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OSMOTIC FRAGILITY OF LIPOSOMES AS AFFECTED BY ANTIHEMOLYTIC COMPOUNDS

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SUMMARY

1. Liposomes prepared by vigorous shaking undergo hypotonic lysis in water. The extent of osmotic fragility was followed by the release of the trapped markers dinitrodithiobenzoic acid and $^{22}Na^{+}$.

2. Phosphatidylcholine liposomes were stabilized against hypotonic lysis by incorporated cholesterol. The maximal stabilization was obtained at a phosphatidylcholine: cholesterol molar ratio of 1:1 as already observed for erythrocytes.

3. Stearic, linolenic, linoleic and oleic acids reduced the osmotic fragility of liposomes in an increasing order and in accordance with their antihemolytic effects.

4. The antihemolytic hashish components, cannabidiol and Δ^{1}-tetrahydrocannabinol, stabilized liposomes against osmotic lysis. While phosphatidylcholine and phosphatidylcholine:cholesterol (1:1) were only slightly affected, liposomes made of erythrocyte lipids and particularly of brain lipids were markedly stabilized by the cannabinoides, possibly indicating some specific interactions.

5. The phenothiazines promethazine, chlorpromazine and fluphenazine promoted the hypotonic lysis of liposomes with a relative effectiveness correlated with the clinical and antihemolytic potency of the tranquilizers.

6. It is concluded that lipid–lipid interactions are involved in the antihemolytic effects of the fatty acids and the cannabinoids, while membrane proteins are apparently required for the stabilization effect of the phenothiazine tranquilizers.

INTRODUCTION

Stabilization of erythrocytes against hypotonic hemolysis by various compounds has been extensively investigated, particularly as a model system for the study of the mode of action of drugs [1, 2]. The use of red blood cell as a model system attracts interest in view of the correlation between the antihemolytic activity of various anesthetics and tranquilizers and their clinical potencies [3, 4]. The site and

Abbreviations used: DTNB, 2,2′-dinitro-5,5′-dithiobenzoic acid, PC, phosphatidylcholine.

mode of action of the antihemolytic compounds are not known, but several possibilities have been advanced [5].

The fluid mosaic model of the structure of cell membrane assumes a lipid matrix in which integral proteins are embedded [6]. The capacity of the erythrocyte to withstand an expansion in hypotonic medium may thus be an intrinsic property of the lipid matrix and the antihemolytic compounds are expected to interact with the bilayer network. The liposome system could aid in testing this supposition, provided they undergo osmotic lysis. Erythrocyte membranes and liposomes indeed show similar properties in terms of thickness [7] and permeability to water [8], ions [8, 9] and other molecules [10, 11]. Both systems respond to pharmacological agents [12] and, furthermore, both behave as osmometers [11, 13]. In the present study we have attempted to quantitate the osmotic fragility of liposomes and to evaluate the effect of various antihemolytic compounds on the liposome system.

MATERIALS AND METHODS

Lipids

Phosphatidylcholine was extracted and purified from egg yolks according to Panghorn [14]. It appeared as a single peak following thinlayer chromatography, using the solvent mixture of chloroform–methanol–water (65:25:4, v/v/v).

Lipid extraction of red blood cells was performed as described by Kuiper et al. [15]. Lamb brain, obtained within 30 min after slaughter, was homogenized in 4 °C in a Waring blender and the lipids were extracted as described for the erythrocyte lipids [15]. All the lipid extracts were stored in chloroform under N_2 at −18 °C and were used within 6 weeks after the extraction. Cholesterol was thrice crystallized from warm ethanol solutions, dried and stored at room temperature (22 °C).

Other materials

The following materials were obtained as indicated: Fatty acids, GSH and dicetylphosphate from Sigma Chemical Co., St. Louis; 2,2′-dinitro-5,5′-dithiobenzoic acid (DTNB) and cholesterol, from Merck, Darmstadt; Sephadex G-25, from Pharmacia Fine Chemicals, Uppsala; Triton X-100, from Agan, Tel Aviv; ^{22}NaCl from the Radiochemical Centre, Amersham; phenothiazines were donated by Taro Ltd., Haifa; cannabidiol and Δ^1-tetrahydrocannabinol were kindly supplied by Professor R. Mechoulam from the School of Pharmacy, the Hebrew University, Jesusalem.

