. Using the values of the extinction coefficient for cytochrome *c*, E_{550}, of $2.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, the phosphatidylcholine content in the sample of $2.7 \cdot 10^{-3}$ M and the inner volume of the liposomes of 2.4 μl/μmol of lipid [12], we determined the cytochrome *c* concentration in the inner volume of the liposomes ($2 \cdot 10^{-4}$ M).

Penetration of O_2^- across liposomal membranes

Generation of O_2^- in a xanthine-xanthine

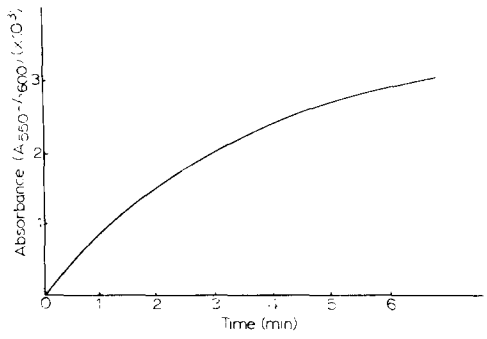

Fig. 1. Kinetics of cytochrome *c* reduction in the liposomes. Incubation medium: 20 mM Tris-acetate buffer (pH 8.5), 100 mM NaCl, $5 \cdot 10^{-5}$ M xanthine, $1 \cdot 10^{-8}$ M xanthine oxidase, $5 \cdot 10^{-8}$ M catalase, cytochrome *c*-loaded liposomes. Phosphatidylcholine concentration = $2.7 \cdot 10^{-3}$ M.

oxidase system in the external volume resulted in the reduction of cytochrome *c* captured into the liposomes (Fig. 1). After completion of the reaction, the potassium ferricyanide was added to the liposomal suspension. The absence of changes in the reduced cytochrome *c* spectrum after addition of ferricyanide is a proof that O_2^- generation in the external volume causes cytochrome *c* reduction in the inner volume of the liposomes and that the cytochrome itself does not penetrate into the solution from the liposomes.

Since neither cytochrome *c* nor xanthine oxidase can penetrate across the liposomal membrane, it may be assumed that cytochrome *c* reduction inside the liposomes is mediated by O_2^-. The cytochrome *c* reduction in the inner liposome volume was completely inhibited by the superoxide dismutase incorporated into the liposomes.

According to the data from gas-liquid chromatography, no marked oxidative destruction of phosphatidylcholine took place under these conditions (data not shown).

Determination of permeability coefficient of bilayer lipid membranes for O_2^-

When calculating the value of the apparent permeability coefficient of bilayer lipid membranes for O_2^- ($P'_{O_2^-}$), it was assumed that: (i) cytochrome *c* reduction in the liposomes is limited by the $P'_{O_2^-}$ value and the rate of O_2^- penetration across the liposomal membrane is equal to that of cytochrome *c* reduction; (ii) O_2^- concentration inside the liposomes (C) is much less than the concentrations of cytochrome *c* inside the liposomes and O_2^- in the external solutions (C_o).

In accordance with the first Fick's law, the value of $P'_{O_2^-}$ can be determined as:

$$P'_{O_2^-} = \frac{V}{S(C_o - C)} = \frac{V}{S \cdot C_o} \quad (1)$$

where V is the experimentally determined initial rate of cytochrome *c* reduction in the liposomes (mol/s), S is the total area of the liposome surface in 1 ml of suspension (cm^2) calculated from the total content of phosphatidylcholine in the liposomes and the area occupied by one phosphatidylcholine molecule in the membrane [23].

In the absence of the O_2^- acceptor (cytochrome) outside the liposomes, the main pathway of O_2^- destruction is spontaneous dismutation. Hence:

$$\frac{dC_o}{dt} = W_g - W_d$$

where W_g and W_d are the rates of generation and spontaneous dismutation of O_2^- (mol/s), respectively.

