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ULTRASONIC ATTENUATION MEASUREMENTS IN PHOSPHOLIPID DISPERSIONS

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SUMMARY

Ultrasonic attenuation measurements have been used to show that relaxation processes occur in the time range $1 \cdot 10^{-7}$–$1 \cdot 10^{-8}$ sec in dispersions of phosphatidylserine and, to a lesser extent, in phosphatidylcholine dispersions. The ultrasonic attenuation is modified by changes in phospholipid concentration, pH, cholesterol and divalent metal ion content. A quantitative analysis of the data is not possible, but the most probable mechanisms for the relaxation processes are intraliposome conformational changes and/or solvation equilibria.

INTRODUCTION

An appealing approach to the investigation of the structure and function of membranes in biological systems is the use of model membranes. Such models are generally considerably simpler in composition than true membranes and have been extensively studied with the hope that an understanding of the behavior of these models will provide helpful guidelines to understanding the properties of native membranes.

Phospholipids constitute a major fraction of most cell membranes, and phospholipid monolayer films have been used frequently as membrane models[1,2]. Although a simple bilayer structure for membranes[3] is not entirely consistent with recent experimental findings[4], phospholipids in membranes probably exist to a significant extent as bimolecular lamellae. Therefore, an extremely attractive model consists of closed, concentric bilayers (liposomes), which can be formed in dilute aqueous or salt solutions of phospholipids and possess permeability properties closely analogous to those of native membranes[5,6]. The liposome composition can be readily varied and the behavior of liposomes has been investigated in considerable detail[7]. Phosphatidylcholine vesicles of uniform size have been isolated recently[8] so that a well-defined liposome system is now available.

Although a variety of physical techniques has been used to characterize the properties of liposomes, relatively little information is available about their dynamic properties. In this work ultrasonic attenuation measurements have been made in suspensions of phospholipids. The results indicate that relaxation processes occur in the time range $1 \cdot 10^{-7}$–$1 \cdot 10^{-8}$ sec. A definitive molecular interpretation for the observed relaxation process is not possible, but a consideration of the effects of phospholipid concentration, pH, cholesterol and divalent metal ions has permitted some reasonable mechanisms to be advanced.

EXPERIMENTAL METHODS

Materials

Phosphatidylserine, *ex* bovine brain, was obtained from Pierce Chemicals (Folch Fraction III with extra washings) and used without further purification. Thin-layer chromatography on silica gel plates with a chloroform–methanol–acetic acid–water (25:15:4:2, by vol.) mixture as the solvent revealed the presence of small amounts of phosphatidylethanolamine and neutral lipids. Elemental analysis indicated the presence of 0.013 mole of Ca^{2+} per mole of phosphatidylserine; the amount of Mg^{2+} was negligible. (Metal ion determinations were performed in Dr. G. Morrison's laboratory using atomic absorption spectrometry.)

Phosphatidylcholine was purified from separated egg yolks. The egg yolks were shaken with methanol–chloroform (1:2, by vol.), filtered and the filtrate treated with 0.01 M $CaCl_2$. After evaporation of the solvent the residue was taken up in acetone and three diethyl ether–acetone precipitations were performed. Equal volumes of hexane and 87 % ethanol were shaken together and separated into an upper, hexane-rich phase and a lower, ethanol-rich phase. The crude phospholipid was shaken with equal volumes of upper and lower phases. The upper phase was discarded, a further volume of upper phase added and the procedure repeated 3 times. Evaporation of the lower phase on a rotary evaporator was followed by the addition of absolute ethanol to remove water. The phosphatidylcholine was dissolved in methanol–chloroform (1:1, by vol.) and was eluted with the same solvent from a neutral alumina column. Two further acetone precipitations yielded phosphatidylcholine which was shown by thin-layer chromatography to be almost completely free of phosphatidylethanolamine and neutral lipids. The purified lipid was stored in acetone solution at $-10°$ under N_2.

All other reagents were of commercial analytical grade.