Preparation of liposomes

Liposomes were prepared according to the methods described by Bangham et al. [8] and Kleinschmidt et al. [16]. To prepare phosphatidylcholine or phosphatidylcholine:cholesterol liposomes, chloroform solutions containing 0.6 μmole dicetylphosphate, 10 μmoles phosphatidylcholine and, as specified, 0–20 μmoles cholesterol, were transferred to a 15-ml round-bottom flask. Chloroform solutions containing 10 mg of either erythrocyte lipids or brain lipids were used for the preparation of the respective liposomes. The solvent was removed under reduced pressure in a rotatory evaporator, leaving a thin lipid layer. A stream of N_2 was passed into the flask for about 20 s to assure the removal of chloroform. A volume of 0.5 ml of a suspension containing 50 μmoles DTNB in 0.1 M Tris–HCl buffer, pH 8.0, was added. Where

indicated, 20 μl ^{22}NaCl (1.3 μg ^{22}Na/ml, 0.24 Ci/ml in aqueous solution) was also added. The flask was stoppered while containing N_2 atmosphere and was gently agitated for about 5 min at 37 °C until the entire lipid layer was suspended. The dispersion was vigorously shaken with a Vortex mixer for 1 min and then immersed at 37 °C for 1 min. This procedure was repeated 5 times and then the suspension was kept for 20 min at room temperature (22 °C). In order to remove the untrapped marker, the liposome suspension was passed through a 0.8 cm × 15 cm column of Sephadex G-25 (medium) previously equilibrated with a solution of 75 mM KCl, 75 mM NaCl in 0.1 M Tris–HCl buffer, pH 8.0. The liposomes, eluted with this solution ("elution medium"), were recovered just past the void volume. Phosphatidylcholine:cholesterol molar ratio of the eluted liposomes differed insignificantly from the molar ratio employed for their preparation.

Sonicated liposomes were similarly prepared except that the shaking lasted for 1 min only, followed by sonic-irradiation with an MSE, 100-W ultrasonic disintegrator at maximal output (8 μM amplitude) at 25 °C for a total of 5 min at 1-min intervals for thermal equilibration.

Determination of osmotic fragility

All steps were conducted at 22 °C. An aliquot of 40 μl of the stock (gel-filtered) liposome suspension was rapidly mixed with 2.5 ml of either distilled water or the elution medium and the absorbance at 420 nm was measured after 10 min incubation at 22 °C. Then, 25 μl of 0.1 M GSH in 0.1 M Tris–HCl, pH 8.0, were added and the absorbance recorded again. The difference in absorbance measures the net release of DTNB, expressed as percentage of total DTNB trapped. The total amount of the trapped marker was determined following liposome disruption by the addition of 20 μl of 10% Triton X-100 in 0.1 M Tris–HCl buffer, pH 8.0.

For kinetic measurements of osmotic lysis, a hypertonic solution (0.5 ml of 0.45 M KCl, 0.45 M NaCl in 0.6 M Tris–HCl, pH 8) was added at various time intervals after mixing the liposomes with water, to obtain final salt concentration as in the elution medium. When $^{22}Na^+$ was used as an additional marker, the liposome stock suspension was centrifuged at 17 000 × *g* for 15 min, the pellet suspended in the elution medium and aliquots were taken for the kinetic measurements. After the addition of the hypertonic solution, the mixtures were centrifuged at 27 000 × *g* for 30 min and DTNB and $^{22}Na^+$ were determined in both the pellet and the supernatant. $^{22}Na^+$ was measured with a Packard Tri-Carb γ well counter.

The tested antihemolytic compounds, dissolved in ethanol or water, were mixed with 2.5 ml water just prior to the addition of liposome suspension. Up to 20 μl ethanol were added, which did not affect the osmotic fragility of the control liposomes.

Electron microscopy

Phosphatidylcholine liposomes, diluted as described above in either water or the elution medium, were negatively stained with 2% phosphotungstic acid and observed in a Phillips electron microscope, model EM 300.

