# Central Asian Cobra Venom Cytotoxins-induced Aggregation, Permeability and Fusion of Liposomes

T. F. ARIPOV, S. E. GASANOV, B. A. SALAKHUTDINOV, I. A. ROZENSHTEIN and F. G. KAMAEV

*Institute of Bioorganic Chemistry, Uzbek Academy of Sciences, Gorki prosp. 83, 700143 Tashkent, USSR*

**Abstract**. The processes of membrane aggregation, permeability and fusion induced by cytotoxins from Central Asian cobra venom were investigated by studying optical density of liposome samples, permeability of liposome membranes for ferricyanide anions and exchange of lipid material between the membranes of adjacent liposomes. Cytotoxins $V_c5$ and $V_c1$ were found to induce aggregation of PC + CL and PC + PS liposomes. Cytotoxin $V_c5$ increased also the permeability of the liposomes for $K_3[Fe(CN)_6]$ and enhanced their fusion. Cytotoxin $V_c1$ increased membrane permeability and enhanced fusion of PC + CL samples only. The changes in membrane permeability and fusion were found to occur within a single value of cytotoxin concentrations. The fusogenic properties of the cytotoxins studied are supposed to be due to the ability to dehydrate membrane surface and to destabilize the lipid bilayer structure. Fusion probability is largely defined by the phospholipid composition of the membranes. A model of interaction of cytotoxins with cardiolipin-containing membranes is offered.

**Key words**: Cytotoxin — Phospholipid liposomes — Aggregation — Permeability — Fusion

## Introduction

The fusion of cells and cellular organelles is a necessary stage of numerous biological processes (Elsbach et al. 1969) and it involves aggregation of membranes and aqueous bulks restricted by the membranes (Chernomordik et al. 1986). A usual approach to study this phenomenon are model lipid bilayer systems (Düzgünes 1985). A number of experimental (Houg and Vacquier 1986; Papahadjopoulos et al. 1978) as well as theoretical (Chernomordik et al. 1986; Markin and Kozlov 1984) papers dealing with these processes have been published so far. The conditions required for the membrane to fuse have been

reported to concern both the properties of the membranes themselves and external environmental trigger effect (Markin and Kozlov 1984). Membrane fusion is accompanied by aggregation of vesicles, by leakage of the vesicle contents into the external solution and, in some cases, by heat release (Wilshut et al. 1980; Portis et al. 1979).

The aim of the present study was to obtain more information concerning the nature of the fusion process by studying processes accompanying membrane fusion: membrane aggregation (Markin and Kozlov 1984) and changes in permeability (Shragin et al. 1986), exchange of lipid material between the membranes of neighbooring liposomes (Papahadjopoulos et al. 1979). Cytotoxins $V_c5$ and $V_c1$ of Central Asian cobra venom were used as the trigger agents. The cytotoxins are polycationic amphiphilic polypeptides (m.w. about 7000 D, pI 11 and pI 9.5 for $V_c5$ and $V_c1$, respectively) (Grishin et al. 1974; Yukelson et al. 1974). The cytotoxins used have all the properties typical of fusogenic proteins (Markin and Kozlov 1984): they strongly change the bilayer order of phospholipids (Aripov et al. 1986; Gasonov et al. 1988), neutralize the surface charge of bilayers (Salakhutdinov and Aripov 1981) and dehydrate the polar area of membranes (Gasanov and Gasanov 1988). As models lipid membranes sonicated phosphatidylcholine liposomes containing acid lipids (cardiolipin or phosphatidylserine) were used.

## Materials and Methods

*Substances used*: phosphatidylcholine (PC) and cardiolipin (CL) (Kharkov Factory, USSR), phosphatidylserine (PS) from bovine brain, dipalmitoylphosphatidylcholine (DPPC) and dimiristoylphosphatidylcholine (DMPC) (Sigma, USA). The phospholipids were purified on silica columns. Liposomes were prepared by 10-min sonication of lipid suspensions in 10 mmol/l Tris-HCl buffer (pH 7.5), 1 mmol/l EDTA at 2–4 °C in helium media in an ultrasonic disperser USDN-1 (USSR) at a frequency of 22 kHz during 20 min, followed by centrifugation at 200 × *g* for 60 min; the liposome samples were incubated in helium atmosphere during 20 hours at 10 °C (natural lipids) or at 45 °C (synthetic lipids). Liposome samples for calorimetric measurements were prepared by mixing synthetic lipid and natural acid lipids (either PS or CL). Liposome samples for $^1$H-NMR analysis were prepared with $D_2O$. Cytotoxins (CT) $V_c5$ and $V_c1$ were extracted from the Central Asian cobra (*Naja naja oxiana*) venom according to Grishin et al. (1974). Erythrosine, pyrene and perylene (Fluka, FRG) were used as luminescent probes. Ferrocene (Sigma, USA), a hydrophobic probe, was used as the phosphorescence quencher. The probes were incubated with liposomes during 24 hours at 4 °C at $5 \times 10^{-6}$ mol/l (erythrosine), $10^{-5}$ mol/l (ferrocene), $5 \times 10^{-6}$ mol/l (pyrene) and $5 \times 10^{-8}$ mol/l (perylene). To remove free probe the liposome samples were chromatographied on Sephadex G 50 columns (1.8 × 75 cm). Following chromatography the concentration of lipids was assessed spectrophotometrically. From liposome samples for luminiscent analyses, oxygen was enzymatically removed by glucoseoxidase (Mekler et al. 1982). The concentrations of the luminiscent probes, glucoseoxidase and glucose used in the liposome samples did not change EPR spectra of doxylstearic acid and $^1$H-NMR spectra of liposomes during 24 hours. The detection of erythrosine phosphorescence quenching by ferrocene and annihilation of retarded perylene

