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DISRUPTION OF LIPOSOMES BY TETRACAINE MICELLES

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The effect of the local anesthetic tetracaine hydrochloride on the stability of egg lecithin vesicles has been studied by means of a filtration procedure using Pellicon membranes with an average molecular weight limit of retention of 10^5. These filters were efficient to retain liposomes and allowed the free passage of disrupted vesicles as detected by measuring the phospholipid content in the filtrates. It has been found that at concentrations above 0.05 M, the anesthetic induces disruption of liposomes presumably through formation of mixed micelles of tetracaine and phosphatidylcholine.

Introduction

The local anesthetic tetracaine hydrochloride has been shown to interact with both, natural and artificial membranes [1,2], the interaction being dependent in part on hydrophobic binding [3–5].

In a recent paper from this laboratory [6] it has been demonstrated that this amphipathic anesthetic is hydrophobic enough to self-associate and form micelles. Upon formation of micelles, tetracaine induces an abrupt decrease in the light scattering of the lipid dispersions which might be attributed to vesicle damage [6].

The purpose of the present work was to prove that the decrease in light scattering of the lecithin dispersion is associated to the disruption of the liposomal structure.

The experimental procedure was based on the study of the filtrability of liposomal samples at various anesthetic concentrations through Pellicon membranes of low molecular weight limit of retention (10^5). These filters could be expected to retain liposomes and to allow the free passage of disrupted vesicles.

Materials and Methods

Chemicals. Egg lecithin from Sigma was purified by column chromatography on alumina [7]; the purified lecithin gave a single spot on thin-layer chromatography. Crystalline tetracaine hydrochloride (2-dimethylaminoethyl-*p*-butylaminobenzoate hydrochloride) purchased from Sigma, was used as received. The fluorescent probe (ANS (8-anilino-1-naphthalene sulfonic acid, sodium salt) was from Eastman. All other reagents were analytical grade. Glass redistilled water was used throughout.

Preparation of liposomes. Liposomes (mostly unilamellar) were prepared as described previously [6] except that and imidazol buffer was used instead of the phosphate buffer to avoid interferences in the determination of phosphatidylcholine by phosphorus assay. A 1% dispersion of egg lecithin in aqueous buffer (0.05 M imidazole/0.10 M NaCl/HCl, pH 6.5) was sonicated for 30 min under nitrogen using a Branson Sonifier (Model B 12) equipped with a

Abbreviation: ANS, 8-anilino-1-naphthalene sulfonic acid, sodium salt.

titanium microtip, at a power output of 60 watts. During sonication the sample was surrounded by ice water. Titanium particles and large vesicles were eliminated from the sonicated dispersion by centrifugation at 100 000 × *g* for 1 h.

Filtration procedure. Different amounts of crystalline tetracaine hydrochloride were dissolved in aliquots of the liposomal dispersion, previously diluted to a final lipid concentration of $(6–8) \cdot 10^{-4}$ M with the same buffer used in the preparation of liposomes.

Each aliquot was filtered through a 25 mm diameter Pellicon PTHK02510 membrane disc (Millipore) having a nominal molecular weight limit of retention of 10^5. The filter was placed in a Swinnex-25 filter holder (Millipore) attached to a 5 ml syringe as source of liquid for filtering. The plunger of the syringe was driven by applying a pressure of approx. 14 lb/inch^2.

Light scattering determinations. The 90° light scattering of the liposomal dispersions in the presence of various anesthetic concentrations, was measured at 600 nm using an Aminco Bowman spectrophotofluorometer. Measurements were performed before and after filtration of samples.

Thin-layer chromatography. A qualitative determination of the presence of lipid in filtrates was made by thin-layer chromatography. Samples were taken both before (as control) and after filtration and applied to Silica gel 60 plates (layer thickness 0.25 mm). Due to the aqueous nature of the samples, spotting was followed by careful drying to eliminate most of the water. The developing solvent consisted of chloroform/methanol/acetic acid/water (35 : 10 : 4 : 2, v/v). In order to visualize the spots the plates were sprayed with aqueous 0.1% ANS and illuminated by long-wave ultraviolet light [8].

Phosphorus assay. The percentage of filtrable phospholipid was determined by analyzing the phosphorus content of the dispersions before and after the filtration procedure. Phosphorus was assayed according to the method of Bartlett [9] except that a longer period of digestion and a larger volume of 10 M H_2SO_4 (0.75 ml instead of 0.5 ml) were used. These modifications were required to obtain complete combustion due to the large amount of organic material (i.e. imidazole and tetracaine) present in the samples.

Critical micelle concentrations of tetracaine hydrochloride in the imidazole buffer. The critical micelle concentration of tetracaine hydrochloride in the buffer used in this work (0.05 M imidazole/0.10 M NaCl/HCl, pH 6.5) was determined by the enhancement of ANS fluorescence, as described previously [6]. A critical micelle concentration of 0.061 M was found by this method.