RESULTS AND DISCUSSION

Osmotic fragility of liposomes

Liposomes behave as an almost perfect osmometer [11, 13]. It was therefore expected that swelling of the liposomes in an increasingly diluted solution would eventually cause a rupture of the lipid membranes. The release of a trapped marker due to such rupture should aid in quantitation of osmotic lysis. DTNB was chosen as a marker for osmotic fragility measurements in view of its low permeability across the liposomal bilayer membrane [16, 17]. The procedure of Kleinschmidt et al. [16] was adopted to follow the rupture in water of liposomes loaded with buffer and DTNB. Fig. 1 shows that liposomes prepared from various lipid sources released in water 41–64% of their trapped marker. The release could be due to either a diffusion of DTNB from intact liposomes or to an osmotic rupture of the membrane. Several lines of evidence indicate that an osmotic rupture is indeed the major cause for the marker release.

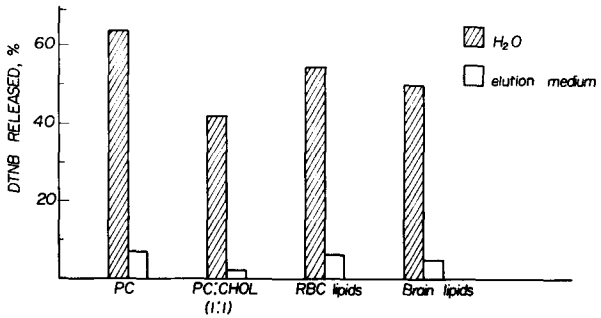


Fig. 1. Release of DTNB from various liposomes in either water or the elution medium. PC, phosphatidylcholine; CHOL, cholesterol; RBC, erythrocyte.

(a) The release of DTNB from liposomes following an incubation for 30 min in a solution containing 75 mM KCl and 75 mM NaCl in 0.1 M Tris–HCl, pH 8.0, amounted to less than 7% of the trapped marker. This release may be accounted for by a non-osmotic rupture of a minor portion of the liposomes, as suggested for the release of $^{86}Rb^{+}$ from phosphatidylcholine liposomes [18].

(b) If considerable diffusion of DTNB across the membrane takes place, it is expected to appear linearly with time [8, 19]. Fig. 2 shows that actually the release of DTNB in water is non linear and is very fast. $t_{\frac{1}{2}}$, the time required for the release of 50% of the marker that is being discharged within 5 min, is 1.2 s. After 5 min no more DTNB is released.

(c) The permeability of PC liposomes to cations is known to be very low [8, 9]. Yet, the kinetics of release of trapped $^{22}Na^{+}$ and DTNB is essentially the same (Fig. 3). The relatively high level of marker release recorded initially is apparently due to the particular treatment in this experiment (note Materials and Methods).

(d) Observation by electron microscopy verified that the liposome population, typified by a vesicular shape, markedly diminished after suspension in water.

(e) Sonicated phosphatidylcholine liposomes, unlike the mechanically prepared liposomes, released altogether only 6–8% of the trapped DTNB even after sus-

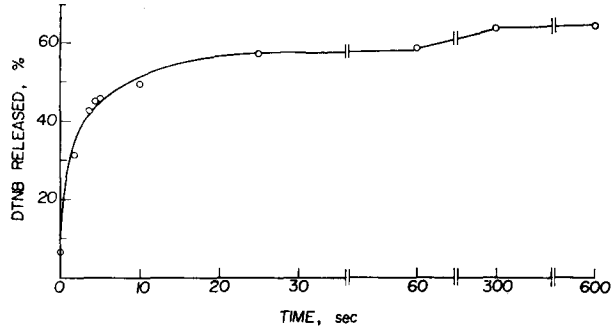


Fig. 2. Time course of DTNB release from phosphatidylcholine liposomes suspended in water. The time required for the release of 50 % of the marker that is being discharged within 5 min ($t_{\frac{1}{2}}$) is 1.2 s.