Under steady-state conditions (i.e., at the initial steps of the reaction):

$$\frac{dC_o}{dt} = 0, \text{ and } W_g = W_d$$

The rate of O_2^- generation in a xanthine-xanthine oxidase system (W_g) was determined in parallel experiments with excess cytochrome *c* ($2 \cdot 10^{-5}$ M) in liposome-free media. In this case, W_g was coincident with the rate of cytochrome *c* reduction which was constant within the first 3–5 min.

Spontaneous dismutation, W_d, can be determined from:

$$W_d = K_{app}([HO_2^\cdot] + [O_2^-])^2$$

where $K_{app} \simeq 1 \cdot 10^{-4}\ M^{-1} \cdot s^{-1}$ (pH 8.5) [20]. The pH-dependent ratio of $[HO_2^\cdot]$ and $[O_2^-]$ was determined from the equation for $HO_2^\cdot$ dissociation:

$$HO_2^\cdot \rightleftarrows H^+ + O_2^-$$

The equilibrium constant for this reaction, $K_{HO_2^\cdot}$,

is equal to $2.05 \cdot 10^5$ M [20], hence, the concentration of HO_2^- in comparison with O_2^- at pH 8.5 is negligible. Consequently:

$$C_o = \left(\frac{W_d}{K_{app}}\right)^{1/2} \quad (2)$$

By substituting the numeric values of $[O_2^-]$ (C_o) determined from Eqn. 2 into eqn. 1, we can calculate the value of $P'_{O_2^-}$. Thus, if $W_g = 1.44 \cdot 10^{-8}$ $M^{-1} \cdot s^{-1}$, the $C_o = 1.2 \cdot 10^{-6}$ M. If $V = 5.16 \cdot 10^{-10}$ $M^{-1} \cdot s^{-1}$, and phosphatidylcholine concentration in the liposomes is $2.7 \cdot 10^{-3}$ M, the $P'_{O_2^-}$ value can be calculated after determination of the liposome surface area in 1 ml of suspension as:

$$P'_{O_2^-} = \frac{5.16 \cdot 10^{-13}(\text{mol/s})}{1.2 \cdot 10^{-9}(\text{mol/cm}^3) \cdot 5.7 \cdot 10^3(\text{cm}^2)}$$

$$= 7.6 \cdot 10^{-8}\ \text{cm/s}$$

The $P'_{O_2^-}$ value is equal to $(7.6 \pm 0.3) \cdot 10^{-8}$ cm/s (the mean $\pm$S.E. of seven experiments), which agrees well with the values of permeability coefficient of bilayer lipid membranes for other anions found from isotope experiments [24].

Apparent activation energy of O_2^- penetration across lipid membrane

In order to determine the value of the apparent activation energy, E'_{act}, for the O_2^- transfer across the liposomal membrane, we studied the temperature dependence of $P'_{O_2^-}$ at 23–39 °C. In parallel experiments, the external concentration of O_2^- was determined from Eqn. 2 at each temperature studied (Table I). In this experiment, the concentration of xanthine oxidase was adjusted so as to achieve the linearity of cytochrome *c* reduction at the initial steps of the reaction at highest temperature values. The E'_{act} value was determined from the Arrhenius plots, using the equation $\ln P'_{O_2^-} = \ln A - E'_{act}/RT$. The straight lines were plotted by the least-squares technique. The E'_{act} value was equal to 11.5 kcal/mol (correlation index, r, = -0.98), which is also consistent with the values of effective activation energies for the transfer of other anions across the lipid bilayer [25].

Effect of membrane modification on $P'_{O_2^-}$

Since the changes in the physical properties of the lipid bilayer may affect the parameters of ion transfer across the membrane, we investigated the effects of some membrane modifiers on the rate of O_2^- penetration across the liposomal membrane. When cholesterol was used as a modifier, the $P'_{O_2^-}$ value was decreased from $7.5 \cdot 10^{-8}$ to $3.5 \cdot 10^{-8}$ cm/s. This was correlated with the decrease in the mobility of membrane lipids, because according to the EPR spectroscopy data, the values of the order parameter and activation energy for the rotation of the spin-probe 6-doxylpalmitic acid incorporated into the liposomal membrane, increased from 0.350 and 1.3 kcal/mol up to 0.363 and 1.9 kcal/mol, respectively.