fluorescence sensibilized by pyrene were detected in a pulsed laser equipment according to Kotelnikov et al. (1979). The phosphorescence emission was detected through a light filter passing light at $\lambda > 700$ nm, and that of the sensibilized annihilation retarded fluorescence through a light filter passing light at $\lambda > 420$ nm. The excited state lifetime of probes was estimated from the dependence of attenuation of the probe glow on time in semilogarithmic coordinates. EPR spectra were recorded with a Varian E-4 spectrometer (USA) at modulation amplitudes not exceeding $2 \times 10^{-4}$ T and resonator input power exceeding 20 mW. $^1$H-NMR spectra were recorded with a Varian XL-200 spectrometer (USA) with an operating frequency of 200 MHz. The width of the 90° pulse was 8.7 $\mu$s, the acquisition time for free induction signal was 1 s. The optical density was continuously monitored at $\lambda = 500$ nm using a SF-26 spectrophotometer (USSR). Calorimetric measurements were performed at a recording rate of 1 °C per minute using a differential scanning microcalorimeter DASM-4 (USSR).

## Results and Discussion

### 1. *Cytotoxin-induced turbidity of liposome samples*

The turbidity of sonicated vesicle solution induced by cations (Lansman and Haynes 1979; 1975) or proteins (Düzgünes 1985) has been accounted for by membrane aggregation. We believe that CT-induced turbidity of liposome samples is due to the aggregation of the liposomes. The degree of turbidity (aggregation) was estimated by measuring the optical density of samples. Fig. 1*a* shows changes in optical density of PC + PS liposome solution with time at different concentrations of $V_c5$ and $V_c1$. The initial $V_c5$ concentration slightly increased the optical density (to 0.04) which remained then stable for a long period of time. The first addition of $V_c1$ did not really affect the optical properties of the liposome solution. The subsequent additions of CT induced a pronounced aggregation, with the increasing concentration further enhancing aggregation. $V_c5$ showed a stronger capacity to induce aggregation of PC + PS liposomes than did CT $V_c1$.

The change in optical properties of PC + CL liposome solution induced by cytotoxins is of a different nature than that induced in PC + PS liposomes (Fig. 1*b*). The introduction of $V_c5$ into a PC + CL liposome sample enhanced membrane aggregation followed by an exponential decrease in the optical density level of the solution. The drop of optical density may be connected with membrane fusion, the process of liposome desaggregation or with precipitation of liposome aggregates. The latter possibility was tested, and the shaking of the liposome sample after clearing did not result in a rise in the optical density. For $V_c1$ there were almost no changes in turbidity over time. This can be explained in two ways: either $V_c1$ does not induce liposome aggregation or the aggregation rate correlates with the membrane fusion rate, resulting in effectively no change in optical properties of the sample.

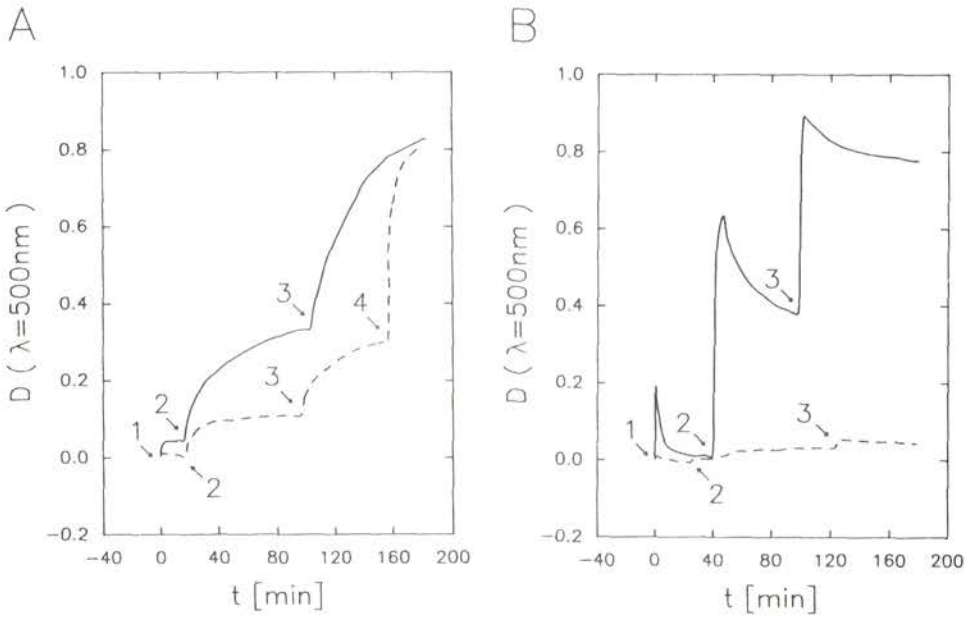


**Fig. 1.** Changes in optical density induced by cytotoxins $V_c5$ (solid line) and CT $V_c1$ (dashed line) in liposome samples: *A* — PC + 10 mol% PS liposomes, *B* — PC + 10 mol% CL liposomes. CT — lipid molar ratios 0.005(1); 0.01(2); 0.015(3); 0.02(4).

## 2. *Liposome fusion studied with luminescent probes*

Two approaches were adopted to study the CT-induced process of membrane lipid material exchange using luminescent probes: estimation of desactivation of erythrosine triplet excited state by a hydrophobic probe (ferrocene) and measurement of annihilation retarded fluorescence of perylene induced by triplet-triplet transfer of excitation energy from pyrene to perylene.