Results and Discussion

Fig. 1 shows the effect of filtration on the light scattering of liposomes dispersed in imidazole-NaCl-HCl buffer (pH 6.5) at various anesthetic concentrations. The scattering curve corresponding to dispersions prior to filtration, which is similar to the one obtained previously in a different buffer system (phosphate, pH 6.5 [6]), is also included for comparison. The light scattering of unfiltered samples increases with the addition of anesthetic up to 0.05 M but drops sharply at 0.06 M to remain constant at higher drug concentrations. The abrupt decrease observed has been attributed to liposome

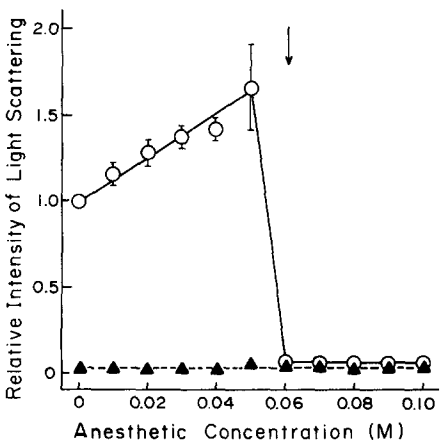

Fig. 1. Comparison of the light scattering of liposomes in the presence of various anesthetic concentrations before and after filtration. Increasing quantities of crystalline tetracaine hydrochloride were dissolved in aliquots of a $(6–8) \cdot 10^{-4}$ M egg lecithin liposomal dispersion prepared in imidazole-NaCl-HCl buffer (pH 6.5). The 90° light scattering of the samples was measured at 600 nm before (o———o) and after (▲- - - - ▲- - - - -▲) being filtered through Pellicon membranes (molecular weight limit of retention: 10^5). The values plotted are the means ± S.D. of five experiments (S.D. bars smaller than symbols not shown). The arrow indicates the anesthetic critical micelle concentration in the imidazole buffer as determined by use of the fluorescent probe ANS.

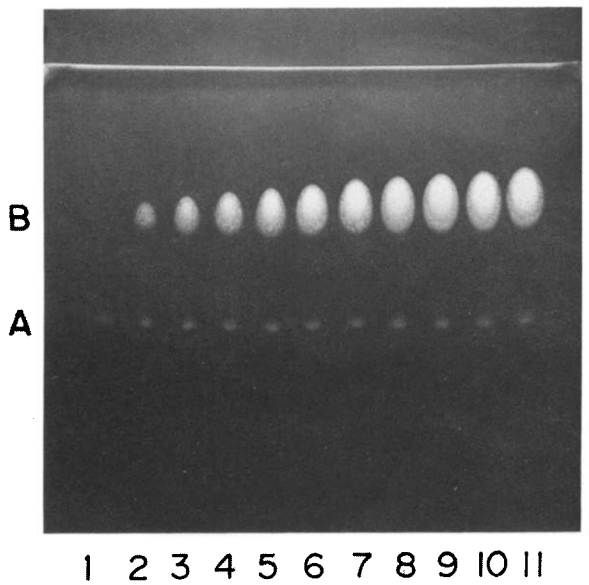

Fig. 2. Thin-layer chromatogram of liposomes in the presence of various anesthetic concentrations prior to filtration. Samples were prepared by dissolving increasing quantities of crystalline tetracaine hydrochloride in aliquots of a $(6-8) \cdot 10^{-4}$ M egg lecithin dispersion prepared in imidazole-NaCl-HCl buffer (pH 6.5). Anesthetic concentrations were: zero (1); 0.010 M (2); 0.020 M (3); 0.030 M (4); 0.040 M (5); 0.050 M (6); 0.060 M (7); 0.070 M (8); 0.080 M (9); 0.090 M (10); 0.100 M (11). Layer: Silica gel 60. Developing solvent: chloroform/methanol/acetic acid/water (35 : 10 : 4 : 2, v/v). Detection: the plate was sprayed with aqueous 0.1% ANS and photographed by long-wave ultraviolet light. Spots in position A correspond to phosphatidylcholine and those in position B to tetracaine.

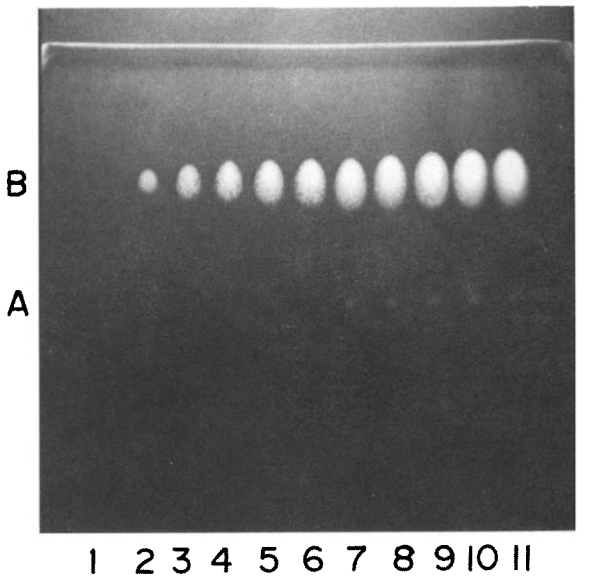

Fig. 3. Thin-layer chromatogram of filtrates from liposome dispersions containing various anesthetic concentrations. Filtrates were obtained by passing the samples prepared as described in Fig. 2 through Pellicon membranes (molecular weight limit of retention: 10^5). The rest of conditions as well as the sequence and symbols are also as in Fig. 2.

damage [6]. By contrast, all the filtrates, independently of the anesthetic concentration, show a constant value of scattering as low as the one corresponding to unfiltered samples containing tetracaine at concentrations equal to or higher than 0.06 M. These results suggest that the filters seem to retain liposomes efficiently.