Preparation of liposomes

A chloroform solution of the phospholipid was evaporated on a rotary evaporator until a thin film of the phospholipid was laid down on a round-bottom flask. Sufficient 0.005 M NaCl solution was added to yield the desired concentration, usually 1 % by wt. of phospholipid. The film was freed from the glass by shaking, allowed to swell and sonicated at low power by means of a 20 kcycles Heat Systems sonifier. Phosphatidylserine gave optically clear solutions almost at once, but phosphatidylcholine required longer sonication times and usually formed slightly cloudy dispersions. The pH was then adjusted to the desired value, usually 6.8. Mixed phospholipid and phospholipid–cholesterol dispersions were prepared by laying down a film of the mixtures from chloroform solution. Divalent metal ions were added to the phospholipids by pipetting stock chloride solution into a prepared dispersion and re-sonicating the resulting cloudy dispersion. Dispersions were stored only for short periods, under N_2 at 4°. A few deviations from this procedure are detailed at appropriate points in the text.

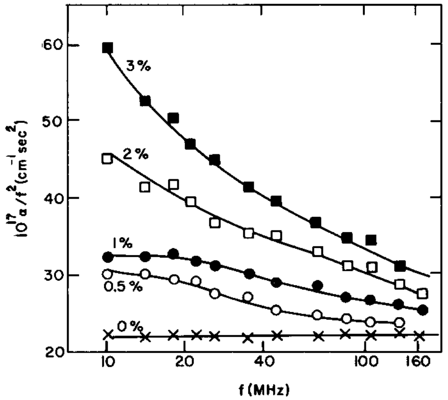
Ultrasonic measurements

Before the ultrasonic attenuation spectrum of a dispersion was measured the dispersion was allowed to equilibrate for at least 45 min. The ultrasonic attenuation spectrum at 25° was measured at eleven or twelve frequencies in the range 10–165 MHz using previously described methods[9]. The original pulse generator and receiving equipment, however, were replaced by a Matec Model 6000 pulse generator and receiver and a Model 1235A pulse amplitude monitor.

RESULTS

The presence of chemical relaxation processes occurring in a given frequency range is most easily detected by consideration of the attenuation of the sample as a function of frequency. In the absence of chemical relaxation processes the value of α/f^2 (where α is the pressure amplitude absorption coefficient and f is the frequency) is independent of frequency. Occurrence of a single chemical relaxation yields a sigmoidal plot of α/f^2 *versus* f, with an inflection point at the relaxation frequency[10]. The relaxation spectra for phosphatidylserine dispersions at various phosphatidylserine concentrations in 0.005 M NaCl solution are shown in Fig. 1; the constant value of α/f^2 in the same frequency range for the solvent alone is also indicated. An increase in the phosphatidylserine concentration increases the amplitude of the observed relaxation effect. Curves of a similar form were obtained for all the dispersions tested. These results indicate that a broad distribution of relaxation times exists, with the mean relaxation time probably occurring in the approximate range $1 \cdot 10^{-7}$–$1 \cdot 10^{-8}$ sec.

A series of control experiments were performed: no excess attenuation is observed in solutions of the inorganic salts in the absence of phospholipids; the size of the liposomes makes a negligible difference to the spectrum obtained (to vary the liposome size the dispersions were prepared by mechanical shaking, the solvent evaporation method[6] and sonication for short and long periods); finally, leaving the sus-



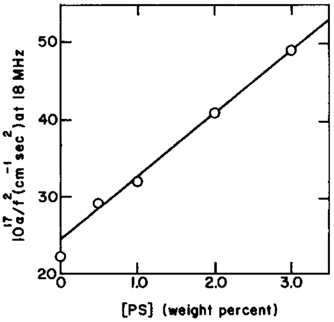
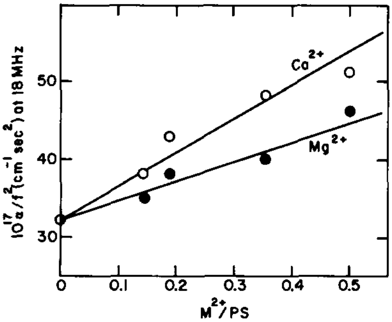


Fig. 1. Ultrasonic relaxation spectra at 25° for the following phosphatidylserine concentrations by wt. in 0.005 M NaCl solution: ■—■, 3%; □—□, 2%; ●—●, 1%; ○—○, 0.5%; ×—×, 0%.