### *a) Erythrosine phosphorescence quenching by ferrocene*

Mixing two liposome samples, one containing erythrosine and the other one ferrocene, resulted in no changes of erythrosine phosphorescence lifetime. This suggested that there is no lipid or probe exchange between liposomes or that these processes are very slow. The addition of $V_c5$ to PC + PS or PC + CL liposomes, or of $V_c1$ to PC + CL samples resulted in phosphorescence quenching suggesting effective probe exchange. When $V_c1$ was added to PC + PS liposomes, no phosphorescence quenching occurred. To assess the quenching

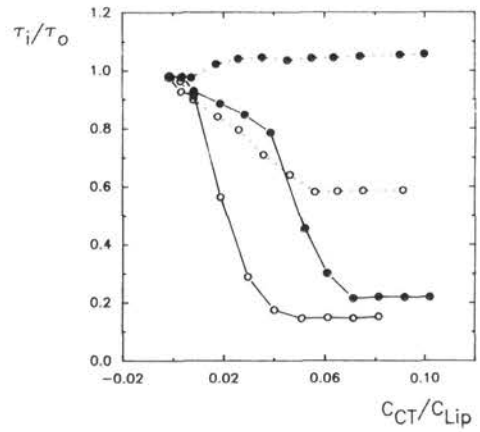


**Fig. 2.** Dependence of erythrosine phosphorescence quenching by ferrocene on relative concentrations of $V_c5$ (empty symbols) and $V_c1$ (filled symbols). $\tau_o$ — lifetime of erythrosine phosphorescence in liposome samples without CT. $\tau_i$ — lifetime of erythrosine phosphorescence with defined CT concentrations. The solid line shows results for PC + 10 mol% CL liposomes, the dashed line those for PC + 10 mol% PS liposomes.

efficiency at different CT concentrations in liposome samples relative lifetime changes or erythrosine phosphorescence — $\tau_i/\tau_o$ were calculated ($\tau_i$ is the lifetime of erythrosine phosphorescence in the presence of a given CT concentration, $\tau_o$ is erythrosine phosphorescence lifetime in the sample without CT). In cardiolipin-containing liposomes, $V_c5$ induced quenching at lower concentrations than did $V_c1$ (Fig. 2). For both PC + PS and PC + CL liposomes the quenching was almost identical at initial concentrations of $V_c1$. However, the phosphorescence lifetime in cardiolipin-containing liposomes dropped sharply as soon as $C_{CT}/C_{lip}$ exceeded 0.04, whereas it reached a plateau in PC + PS liposomes. The lower $\tau_i/\tau_o$ values for PC + CL samples in the presence of $V_c1$ and $V_c5$ as compared with those for PC + PS samples in the presence of $V_c5$, can be explained by a more effective diffusion of probes in the cytotoxin modified lecithin-cardiolipin membrane.

With a more rigorous approach, erythrosine phosphorescence quenching by ferrocene may be explained not only by liposome fusion. Erythrosine in membranes is known to be localized in the area of polar lipid heads (Mekler et al. 1982), and the close contact under conditions of aggregation of neighbouring liposome bilayers may provide the interaction of erythrosine with ferrocene. To investigate in detail the conditions of erythrosine phosphorescence quenching by ferrocene an experiment was performed to study the energy transfer of triplet

excitation from pyrene to perylene, known to be localized deep in the membrane hydrophobic region (Wallach et al. 1974; Podo and Blasie 1977).

*b) Triplet-triplet energy transfer from pyrene to perylene*

The quantum emission of annihilation retarded fluorescence (ARF) occurs at the interaction of excited triplet molecules: one molecule passes to the basic state while the other to the excited singlet state (Parker 1972). The quantum emissions of ARF are known both for pyrene and perylene (Mekler et al. 1983). However at certain chromophore concentrations and with insufficient power of excitation laser no ARF of pyrene and perylene may occur. We established the necessary conditions for detection and defined the chromophore concentrations in liposome solutions required for no ARF for pyrene or perylene to occur. When both liposome systems were sonicated in the presence of pyrene or perylene, glow of a sufficient intensity was observed with a lifetime of $4.5 \times 10^{-5}$ s. The processes of the given fluorescence may be described as follows:

1) $\mathrm{pyr} + h\nu \rightarrow \mathrm{pyr}^3$
2) $\mathrm{pyr}^3 + \mathrm{per} \rightarrow \mathrm{pyr} + \mathrm{per}^3$
3) $\mathrm{per}^3 + \mathrm{per}^3 \rightarrow \mathrm{per}^1 + \mathrm{per}$
4) $\mathrm{per}^1 \rightarrow \mathrm{per} + h\nu$

Pyrene has a somewhat higher triplet level than does perylene (Parker 1972). This allows an efficient occupation of the perylene triplet level by triplet-triplet energy transfer $\mathrm{pyr}^3 + \mathrm{per} \rightarrow \mathrm{pyr} + \mathrm{per}^3$; i.e., pyrene acts as a sensibilizer of perylene ARF. For the first time such a sensibilized retarded fluorescence in a solution was observed in phenanthrene (donor) + anthracene (acceptor) system (Parker and Hatchard 1962), and the theory thereof was developed by Parker (1972). This approach was used to study the cytotoxin-induced process of lipid exchange for different membranes. The quantum emission of ARF in mixture of two liposome samples containing different chromophores was found to occur in PC + CL vesicles at values of $C_{CT}/C_{lip} > 0.01$ for $V_c5$; the corresponding value for $V_c1$ was $> 0.0175$, and that for PC + PS vesicles and $V_c5 > 0.015$. No fluorescence was in PC + PS vesicles $C_{CT}/C_{lip}$ between 0 and 0.1 for $V_c1$.