Modification of liposomes with α-tocopherol also caused a decrease in the membrane permeability for O_2^- (from $7.1 \cdot 10^{-8}$ to $2.5 \cdot 10^{-8}$ cm/s); this effect was not due to the chemical interaction of O_2^- with α-tocopherol, for after the exposure of liposomes containing α-[^{3}H]tocopherol to super-

TABLE I

TEMPERATURE DEPENDENCE OF THE PERMEABILITY COEFFICIENT ($P'_{O_2^-}$) OF THE LIPID BILAYER FOR O_2^-

Temperature (°C)	Rate of O_2^- generation (W_g) [a]		O_2^- concentration outside liposomes (C_o) ($mol \cdot l^{-1}$)	Initial rate of cytochrome *c* reduction in liposomes (V)		$P'_{O_2^-}$ (cm/s)
	D_{550} (min^{-1})	C ($mol \cdot l^{-1} \cdot s^{-1}$)		D_{550} (min^{-1})	C ($mol \cdot l^{-1} \cdot s^{-1}$)	
23	$1.8 \cdot 10^{-2}$	$1.4 \cdot 10^{-8}$	$1.2 \cdot 10^{-6}$	$6.5 \cdot 10^{-4}$	$5.2 \cdot 10^{-10}$	$7.6 \cdot 10^{-8}$
28	$2.5 \cdot 10^{-2}$	$2.0 \cdot 10^{-8}$	$1.4 \cdot 10^{-6}$	$9.6 \cdot 10^{-4}$	$7.6 \cdot 10^{-10}$	$9.6 \cdot 10^{-8}$
32	$2.9 \cdot 10^{-2}$	$2.3 \cdot 10^{-8}$	$1.5 \cdot 10^{-6}$	$1.6 \cdot 10^{-3}$	$1.3 \cdot 10^{-9}$	$1.5 \cdot 10^{-7}$
37	$3.7 \cdot 10^{-2}$	$3.0 \cdot 10^{-8}$	$1.7 \cdot 10^{-6}$	$2.4 \cdot 10^{-3}$	$1.9 \cdot 10^{-9}$	$1.9 \cdot 10^{-7}$
39	$4.2 \cdot 10^{-2}$	$3.3 \cdot 10^{-8}$	$1.8 \cdot 10^{-6}$	$2.6 \cdot 10^{-3}$	$2.1 \cdot 10^{-9}$	$2.0 \cdot 10^{-7}$

[a] Measured in parallel experiments with cytochrome *c* reduction in a liposome-free medium.

oxide radicals, no further accumulation of the α-tocopherol degradation products was observed (data not shown). This suggests that in the tested system, lipid peroxidation occurs at a relatively low level. Thus, the decrease of $P'_{O_2^-}$ during modification of liposomes by α-tocopherol may result from the changes in the fluidity of the lipid bilayer [26].

We also checked the effect of Ca^{2+} on the permeability of liposomes for O_2^-. An addition of 50–200 μM Ca^{2+} to the external solution resulted in a decrease in the $P'_{O_2^-}$ value from $7.1 \cdot 10^{-8}$ to $3.8 \cdot 10^{-8}$ cm/s; this effect was independent of Ca^{2+} concentration. In control experiments, it was found that, at the concentrations used, Ca^{2+} did not affect the rate of O_2^- generation by xanthine oxidase or cytochrome *c* reduction in the presence of liposomes that were not loaded with cytochrome *c*.

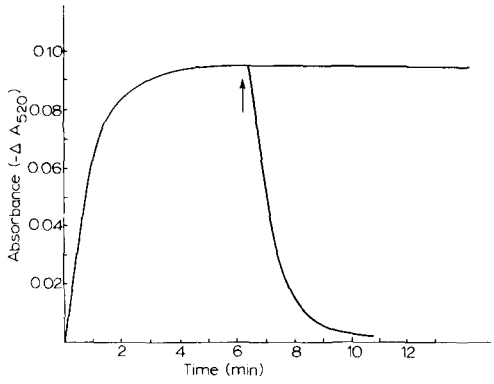

Fig. 2. Kinetics of absorbance response of safranin after addition of valinomycin to the liposome suspension. Incubation medium: 20 mM Tris-HCl buffer (pH 8.5), 33 μM safranin, 4 mM KCl, 96 mM choline chloride, liposomes (40 μm phosphatidylcholine), $1 \cdot 10^{-8}$ M valinomycin. The inner liposomal solution: 20 mM Tris-HCl buffer (pH 8.5), 100 mM KCl. The arrow designates an addition of carbonylcyanide *m*-chlorophenylhydrazone ($4.8 \cdot 10^{-7}$ M).