Fig. 2. Dependence of α/f^2 at 18 MHz on phosphatidylserine (PS) concentration; the solid line has no theoretical significance.

pensions overnight causes an increase in α/f^2 similar in magnitude to the experimental uncertainties, estimated at ± 5 %.

For the sake of brevity, the complete relaxation spectra for the various dispersions are not shown. The effects of phospholipid concentration, pH, cholesterol and divalent metal ions on phosphatidylserine dispersions are summarized in Figs. 2–4,



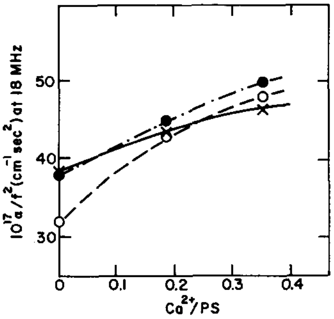


Fig. 3. The variation in α/f^2 at 18 MHz with changes in the ratio of divalent metal ion to phosphatidylserine (PS) molalities: ○—○, Ca^{2+}; ●—●, Mg^{2+}. The phosphatidylserine concentration was 1 % by wt. (11.8 mM). The lines have no theoretical significance.

Fig. 4. The effect on α/f^2 at 18 MHz of initial bulk solution pH and cholesterol for various Ca^{2+}/phosphatidylserine (PS) molal ratios: ○--○, pH 6.8, no cholesterol; ●-·-●, pH 4.0, no cholesterol; ×—×, pH 6.8, 30 mole % cholesterol. The phosphatidylserine concentration was 1 % by wt. (11.8 mM). The lines have no theoretical significance.

in which α/f^2 at 18 MHz is indicated for a variety of conditions. An increase in α/f^2 at 18 MHz monitors an increase in the amplitude of the observed relaxation (*cf.* Figs. 1 and 2). The solid connecting lines in Figs. 2–4 have no theoretical significance. Addition of divalent metal ions, particularly Ca^{2+}, or 30 mole % chloresterol to 1 % by wt. phosphatidylserine dispersions causes an increase in α/f^2 at 18 MHz, as did lowering the bulk solution to an initial pH of 4.0. The addition of Ca^{2+} was less effective when added to cholesterol-containing dispersions or dispersions at the lower pH.

In more concentrated NaCl solution (0.1 M), α/f^2 at 18 MHz increases slightly (approx. 8 %) for phosphatidylserine dispersions but decreases (approx. 12 %) for phosphatidylserine–Ca^{2+} dispersions compared with the values in dilute salt.

Dispersions of phosphatidylcholine possess a far smaller relaxation effect in the considered frequency range than did those of phosphatidylserine and little enhancement of the relaxation effect occurs upon addition of Ca^{2+} to phosphatidylcholine. A 1:1 mixture of phosphatidylserine and phosphatidylcholine behaves in a manner intermediate between that of the two individual phospholipids.

DISCUSSION

The relaxation processes observed are clearly associated with the phospholipids since excess attenuation is seen only in the presence of the phospholipids. Quantitative analysis of the data is hindered by the lower frequency bound of the instrumentation, which does not allow the complete spectra to be measured, and the distribution of relaxation times which is observed. This distribution may be due to the heterogeneity of the liposomes or may simply reflect a multiplicity of relaxation mechanisms. For multiple relaxation processes

$$\alpha/f^2 = B + \sum_{i=1}^{N} \frac{A_i\tau_i}{1+\omega^2\tau_i^2}$$

where ω is the angular frequency, B is the constant residual value of α/f^2 at high frequencies, τ_i is the relaxation time for the ith process and A_i is the associated amplitude parameter[10]. Accurate estimates of the relaxation times cannot be made from the data obtained so that variations in α/f^2 may be due to changes in either the A_i or τ_i. Impurities in the lipid preparation (*e.g.* oxidation products) may influence the results reported, although the fact that quantitatively similar results were obtained over an extended time period, during which the amount of oxidation would vary, suggests this is not a dominant factor.