## 3. *Calorimetric study of liposome fusion*

The differential scanning microcalorimetry was used as an additional method for the study of lipid exchange processes between membranes. The capacities of the method in studies of liposome fusion processes have been discussed by Papahadjopoulos et al. (1977).

The phase transition temperature is known to depend on the hydrocarbon chain length, polar head structures and charge (Ivkov and Berestovsky 1981) and may provide information about the character of bilayers composed of a

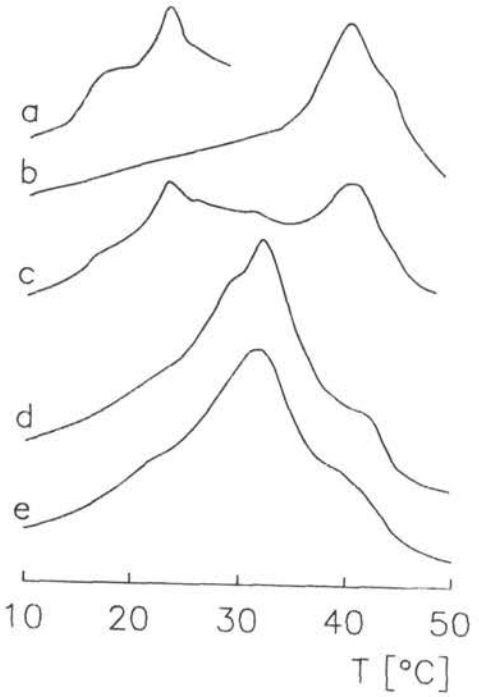


**Fig. 3.** Calorimetric curves for liposome samples: *a* — DMPC + 2.5 mol% CL liposomes; *b* — DPPC + 2.5 mol% CL liposomes; *c* — a mixture of two liposome populations; *d* — a mixture of two liposome populations containing $V_c1$; *e* — a mixture of liposome populations containing $V_c5$.

single phospholipid type. For a mixture of two phospholipids the calorimetric curve would show a peak of heat absorbance corresponding to the phase transition of the two-component system (Ivkov and Berestovsky 1981). Hence the transition temperature parallels a portion of each component in the mixture (Ivkov and Berestovsky 1981).

In the present work DMPC and DPC, with a difference in transition temperatures of 18 °C, were chosen. These phospholipids are also known as ideally mixing systems both in solid and liquid phase (Ivkov and Berestovsky 1981). The calorimetric curve of a mixture of separately sonicated DMPC and DPPC showed two transitions at 23 °C and 41 °C which corresponded to phase transition of DMPC and DPPC respectively. When the two lipids were sonicated together, one peak of heat absorbance appeared, and at an equimolar concentration the temperature was equal to half the sum of DMPC and DPPC phase transition temperatures. The lipid systems used for the study of interactions between membranes and CT included negatively charged phospholipids (5 mol% PS and 2.5 mol% CL). The comparatively lower proportion of CL

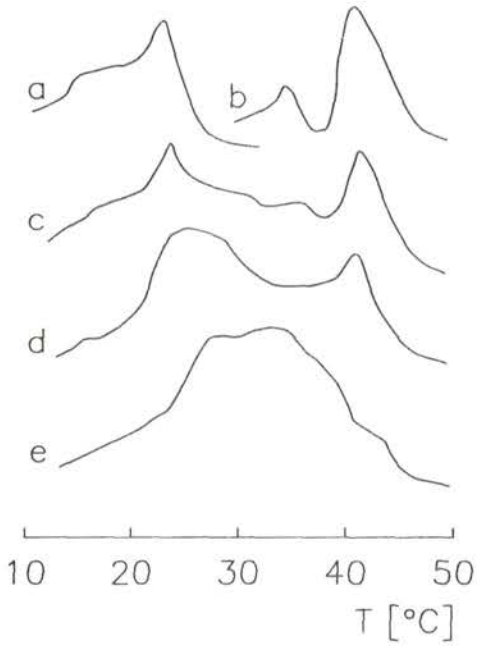


**Fig. 4.** Calorimetric curves for liposome samples: *a* — DMPC + 5 mol% PS; *b* — DPPC + + 5 mol% PS; *c* — a mixture of two liposome populations; *d* — a mixture of two liposome populations containing $V_c1$; *e* — a mixture of two liposome populations containing $V_c5$.

used was due to the fact that CL appreciably disturbs the ordered packing of hydrocarbon chains, this notably reducing the degree of transition cooperativity. It is responsible for considerable broadening of calorimetric curves and makes difficult (or even impossible) interpretation of experimental data. In our experiments, the concentrations of CL and PS used only insignificantly broadened and shifted phase transition peaks. The calorimetric curves for DMPC + CL and DPPC + CL liposomes each have two peaks of basic phase transitions (Fig. 3*c*) corresponding to phase transitions of two liposome populations. The calorimetric curves of liposome mixtures containing $V_c5$ (Fig. 3*d*) and $V_c1$ (Fig. 3*e*) show one phase transition peak. According to Papahadjopoulos et al. (1979) this should be taken as evidence for complete mixing of different system liposome lipids and considered as reflecting fusion of two liposome populations. The addition of $V_c1$ to a sample of two liposome populations prepared of DMPC + PS and DPPC + PS (Fig. 4*d*) resulted in a broadening and a shift by 0.7 °C of the lower phase transition temperature peak towards higher temperatures, and a shift by 0.9 °C of the higher transition temperature peak towards lower temperatures; most likely, this suggests some mixing of