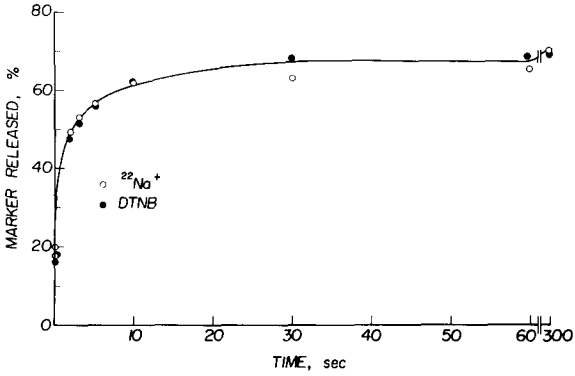


Fig. 3. Time course of simultaneous release of $^{22}Na^+$ and DTNB from phosphatidylcholine liposomes suspended in water.

pension in water for 16 h. This osmotic resistance agrees well with the recent report that upon sonication liposomes lose their osmotic properties [20].

It is concluded that release in water of trapped DTNB from mechanically prepared liposomes represents primarily an osmotic rupture of the liposome membrane.

Cholesterol and osmotic fragility of liposomes

Removal of cholesterol from erythrocytes increases their osmotic fragility [21]. Cholesterol is known as a membrane stabilizing compound in complexes with phospholipids [22]. Cholesterol incorporated into phosphatidylcholine bilayer membrane also causes a decrease in water permeability [23, 24]. Therefore, the effect of cholesterol was tested with respect to both the kinetics and extent of liposome lysis. Fig. 4 shows that incorporated cholesterol lowers the rate of liposome lysis in water. The $t_{\frac{1}{2}}$ value changes from 1.2 to 3.8 s for saturating level of cholesterol. Cholesterol in up to 1:1 molar ratio (phosphatidylcholine:cholesterol) also reduces the osmotic fragility of the liposomes (Fig. 5).

A 1:1 molar ratio of phosphatidylcholine to cholesterol is found in erythrocyte and myelin membranes [22, 25]. A mole per mole steric interaction of phospholipid

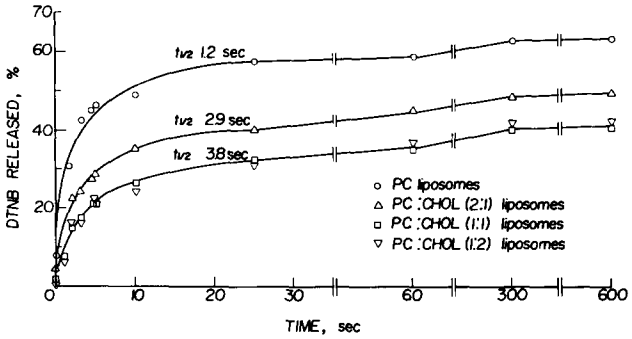


Fig. 4. Time course of DTNB release from liposomes made of phosphatidylcholine and cholesterol at varying molar ratio ($t_{\frac{1}{2}}$: as defined in Fig. 2).

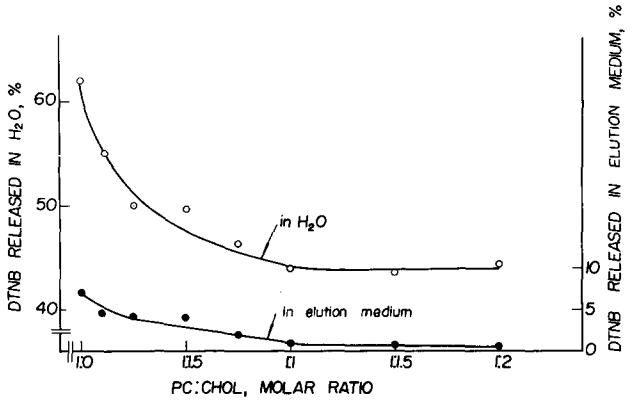
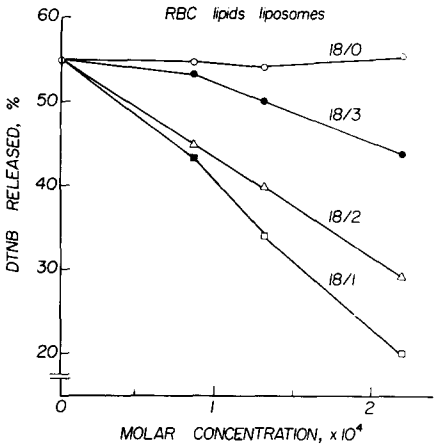