To investigate whether the decrease in light scattering induced by tetracaine concentrations higher than 0.05 M is due to disruption of liposomes, the lipid content of samples was assayed both, before and after filtration.

A qualitative test of the presence of phosphatidylcholine in the filtrates was performed by thin-layer chromatography. To visualize the compounds, the plates were sprayed with an aqueous solution of the fluorescent dye ANS and illuminated with ultraviolet light. It is worth mentioning that by use of this procedure, which was introduced by Gitler for the detection of lipids [8], not only lecithin but also tetracaine can be visualized. In a control experiment illustrated in Fig. 2, samples of liposomes in the presence of various anesthetic concentrations, were chromatographed prior to filtration. It can be appreciated that the chromatographic procedure is suitable for separating phosphatidylcholine and tetracaine and that the presence of lipid can be clearly ascertained. Fig. 3 shows the thin-layer chromatography of filtrates. It can be observed that whereas lecithin can not be detected in filtrates from samples containing up to 0.05 M tetracaine, it is still present in those filtrates from samples containing higher drug concentrations. By contrast, the anesthetic spots remain similar to those in the control plate.

The quantitative evaluation of the ability of the liposomal lipid to pass through the filters was performed by assaying the phospholipid content of samples before and after filtration. Fig. 4 shows that the

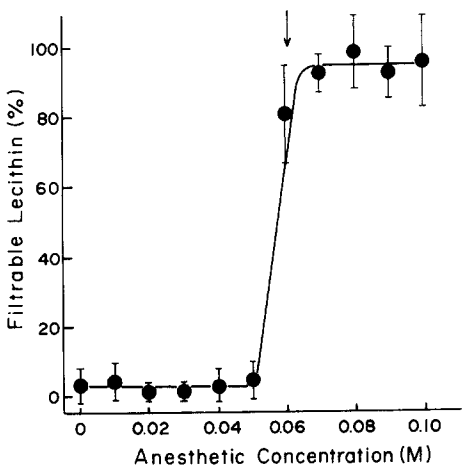

Fig. 4. Percentage of phosphatidylcholine in filtrates from liposome dispersions containing different anesthetic concentrations. Increasing quantities of crystalline tetracaine hydrochloride were dissolved in aliquots of a $(6-8) \cdot 10^{-4}$ M egg lecithin liposomal dispersion prepared in imidazole-NaCl-HCl buffer (pH 6.5). The percentage of filtrable phospholipid was obtained for each sample by determining its phosphorus content before and after filtration through Pellicon membranes (molecular weight limit of retention: 10^5). The values plotted are the means ± S.D. of five experiments. The arrow indicates the anesthetic critical micelle concentration in the imidazole buffer as determined by use of fluorescent probe ANS.

lipid from samples containing up to 0.05 M tetracaine is almost completely retained by the filters: a maximum of 3% of filtrable phospholipid is present in these dispersions. By contrast, almost 95% of the lecithin from samples containing more than 0.05 M anesthetic, is filtrable lipid.

The critical micelle concentration of the anesthetic in the imidazol buffer used in this work is 0.061 M. It is evident that liposomes show high scattering readings and are retained by the filters when interacting with tetracaine concentrations below the critical micellar value whereas at higher anesthetic concentrations the light scattering becomes very low and almost all the lipid can be filtered. Moreover, the lipid filtrability is triggered at the same anesthetic concentration (0.06 M) at which the abrupt decrease in scattering appears.

Taking into account that the filters have a nominal molecular weight limit of retention of 10^5 and that the minimum vesicle weight estimated for liposomes, both theoretically and experimentally, is $2 \cdot 10^6$ [10–12], it can be concluded that the filtrable lipid is not organized in a liposomal structure but corresponds to disrupted vesicles. Since the lipid filtrability appears abruptly at an anesthetic concentration very similar to the critical micellar value of the drug it is proposed that the disruption of liposomes occurs via formation of mixed micelles of tetracaine and phosphatidylcholine. It is worth mentioning that the existence of mixed micelles of phospholipids and conventional detergents such as Triton X-100, has been described by several authors [13,14].

The detergent behavior of tetracaine is an important aspect to be taken into account in the studies of the interaction of this drug with membranes in view of the deleterious effect on the liposomal structure shown by anesthetic concentrations above the critical micellar value.

Acknowledgements

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