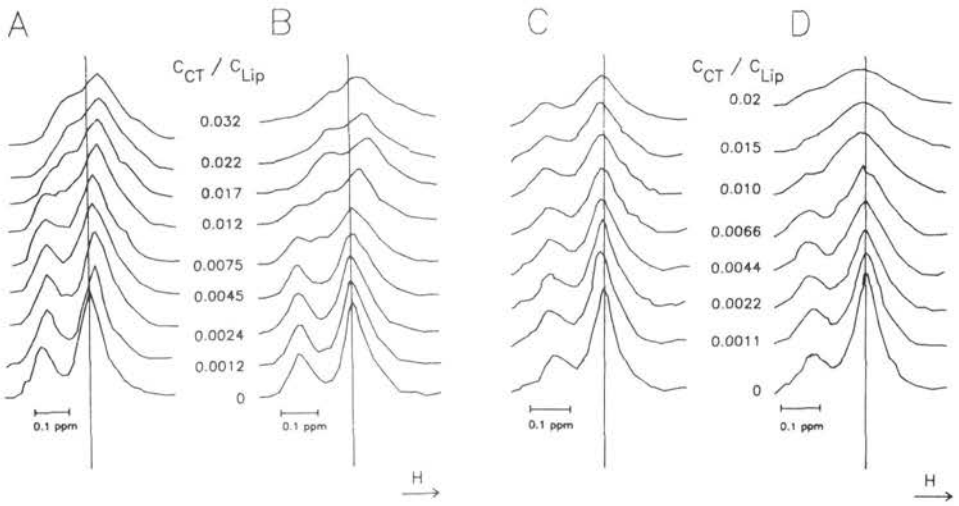


**Fig. 5.** $^1$H-NMR signals of trimethylammonium groups of PC liposomes interacting with increasing CT concentrations in the presence of potassium ferrycianide. *A* — $V_c1$ and *B* — $V_c5$ both in PC + + 10 mol% CL; *C* — $V_c1$ and *D* — $V_c5$ both in PC + 10 mol% PS.

lipids of the two liposome populations. However the degree of the association is insufficient for fusion as indicated by the calorimetric peak of two liposome system preserved at least during 24 hours. The addition of $V_c5$ to a mixture of two liposome populations DMPC + PS and DPPC + PS induced considerable changes of the calorimetric curves. The melting curve of the sample represents three superposed peaks (Fig. 4*e*), two of which correspond to the melting of two initial liposome populations, with the third one (an intermediate peak) corresponding to melting of fused liposomes. This fact is rather unequivocally indicative of an effective lipid material exchange between membranes.

## 4. *$^1$H-NMR studies of cytotoxin-lipid interactions*

$^1$H-NMR spectroscopy allows detailed investigations of packing and movement of polar groups and hydrocarbon chains in a "liquid" bilayer (Ivkov and Berestovsky 1982). As for sonicated vesicles, paramagnetic metal ions used as shift reagents considerably extend the capacity of the method (Bergelson 1976). Paramagnetic ions allow to separate NMR signals of polar lipid groups on outer and inner membrane surfaces. It makes possible the observation of the state of two monolayers and the examination of the membrane structure stability (Ivkov and Berestovsky 1982). This approach was used to study the annealing process of small lecithin vesicles (Lawaczech et al. 1975) as well as the process of fusion

of membranes induced by sonication at temperatures below the phase transition (Lawaczech et al. 1976).

In the present work ferricyanide anion $[Fe(CN)_6]^{3-}$ was used as the shift reagent: its addition to samples of sonicated vesicles resulted in a shift of the signal of trimethylammonium groups of the outer lecithin monolayer towards higher values. It is a characteristic of the ferricyanide anion that within a defined concentration range it does not induce membrane fusion (Ivkov and Berestovsky 1982) and allows to study bilayer permeability for paramagnetic ions under conditions of cytotoxin induced liposome samples broadened the signal, while relative $V_c5$ concentrations equal to or exceeding 0.01 shifted signals of the inner monolayer $N^+(CH_3)_3$ groups from the weak field towards the resonance domain of the trimethylammonium groups of the outer monolayer (Fig. 5*D*). The broadening of $^1$H-NMR signals may be explained by membrane aggregation of fusion (Smith et al. 1981) and/or by limited mobility of the phospholipid molecules (Hauser and Phillips 1973). The shift of the inner monolayer $N^+(CH_3)_3$ group signal towards the stronger field suggests that the membrane is permeable for ferricyanide ions. The ratio of intensities of inner ($I_i$) and outer ($I_o$) monolayer $N^+(CH_3)_3$ signals was used to assess the rate of ferricyanide penetration into the internal volume of liposomes (Fig. 6). Upon increasing the concentration of $V_c5$ up to a value of $C_{CT}/C_{lip}$ of 0.03 the ratio $I_i/I_o$ also increased. Further increasing $V_c5$ concentration was associated with a drop of the $I_i/I_o$ ratio. The increase of $I_i/I_o$ indicates that $V_c5$ induces a greater broadening, and hence decrease, in the outer monolayer signal intensity as compared to that of the inner monolayer signal. This may be due to a cytotoxin-induced reduction of the mobility of the outer monolayer phospholipid since membrane aggregation and fusion broaden both signals leaving the intensity ratio unchanged. The reduction of the $I_i/I_o$ value suggests that the ferricyanide ion penetrates into the liposomes and shifts the resonance of $N^+(CH_3)_3$ group towards stronger fields. Fig. 6 illustrates the dependence of the change of $^1$H-NMR signal half-width $\Delta\nu$ of hydrocarbon chain $CH_2$-groups on CT concentration. For $V_c5$ in PC + PS liposomes $\Delta\nu_i/\Delta\nu_o$ starts increasing within the same CT concentration range as does the process of ferricyanide ion penetration into the liposome interior start. Hence, the disturbance of the bilayer structure and the permeability enhancement coincide with the process of membrane fusion or aggregation.