Fig. 5. Effect of phosphatidylcholine: cholesterol molar ratio of liposomes on the release of trapped DTNB in either water or the elution medium.

hydrocarbon chain with cholesterol was inferred [26] from studies of nuclear magnetic resonance [27], electron spin resonance [28], calorimetry and X-ray diffraction [29] of model systems. Rothman and Engelman [30] proposed that "the effect of cholesterol on neighboring hydrocarbon chains makes it act as a universal equalizer by stabilizing the membrane in an intermediate fluid condition", between sol and gel. The data shown in Figs 4 and 5 may be an illustration of this stoichiometric stabilizing effect of cholesterol.

Effect of fatty acids

Fatty acids are known to protect erythrocytes against osmotic hemolysis [31], with varying effectiveness according to number of double bonds [32, 33]. If the interaction of the fatty acids with the erythrocyte membrane is a lipid–lipid one, it should be possible to demonstrate a similar protection with respect to liposomes. The effect of stearic, oleic, linoleic and linolenic acids on the osmotic fragility of erythrocyte lipid liposomes is shown in Fig. 6. The protection potency is in an order already found for red blood cells [32, 33]. Liposomes made of phosphatidylcholine or phosphatidylcholine:cholesterol (1:1) were similarly affected by the added fatty acids. It is concluded that the protection of erythrocytes against osmotic fragility follows a lipid–lipid interaction with the fatty acids.



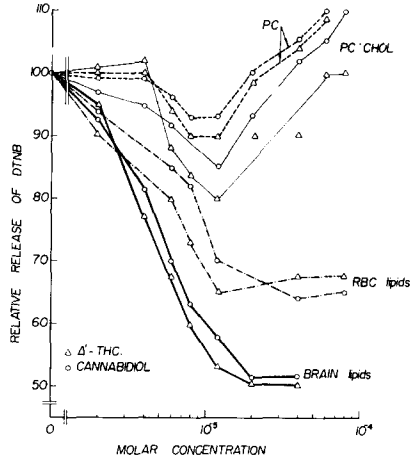


Fig. 6. Effect of stearic (18/0), linolenic (18/3) linoleic (18/2) and oleic (18/1) acids on the release in water of DTNB from liposomes made of erythrocyte lipids.

Fig. 7. Effect of Δ^1tetrahydrocannabinol and cannabidiol on the release of DTNB from liposomes made of either phosphatidylcholine, phosphatidylcholine : cholesterol (1 : 1), red blood cell (RBC) lipids or brain lipids.

Effect of cannabinoids

Δ^1-tetrahydrocannabinol is the major psychoactive hashish compound, while cannabidiol is not psychoactive [34]. The mode of action of the hashish components is not known, but common to both Δ^1-tetrahydrocannabinol and cannabidiol are interactions with various cellular membranes: erythrocytes [35], mitochondria [36] and liver lysosomes [37]. While Δ^1-tetrahydrocannabinol was found more effective than cannabidiol in stabilizing erythrocytes against osmotic hemolysis [35], the two compounds exerted similar effects on lysosome integrity [37]. It was therefore deemed of interest to utilize the liposomes, a much simpler membrane system, to compare the effect of the cannabinoids on membrane integrity. Liposomes of different lipid composition were examined and the protecting effects were compared on relative basis, when the release of DTNB in water from untreated liposomes is taken as 100%. Fig. 7 shows that the osmotic fragility of phosphatidylcholine liposomes is only slightly reduced by the cannabinoids while a greater response is presented by liposomes made of phosphatidylcholine:cholesterol (1:1). In both cases the effect is biphasic. A progressively greater effect is exhibited by liposomes derived from red blood cell lipids and particularly by liposomes from brain lipids.