Effect of transmembrane potential on permeability of liposomes for O_2^-

In order to create the transmembrane potential, the liposomes were prepared in a medium containing 20 mM Tris-HCl (pH 8.5) and 100 mM KCl. Aliquots of samples were diluted with 20 mM Tris-HCl containing 100 mM NaCl or 100 mM choline chloride, so that the external K^+ concentration was 5–10 mM and a 20–10-fold K^+ gradient was formed in the absence of the osmotic gradient outside and inside the liposomes; finally, valinomycin at a concentration of $1 \cdot 10^{-8}$ M was added to the liposomes. To create an oppositely charged field, the liposomes were prepared in a medium containing 20 mM Tris-HCl, 5–10 mM KCl and 95–90 mM NaCl. A small aliquot of the liposome suspension was diluted with a buffer containing 100 mM KCl, until a 20–10-fold K^+ gradient was formed; then valinomycin was added.

The generation of $\Delta\psi$ was controlled by a potential-sensitive dye, safranin [17]. The liposomes prepared in a medium with 100 mM KCl were diluted with 20 mM Tris-HCl containing 33 μM safranin and 100 mM NaCl or 100 mM choline chloride. After addition of valinomycin, safranin absorption was reduced, showing a plateau after 2–3 min (Fig. 2). In the absence of proton uncouplers, the membrane potential remained unchanged for 15–20 min. An addition of dinitrophenol (0.8 mM) or carbonylcyanide *m*-chlorophenylhydrazone ($4.8 \cdot 10^{-7}$ M) caused a rapid fall of the potential value and an increase of absorption at 520 nm.

The experimental results illustrating the effects of the transmembrane potential on the liposome permeability for O_2^- are shown in Table II. These data suggest that the generation of $\Delta\psi$ through the liposomal membrane irrespective of its polarity does not influence the $P'_{O_2^-}$ value. It may thus be assumed that the superoxide radical penetrates across the lipid membrane in an uncharged form.

It is known that there are other ions that also can penetrate nonmodified lipid bilayers in an uncharged form (this is evident from a comparison of results available from isotope and electric experiments). The origin of neutral forms of ions is essentially not clear yet. One may postulate that the superoxide radical penetrates the lipid bilayer in a protonated form, HO_2. On this assumption, we estimated, in a similar manner, the apparent permeability coefficient of the liposomal membrane for HO_2 (P'_{HO_2}). The concentration of HO_2 in the external solution (C'_o) was estimated from the pH used, the HO_2 constant dissociation, and concentration of O_2^- the estimate of which was