The addition of $V_c1$ to PC + PS liposome samples induced broadening of $^1$H-NMR peaks (Fig. 5*C*). Nevertheless, the signal from the inner monolayer choline groups did not disappear suggesting that the bilayer has preserved its integrity. The growing value of the $I_i/I_o$ ratio over the entire range of $V_c1$ concentrations tested (Fig. 6) suggests a limited mobility of the outer monolayer choline groups. The $\Delta\nu_i/\Delta\nu_o$ value most likely reflects two processes: membrane aggregation and compaction of the outer monolayer phospholipid packing.

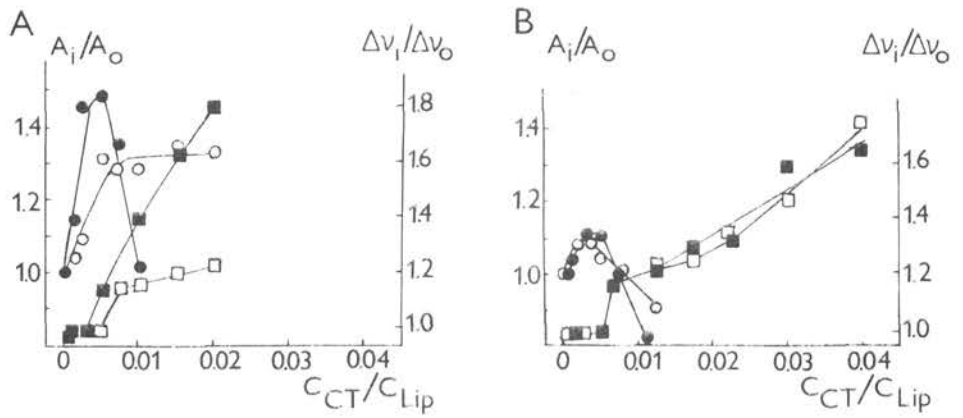


**Fig. 6.** The dependence of the relative changes in the ratio of $^1$H-NMR signal intensity from trimethylammonium groups of inner ($I_i$) and outer ($I_o$) monolayers ($A_i/A_o$, where $A = I_i/I_o$) and the dependence of relative changes of $^1$H-NMR signal halfwidths from $CH_2$-groups of hydrocarbon chains ($\Delta\nu_i/\Delta\nu_o$) on relative concentration of $V_c5$ (filled symbols) and $V_c1$ (empty symbols). *a* — liposomes of PC + 10 mol% PS; *b* — liposomes of PC + 10 mol% CL. Circles — $A_i/A_o$; squares — $\Delta\nu_i/\Delta\nu_o$.

Fig. 5*A*, *B* shows shape changes of $N^+(CH_3)_3$ proton signal from outer and inner monolayers of PC + CL liposomes upon the interaction with $V_c1$ and $V_c5$. The resonance line was broadened with both toxins, while at $C_{CT}/C_{lip}$ equal to or exceeding 0.012, $N^+(CH_3)_3$ signals from the inner monolayer groups were shifted towards stronger fields. The resonance signals of $N^+(CH_3)_3$ groups were thus modified and appeared as a peak with a sufficiently pronounced weak-field shoulder at final CT concentrations. With both toxins added simultaneously the peak maximum was markedly shifted, relative to the signal peaks of choline groups from the outer monolayer of to nonmodified CT membrane, towards stronger fields. This may indicate that the bilayer packing of phospholipids undergoes structural modification responsible for the new conditions of interaction between ferricyanide ions and lecithin choline groups, resulting in a resonance frequency shift.

For PC + CL liposomes variations of $(I_i/I_o)/(I_i/I_o)$ and $\Delta\nu_i/\Delta\nu_o$ values accompanying increasing CT concentrations (Fig. 6) were analyzed. The pattern of changes suggests that also here the cytotoxin-induced permeability enhancement and destabilization of the lipid bilayer packing is accompanied by membrane aggregation and fusion. In this case however, the membrane outer monolayer is compacted to lesser extent as compared with phosphatidylserine liposomes.

The results of the present study and recent reports by other autors allow us

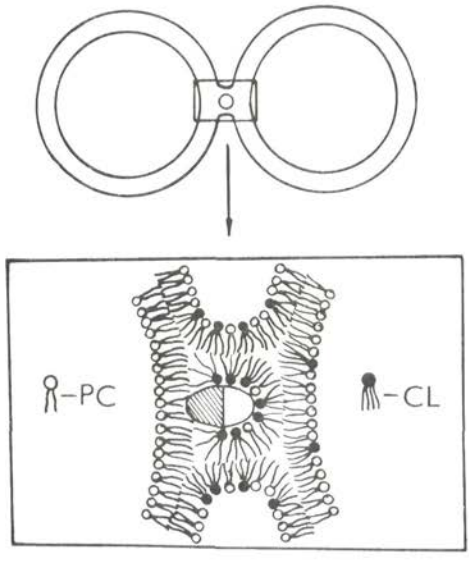


**Fig. 7.** A model of interaction of cytotoxins $V_c5$ and $V_c1$ with cardiolipin-containing membranes. The shape, size and penetration depth of CT molecule into the bilayer are illustrated according to Oimatov et al. (1986). The nonpolar region of the CT molecule is shaded.

to draw conclusions concerning some features of the fusion mechanism in model system discussed above. The major factors determining fusion probability include liposome aggregation (Houg and Vacquier 1986), dehydration of membrane contact area (Sundler et al. 1981; Hoekstra 1982; Hui et al. 1981; Ekerdt and Papahadjopoulos 1982), membrane transition destabilization (Houg and Vacquier 1986; Shragin et al. 1986), all of which lead to the formation of a non-bilayer structure resulting in an increased probability of the fluctuation phenomenon (Shragin et al. 1986; Koter et al. 1978; Shragin et al. 1985). It was shown earlier that cytotoxins considerably reduce the spectral anisotropy of doxylstearic acid in oriented PC + CL membranes (Aripov et al. 1986; Gasanov et al. 1988), due to the formation of lipid phase sites with isotropic orientation of the phospholipid molecule long axes (Aripov et al. 1986). It is probable that similar structures are formed at the contact of two membranes; as a result of the CT-induced dehydration of phospholipid polar heads the CL molecules take the form of reverse wedges which, under conditions of aggregation caused by the polycationic nature of CT, leads to the formation of inverted micelles involving the cytotoxin molecule (Fig. 7). Further evolution of these formations may result in liposome fusion.