Ultrasonic relaxation effects can be observed, in principle, for any chemical reaction having a non-zero enthalpy or volume change. For phospholipid dispersions the mechanistic possibilities can be divided into four main classes: liposomal aggregation, the breaking and reforming of liposomes, intraliposomal conformational changes and equilibria in the hydration sphere[11] of the liposomes. Other reactions may occur in the presence of metal ions and these will be considered shortly. We now discuss the feasibility of the above mechanisms accounting for the observed ultrasonic relaxations in phosphatidylserine dispersions.

Aggregation undoubtedly occurs to some extent. However, the total concentration of the negatively-charged phospholipid is approx. 0.01 M and a fair estimate of the liposome concentration is 1–10 μM (ref. 8). The mean relaxation time must lie near the range 10^{-7}–10^{-8} sec and it appears unlikely that aggregation equilibria would involve sufficiently rapid time constants for such dilute liposome dispersions. Further, the smaller relaxational effect for the zwitterionic phosphatidylcholine dispersions and the independence of the spectrum upon liposome size argue against aggregation being responsible for the data. As shown in Fig. 2, α/f^2 possesses a near linear dependence upon phosphatidylserine concentration. Such a dependency is consistent with the occurrence of a unimolecular process (or processes); for such a reaction, τ is independent of concentration and A is a linear function of concentration. For a single-step mechanism A is proportional to $m\Gamma(\Delta V - \beta\Delta H/\rho C_P)^2$ to a good approximation, where m is the total reactant concentration, β is the thermal expansion coefficient, ρ is the density, C_P is the constant-pressure specific heat and ΔV and ΔH are the volume and enthalpy changes involved in the reaction. The quantity Γ is a function of the equilibrium constant and is independent of concentration only for unimolecular reactions[10]. For a process such as $A \rightleftharpoons B$, $\Gamma = K/(1+K)^2$ where K is the equilibrium constant: $K = p/(1-p)$, where p is the extent of reaction, $(B)/[(A)-(B)]$. Hence the linear relationship observed in Fig. 2 also suggests that aggregation is not responsible for the relaxation effects.

Ion permeability studies have shown that trapped ions diffuse slowly across the bilayers with a high activation energy and a high degree of discrimination between univalent ions[5,6]. These findings are not consistent with an hypothesis in which the liposomes are continuously breaking and reforming. Liposomes are quite stable structures and the critical micelle concentration is known to be very low[12]; thus lipid monomers are unlikely to be breaking away from the liposomes. An inversion between an oil-in-water configuration and its converse has been suggested in the presence of divalent metal ions[13,14]. Such a drastic configurational change must surely be rather slow; moreover X-ray diffraction studies have indicated that the lamellar form is still preferred even in the presence of Ca^{2+} (ref. 15).

Thus the most likely causes of the ultrasonic relaxations are hydration processes and conformational changes within the liposome. Although, in principle, these two types of processes cannot be distinguished, a reasonable explanation of all the data can be given if the relaxation processes are assigned primarily to intraliposome conformational changes. The smaller amplitudes of the relaxations observed with phosphatidylcholine dispersions indicate that the net negative charge present on phosphatidylserine molecules at pH 6.8 is of importance. The effect of divalent metal ions, and of a lower pH, is to neutralize this net charge. Addition of divalent metal ions or lowering the pH causes the value of α/f^2 at 18 MHz to increase markedly (Figs. 3 and 4). Moreover, Ca^{2+} is less effective in increasing α/f^2 at 18 MHz at pH 4.0, where phosphatidylserine is almost neutral, than at pH 6.8, where phosphatidylserine is charged (Fig. 4). Neutralization effects also can account for the decreased attenuation observed in high salt concentrations. The effect of addition of divalent metal ions and lowering pH may be to shift an equilibrium between charged and neutral forms of the liposomes in such a way that α/f^2 at 18 MHz is increased. This effect is less at high salt concentrations because of the decreased interaction between Ca^{2+} and the liposome. A tightening of the liposome structure is known to occur upon charge neutralization and the equilibrium being disturbed could be between loose (negatively-charged) and tight (neutral) forms of the liposomes. Tightening could occur between adjacent lipids, adjacent lamellae or both. The increase in α/f^2 at 18 MHz upon neutralization can be explained if the loose form predominates at pH 6.8 in the absence of divalent metal ions, since neutralization will then increase the amplitude of the relaxation process through the factor Γ. This can be easily visualized by reference to Fig. 5, in which Γ is plotted against the extent of reaction, or fraction of liposomes in the tight form, p; Γ goes through a maximum at $p = 0.5$ so that neutralization, which shifts the loose $\rightleftharpoons$ tight equilibrium towards the tight form, increases Γ and hence α/f^2. (This assumes an approximately constant relaxation time, which is true if one of the rate constants remains essentially constant and appreciably greater than the other when the extent of reaction is altered.)