TABLE II

EFFECTS OF THE TRANSMEMBRANE ELECTRIC POTENTIAL ON THE PERMEABILITY OF LIPID BILAYER FOR O_2^-

Concentrations				Rate of O_2^- generation (W_g) ($mol \cdot l^{-1} \cdot s^{-1}$)	O_2^- concentration outside liposomes (C_0) ($mol \cdot l^{-1}$)	Area of liposomal surface (S) (cm^2)	Initial rate of cyt.*c* reduction in liposomes (V) ($mol \cdot l^{-1} \cdot s^{-1}$)	$P'_{O_2^-}$ (cm/s)
Phosphatidylcholine (M)	Valinomycin (M)	K^+ (M)						
		Inside liposomes	Outside liposomes					
$2.8 \cdot 10^{-3}$	0	0	0	$1.8 \cdot 10^{-8}$	$1.3 \cdot 10^{-6}$	$5.9 \cdot 10^{6}$	$6.2 \cdot 10^{-10}$	$7.8 \cdot 10^{-8}$
$2.8 \cdot 10^{-3}$	$1 \cdot 10^{-8}$	0	0	$1.8 \cdot 10^{-8}$	$1.3 \cdot 10^{-6}$	$5.9 \cdot 10^{6}$	$6.0 \cdot 10^{-10}$	$7.5 \cdot 10^{-8}$
$3.0 \cdot 10^{-3}$	0	0.1	0.005	$1.9 \cdot 10^{-8}$	$1.3 \cdot 10^{-6}$	$6.2 \cdot 10^{6}$	$6.2 \cdot 10^{-10}$	$7.4 \cdot 10^{-8}$
$3.0 \cdot 10^{-3}$	$1 \cdot 10^{-8}$	0.1	0.005	$1.9 \cdot 10^{-8}$	$1.3 \cdot 10^{-6}$	$6.2 \cdot 10^{-6}$	$6.5 \cdot 10^{-10}$	$7.6 \cdot 10^{-8}$
$2.7 \cdot 10^{-3}$	0	0.005	0.1	$1.6 \cdot 10^{-8}$	$1.2 \cdot 10^{-6}$	$5.7 \cdot 10^{6}$	$5.5 \cdot 10^{-10}$	$7.8 \cdot 10^{-8}$
$2.7 \cdot 10^{-3}$	$1 \cdot 10^{-8}$	0.005	0.1	$1.6 \cdot 10^{-8}$	$1.2 \cdot 10^{-6}$	$5.7 \cdot 10^{6}$	$5.2 \cdot 10^{-10}$	$7.4 \cdot 10^{-8}$

[a] Measured in parallel experiments with cytochrome *c* reduction in a liposome-free medium.

given above. Hence:

$$P'_{HO_2} = \frac{V}{S \cdot C'_o}$$

The P'_{HO_2} value thus obtained is equal to $4.9 \cdot 10^{-4}$ cm/s, which is close to the known values of the apparent permeability coefficient of the lipid bilayer for H_2O and some other non-electrolytes [24].

Effect of amphotericin B on permeability of liposomes for O_2^-

According to the current viewpoint, the superoxide radical can penetrate across biological membranes (erythrocytes, granulocytes, etc.) via anionic channels [7,27]. We investigated the possibility of O_2^- penetration via predominantly anionic channels formed in the lipid membrane by means of a cholesterol-amphotericin B complex. To the liposomes modified by cholesterol (15.7 mol%), amphotericin B ($6 \cdot 10^{-5}$ M) was added, and the reaction of O_2^- generation was initiated 5–10 min thereafter. The effect of the amphotericin B concentration mentioned above on cellular and liposomal membranes cause the formation of channels with predominantly anionic conductance [25,28]. Our experimental results indicate that amphotericin B increases the permeability of cholesterol-modified liposomes for O_2^- at $P'_{O_2^-}$ from $3.6 \cdot 10^{-8}$ to $1.1 \cdot 10^{-7}$ cm/s, whereas no significant changes in $P'_{O_2^-}$ for the unmodified liposomes ($7.7 \cdot 10^{-8}$ and $8.0 \cdot 10^{-8}$ cm/s) were observed in the presence of amphotericin B. The latter compound at indicated concentrations did not influence the rate of O_2^- generation by xanthine oxidase or that of cytochrome *c* reduction by superoxide radicals.

Conclusions

The data obtained suggest that in some parameters under study (apparent permeability coefficient, apparent activation energy, effect of modifiers, mode of penetration across the membrane), the O_2^- transfer across the liposomal membranes has much in common with that of simple anions. A comparison of the experimentally obtained permeability coefficient of lipid membrane for superoxide radicals and the relatively low experimental concentrations of superoxide radicals in aqueous media allows to conclude that superoxide radical concentration in the hydrocarbon zone of the membrane is low. It might be expected that physiologically significant membrane and transmembrane events occurring under the influence of the superoxide radicals are more conspicuous in acidic media in the sites and states coupled with the enhanced generation of superoxide radicals.

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