Investigations of CT interactions with membranes of phospholipids form-

ing stable bilayers show that CT induce lipid phase compaction (Aripov et al. 1984; 1987). According to the results of the present study and other reports (Oimatov et al. 1986) CT compacts the outer monolayer only. Markin et al. (1984) provided the theory of stresses and breaks in a membrane resulting from molecule compaction in the outer monolayer. From the theory it follows that stresses arising from molecule crystallization in the contact area of two membranes are capable of breaking membranes in this area, i.e. bringing into operation the adhesive-condensation mechanism of membrane fusion (Markin and Kozlov 1984). Such a fusion mechanism occurs when $V_c5$ interacts with PC + PS liposomes. No fusion occurs on interaction of $V_c1$ with PC + PS liposomes. In contrast to $V_c5$, $V_c1$ does not induce in the given lipid system the necessary destabilization of the phospholipid bilayer structure as suggested by membrane impermeability for the ferricyanide anion. The different levels of PC + PS membrane destabilization by $V_c5$ and $V_c1$ may be accounted for by some differences in the structural arrangement of these cytotoxins (Grishin et al. 1974; 1976; Yukelson et al. 1974).

In summary, both cytotoxins studied are characterized by fusogenic activity within certain lipid systems. The fusion probability is determined not only by natural features of fusogenic substances but, to a great extent, also by the phospholipid composition of the interacting membranes.

## References

Aripov T. F., Salakhutdinov B. A., Salikhova Z. T., Sadykov A. S., Tashmukhamedov B. A. (1984): Structural changes of liposome phospholipid packing induced by cytotoxin of Central Asia cobra venom. Gen. Physiol. Biophys. **3**, 489–496

Aripov T. F., Gasanov S. E., Salakhutdinov B. A., Sadykov A. S. (1986): Studies on the interaction of cobra venom cytotoxin with oriented phospholipid multilayers. Dokl. Akad. Nauk SSSR **288**, 728–730 (in Russian)

Aripov T. F., Rozenstein I. A., Salakhutdinov B. A., Lev. A. A., Gotlib V. A. (1987): The influence of cytotoxin from Central Asian cobra venom and melittin from bee venom on the thermodynamic properties of phospholipid bilayer. Gen. Physiol. Biophys. **6**, 343–357

Bergelson L. D. (1976): Complexes of paramagnetic metals and phospholipid and their application for research of membrane molecular organisation by a NMR method. ZhVHO im. D. I. Mendelejeva **21**, 638–650 (in Russian)

Chernomordik L. V., Kozlov M. M., Leikin S. L., Markin V. S., Chizmadjev Yu. A. (1986): Membrane fusion: local interactions and structural reconstruction. Dokl. Akad. Nauk SSSR **288**, 1009–1013 (in Russian)

Düzgünes N. (1985): Subcellular Biochemistry. Plenum Press, New York

Ekerdt R., Papahadjopoulos D. (1982): Intermembrane contact affects calcium binding to phospholipid vesicles. Proc. Nat. Acad. Sci. USA **79**, 2273–2277

Elsbach P., Holmes K. V., Choppin P. W. (1969): Metabolism of lecithin and virus-induced cell fusion. Proc. Soc. Exp. Biol. Med. **3**, 903–908

Gasanov S. E., Gasanov E. E. (1988): About one fusion mechanism induced by cobra venom

cytotoxin in membranes formed of bilayer stabilising phospholipids. I. Ya. Fiz. Akad. Nauk UzSSR Prepr. P-5 II-356. Tashkent (in Russian)

Gasanov S. E., Aripov T. F., Gasanov E. E. (1988): Study structure of phospholipid membranes modified by membrane-active polypeptide. I. Ya. Fiz. Akad. Nauk UzSSR Prepr. P-5 II-359, Tashkent (in Russian)

Grishin E. V., Sukhikh A. P., Adamovich T. V., Ovchinnikov Yu. A., Yukelson L. Ya. (1974): The isolation and sequence determination of a cytotoxin from the Middle-Asian cobra *Naja naja oxiana*. FEBS Lett. **48,** 179—183

Grishin E. V., Sukhikh A. P., Adamovich T. V., Ovchinnikov Yu. A. (1976): Isolation, properties and amino acid sequence of two cytotoxins from the venom of Central Asian cobra *Naja naja oxiana*. Bioorg. Khim. **2,** 1018—1034 (in Russian)

Hauser H., Phillips M. C. (1973): Structures of aqueous dispersions of phosphatidylserine. J. Biol. Chem. **346,** 8585—8591

Hoekstra D. (1982): Role of lipid phase separations and membrane hydrations in phospholipid vesicle fusion. Biochemistry **21,** 2833—2840

Houg K., Vacquier V. D. (1986): Fusion of liposome induced by a cationic protein from the acrosome granule of abalone spermatozoa. Biochemistry **25,** 543—549