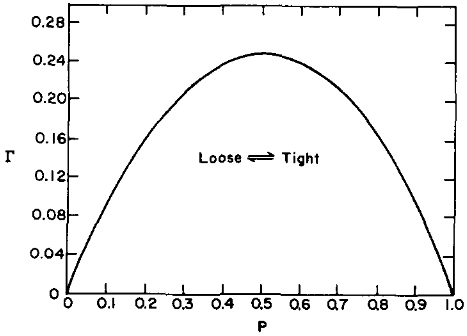


Fig. 5. The variation of Γ (see text and ref. 10) for the unimolecular process loose $\leftrightarrows$ tight as a function of the fraction, p, of liposomes in the tight form.

The effect of cholesterol (Fig. 4) can be interpreted as shifting the equilibrium towards the tight form by "solidifying" the hydrocarbon tails and/or diluting the surface charge. Finally, the small relaxation amplitudes observed with the neutral, zwitterionic phosphatidylcholine liposomes could be due to the fact that the tight form predominates under all conditions, and therefore Γ would be very small (*cf.* Fig. 5). However, the net charge of the liposome cannot be the only factor determining the liposome structure involved in the observed relaxation processes since an appreciable attenuation is observed with phosphatidylserine at pH 4 where it is almost neutral.

The tightened structure brought about by Ca^{2+} that has been postulated here need not be incompatible with the increased ion permeability associated with Ca^{2+} in some instances. PAPAHADJOPOULOS AND OHKI[16] have discussed the often contradictory properties of Ca^{2+}, and BANGHAM AND PAPAHADJOPOULOS[17, 18] earlier illustrated that an increase in the diffusion rates of Na^+ and K^+ only occurs above a critical Ca^{2+} concentration, suggested to be the equivalence point.

Relaxation processes due to metal-ligand formation present an alternative explanation for the effect of divalent metal ions. However, for such reactions, Ca^{2+} and Mg^{2+} generally possess rate constants which differ by two orders of magnitude (*cf.* ref. 10). Since the data obtained suggest that rather similar time constants are involved for the two metals (Fig. 3), direct metal–ligand interactions are probably not the cause of the observed relaxation process. Nevertheless, it cannot be ruled out that the effect of metal ions arises from equilibria involving metal-promoted complexes or aggregates. Such complexes have been shown to occur and a hypothetical structure advanced[2]. The rather greater enhancement of the relaxation effect caused by Ca^{2+} compared with Mg^{2+} may be related to the greater stability of Ca^{2+}–acidic phospholipid complexes[19].

Relaxation spectra due to perturbation of equilibria involving the hydration spheres of macromolecules have been observed[20] and could be responsible for the results reported here. CHAPMAN *et al.*[11] have shown that phospholipids bind a significant amount of highly structured water quite tightly. Since the effect of charge neutralization and cholesterol cannot be predicted for such a mechanism, the plausibility of this mechanism cannot be further assessed.

In conclusion, relaxation processes with associated relaxation times of 10^{-7}–10^{-8} sec have been observed in phospholipid dispersions. Moreover, these processes are modified under conditions which alter membrane properties. A mechanism involving an equilibrium between tight and loose forms of the liposomes is compatible with all the data. However, it is not possible to assign an unambiguous mechanism at present, and other mechanisms (*e.g.* solvation equilibria) are also quite plausible. In any event these results suggest that relaxation methods may permit the dynamic properties of membranes to be probed.

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