The restricted protection afforded to the phosphatidylcholine liposomes may possibly be explained by a rate of hypotonic lysis which surpasses the rate of interaction with the cannabinoids. However, such a possibility is excluded, since the preincubation of the stock suspension of phosphatidylcholine liposomes with Δ^1-tetrahydrocannabinol (5 min, 22 °C) did not modify the eventual hypotonic DTNB release. The effective interaction of the cannabinoids with erythrocyte and brain lipids, as presented in Fig. 7, may indicate an affinity to particular lipids. Cardiolipin has already been pointed out as specifically required for the interaction of Δ^1-tetrahydrocannabinol with phospholipid micelles [38]. Cardiolipin, however, is apparently not

present in the erythrocyte membrane but exclusively associated with mitochondria [22]. Van Deenen and Demel [39] have demonstrated that psychoactive drugs penetrate ganglioside monolayer films but not films of other lipids (cholesterol, cerebrosides, phosphatidylethanolamine, sphingomyelin and lecithin). Thus, since gangliosides are lipid constituents of both brain and erythrocytes, their specific interaction with the cannabinoids should be further explored.

Effect of tranquilizers

Phenothiazines, clinically used as tranquilizers [40], inhibit lysosomal enzyme release, arrest hypotonic swelling of mitochondria and protect erythrocytes against osmotic hemolysis at a certain concentration range. At higher concentrations the phenothiazines lyse these membranes, exhibiting a typical biphasic effect [1]. To examine a possible lipid site of action of these tranquilizers, we tested the effect of phenothiazines on liposome fragility.

Fig. 8 shows that all three phenothiazines failed to confer stability to the phosphatidylcholine: cholesterol liposomes, but rather promoted the lysis of the liposomes. The lytic effect correlated with the clinical and the erythrocyte stabilization potency [3]. Liposomes made of phosphatidylcholine and of erythrocyte and brain lipid extracts showed similar response to the added phenothiazines. Since liposomes, in contrast to erythrocytes, are not protected against osmotic lysis by phenothiazines, it appears that the protecting site for these tranquilizers is comprised of a protein or a lipoprotein. Kriegstein et al. [41] concluded that the interaction of phenothiazines with albumin is mainly hydrophobic, determined by the paraffinic chain of the phenothiazine. We propose that the biphasic effect of the tranquilizers results from two types of interactions: (a) an interaction with a protein or a lipoprotein, leading to a decreased hypotonic hemolysis in erythrocytes, and (b) a lipidic interaction which, at higher concentration of phenothiazines, promotes lysis in liposomes and erythrocytes.

The supplement of a "protection site" in liposomes by added membrane proteins might aid not only in the elucidation of the site of action of the tranquilizers but could also serve as a sensitive tool for reconstitution of the membrane.

In conclusion, a method was adopted in this study for the quantitative evaluation of liposome osmotic fragility. The usefulness of the model system is demonstrated

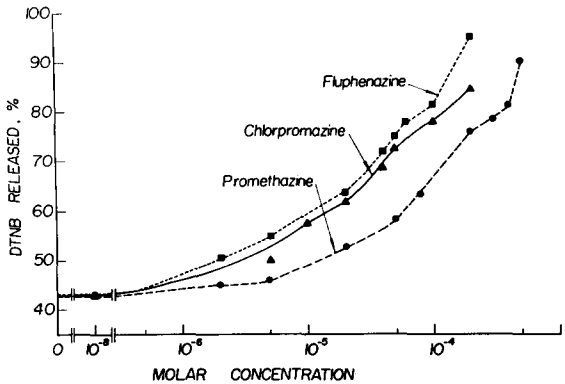


Fig. 8. Effect of phenothiazines on the release of DTNB from phosphatidylcholine : cholesterol (1 : 1) liposomes.

with respect to the effect of known antihemolytic components. Based on the analogous effects of fatty acids and cannabinoids on osmotic fragility of erythrocytes and liposomes, it may be concluded that lipid–lipid interactions underlie the stabilizing effects in both systems. The lytic, but not the stabilizing, effect of phenothiazines can be reproduced with liposomes, indicating that membrane proteins or lipoproteins are apparently required for the stabilizing effect of phenothiazines. This requirement may, in turn, form the basis for an approach to reconstitute the biological membrane.

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