Hui S. W., Stewart T. P., Boni L. T. (1981): Membrane fusion through point defects in bilayers. Science **212,** 921—922

Ivkov V. G., Berestovsky G. N. (1981): The dynamic structure of lipid bilayer. Nauka, Moscow (in Russian)

Ivkov V. G., Berestovsky G. N. (1982): The Lipid Bilayer of Biological Membranes. Nauka, Moscow (in Russian)

Kotelnikov A. M., Kuznetsov S. A., Fogel V. R., Lichtenshtein G. I. (1979): Studies on the microstructure of biological systems by the method of triplet markers. Molekulyarnaya Biologiya **13,** 152—159 (in Russian)

Koter M., De Kruijff B., Van Deenen L. L. M. (1978): Calcium-induced aggregation and fusion of mixed phosphatidylcholine-phosphatidic acid vesicles as studied by $^{31}$P-NMR. Biochim. Biophys. Acta **514,** 255—263

Lansman J. B., Haynes D. H. (1975): Kinetics of a $Ca^{2+}$-triggered membrane aggregation reaction of phospholipid membranes. Biochim. Biophys. Acta **394,** 335—347

Lansman J. B., Haynes D. H. (1979): Charge asymmetry does not affect the rate of $Ca^{2+}$-induced aggregation of phospholipid vesicles. Biophys. J. **26,** 335—337

Lawaczech R., Kainosho M., Girardet J.-L., Chan S. I. (1975): Effects of structural defects in sonicated phospholipid vesicles on fusion and ion permeability. Nature **265,** 584—586

Lawaczech R., Kainosho M., Chan S. I. (1976): The formation and annealing of structural defects in lipid bilayer vesicles. Biochim. Biophys. Acta **443,** 313—330

Markin V. S., Kozlov M. M. (1984): Summaries of Science and Technology. Membrane Biophysics. VINITI, Moscow (in Russian)

Mekler V. M., Kotelnikov A. I., Lichtenshtein G. I., Berkovich M. A. (1982): Application of phosphorescent probes for the study of model and biological membranes. Biofizika SSSR **27,** 641—645 (in Russian)

Mekler V. M., Kotelnikov A. I., Lichtenshtein G. I. (1983): Application of probes emitting annhiliation retarded fluorescence for studies of model and biological membranes. Biofizika SSSR, **28,** 303—305 (in Russian)

Oimatov M., Lvov Yu. M., Aripov T. F., Feigin L. A. (1986): X-ray small-angle scattering structural studies of model membrane complexes with cytotoxin. Stud. Biophys. **112,** 237—241

Papahadjopoulos D., Portis A., Pangborn W. (1978): Calcium-induced lipid phase transitions and membrane fusion. Ann. N. Y. Acad. Sci. **308,** 50—66

Papahadjopoulos D., Poster G., Vail W. I. (1979): Methods in Membrane Biology. Plenum Press,

New York, London
Parker C. (1972): Solution Photoluminescence. Mir, Moscow (in Russian)
Parker C. A., Hatchard C. G. (1962): Delayed fluorescence from solutions of anthracene and phenanthrene. Proc. Roy. Soc. (London) A, **269,** 574–584
Podo F., Blasie J. K. (1977): Nuclear magnetic resonance studied of lecithin bimolecular leaflet with incorporated fluorescent probes. Proc. Nat. Acad. Sci. USA **74,** 1032–1036
Portis A., Newton C., Pangborn W., Papahadjopoulos D. (1979): Studies on the mechanism of membrane fusion: evidence for an intermembrane $Ca^{2+}$-phospholipid complex, synergism with $Mg^{2+}$ and inhibition by spectrin. Biochemistry **18,** 780–790
Salakhutdinov B. A., Aripov T. F. (1981): Spin label studies on change of membrane surface charge induced by cobra venom cytotoxin. Khim. Prir. Soed. **1,** 109–110 (in Russian)
Smith C. F., Lichtenberg D., Thomson T. E. (1981): Vesicle-vesicle interactions in sonicated dispersions of dipalmitoylphatidylcholine. Biochemistry **20,** 4792–4797
Shragin A. S., Vasilenko I. A., Selishcheva A. A., Shvets V. I. (1985): The effect of metabolite phospholipids on fushion of model membranes of different composition. Biol. Membrany **2,** 789–794 (in Russian)
Shragin A. S., Vasilenko I. A., Selishcheva A. A., Shvets V. I. (1986): Membrane permeability and fusion induced by metabolic products of phosphoinosids. Dokl. Akad. Nauk SSSR **287,** 717–720 (in Russian)
Sundler R., Düzgünes N., Papahadjopoulos D. (1981): Control of membrane fusion by phospholipid head groups. II. The role of phosphatidylethanolamine in mixtures with phosphatidate and phosphatidylinositol. Biochim. Biophys. Acta **649,** 751–758
Wallash D. E. H., Verma S. P., Weidekamm E., Bieri V. (1974): Hydrophobic binding sites in bovine serum albumin and erythrocyte ghost proteins. Study by spin-labelling paramagnetic fluorescence quenching and chemical modification. Biochim. Biophys. Acta **356,** 68–81.
Wilshut J., Düzgünes N., Fraley R., Papahadjopoulos D. (1980): Studies on the mechanism of membrane fusion: Kinetics of fusion followed by a new assay for mixing of aqueous vesicles contents. Biochemistry **19,** 6011–6021
Yukelson L. Ya., Sadikov E., Sorokin V. M. (1974): Isolation and characteristics of a "direct" haemolytic factor of Central Asian cobra venom. Biokhimiya **39,** 310–313 (in Russian)

Final version accepted March 23, 1989