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Kinetics of Water Penetration into Unsonicated Liposomes

Effects of *n*-Alkanols and Cholesterol

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Summary. The rate of swelling of egg lecithin liposomes under osmotic shock has been studied employing a stopped-flow spectrophotometer. Incorporation of cholesterol and simple alcohols into the liposomal structure elicits a biphasic response in swelling rate: at low concentrations these additives increase but at high concentrations they decrease water permeabilty. For simple *n*-alkanols, the effects can be correlated with structure. Specifically, the concentration of alcohol required to elicit maximal permeability as well as the maximal permeability decreases with increasing length of the alcohol. These effects are accounted for on the basis of modification of the orientation and packing of lecithin molecules in the bilayer membrane of the liposome.

The closed concentric multilamellar structures formed by mechanical or sonic dispersion of phospholipids in aqueous media, liposomes, have proved to be useful models in studies designed to increase understanding of the permeability properties of biological membranes. For example, the kinetics of transport of water (Bangham, DeGier & Greville, 1967; Hammes & Schullery, 1970; Bittman & Blau, 1972), nonelectrolytes (Bangham, Standish & Watkins, 1965; Demel, Kinsky, Kinsky & Van Deenen, 1968; Cohen & Bangham, 1972), and electrolytes (Bangham *et al.*, 1965; Papahadjopoulos & Watkins, 1967; Vanderkooi & Martonosi, 1971) across the liposomal membrane have been determined and related to corresponding findings obtained with biomembranes. Quite generally, results obtained accord with those expected on the basis of multilamellar lipid bilayers as the permeability barrier (Sessa & Weissmann, 1968; Van Deenen, 1971).

The rate of swelling or shrinking of liposomes following osmotic shock, which may be followed spectrophotometrically, is sufficiently rapid so that best results are obtained employing stopped-flow methods. The earliest studies in this field, carried out by Bangham and his co-workers (1967), employed a simple mechanical means of mixing following osmotic shock

which did not permit measurements to be made for about 2 to 5 sec following initiation of transmembrane water flow. Subsequent measurements have, however, established the utility of stopped-flow methods for collecting data beginning a few milliseconds after osmotic shock (Bittman & Blau, 1972). A related technique has been employed in the studies described in this manuscript.

The basis of the spectrophotometric assay for the kinetics of liposome swelling or shrinking is the assumption that changes in liposomal volume are directly proportional to the first power of the reciprocal of changes in optical absorbance (for those wavelengths at which absorbance is due entirely to scattered light). This was demonstrated to be the case for liposomes by Bangham *et al.* (1967). To relate volume changes in liposomes to water flow across the liposomal membrane it is further required that liposomes behave as ideal osmometers; that is, that the liposomes are permeable only to water and not to solutes. This assumption, too, appears to be valid (Bangham *et al.*, 1967; Rendi, 1967; Bittman & Blau, 1972). Consequently, initial changes in absorbance can be employed as measures of initial liposomal volume changes resulting from water influx or efflux under osmotic shock.

In a recent study, Bittman and Blau (1972) have demonstrated that the permeability of liposomes to water is a sensitive function of the degree of unsaturation of the fatty acyl residues of the constituent phospholipids, the temperature, and the liposomal cholesterol content. It appears likely that other variables which affect the fluidity and organization of the hydrocarbon chains of the lipid bilayer will also affect the rate of water permeation. As discussed in detail later, the nature and concentration of alcohols is such a variable. The bulk of the experimental work described herein is focused on this matter: as anticipated, simple alcohols have been observed to markedly influence the rate at which liposomes swell under hypoosmotic shock.

Materials and Methods

Egg lecithin was prepared by the method of Singleton *et al.* (1965). All preparations employed yielded one single spot following thin-layer chromatography in several solvent systems. Liposomes were prepared from egg lecithin according to the following procedure. All stages of the preparation procedure were carried out in an atmosphere of nitrogen. A known quantity of lecithin dissolved in chloroform was transferred to a round-bottom flask which had been previously flushed with nitrogen. The chloroform was removed through evaporation under a stream of nitrogen; the final traces of this solvent were removed under vacuum. A quantity of degassed 0.05 M KCl solution sufficient to yield a final lecithin concentration of 4 mM was added to the flask along with 4 glass beads of 3 mm diameter. Dispersion of the lecithin was initiated by swirling the flask manually for approximately 3 min. The preparation was then permitted to remain at room tem-

perature for 30 min following which it was shaken vigorously for 30 sec. on a Vortex apparatus at the maximum setting (250 rpm). The milky white preparations of unsonicated liposomes obtained, which had an optical density at 450 nm near 2.6, were permitted to stand at room temperature for 60 to 90 min prior to their use in kinetic experiments. Liposomes once prepared were employed within 8 hr.

Use of liposomes prepared from egg lecithin alone yielded some experimental difficulties. These included a tendency of the liposomes to aggregate on aging and to deteriorate in the presence of added *n*-alkanols. These difficulties could be alleviated by the incorporation of 1 to 4 mole per cent of dicetylphosphate into the liposomes. Kinetic consequences of addition of dicetylphosphate to egg lecithin liposomes are discussed below (*see* Results).

The rate of swelling of egg lecithin following hypoosmotic shock was followed spectrophotometrically at 450 nm employing a Durrum-Gibson stopped-flow spectrophotometer equipped with 10:1 ratio drive syringes (kit No. 13838, Durrum-Gibson, Inc.) (Hammes & Schullery, 1970; Forster, 1972; Bittman & Blau,1972). The nozzle of the smaller of the drive syinges was enlarged to a diameter of 1.5 mm. The rate of change of transmittance was read directly off the oscilloscope screen. The theoretical basis for these measurements has been developed in detail earlier (Bangham *et al.*, 1967; Bittman & Blau, 1972). All kinetic measurements of initial rates were completed within a time period of 5 sec or less; consequently, the formation of unstirred layers around the liposomal membranes is not considered a serious problem; formation of such layers greatly complicates the interpretation of the kinetic data. The use of the 10:1 ratio drive syringes, which permit one volume of liposome suspension to be mixed with 10 volumes of water, was found to be important in obtaining reproducible kinetic data. The use of the usual 1:1 ratio drive syringes provides a much smaller hypoosmotic shock, resulting in a lowered rate of liposomal swelling and less accurate kinetic data.

All kinetic measurements were made at 25 °C. Water which was continuously circulated through a carefully thermostated bath was employed to maintain the drive syringes and their contents at this temperature. Effective temperature control of the mixing system was found to be crucial to obtaining reliable kinetic data: failure to adequately thermostat the system results in light scattering due to temperature-gradient mixing. This is a possible source for spurious optical density changes noted by Bittman and Blau (1972) which occurred within a few milliseconds after the initiation of mixing in their system. All kinetic measurements were followed employing a slit width of 1.0 mm. Control experiments indicated that light transmission at 450 nm through liposome suspensions is nearly independent of slit width from 0.7 to 5.0 mm. Over this range kinetic results are not sensitive to choice of slit width.

Each measurement of the zero-order rate of liposome swelling under hypoosmotic shock was repeated 5 to 8 times. Provided that all precautions mentioned above concerning the details of both preparation of the liposomes and performance of kinetic measurements were observed, variation in sucessive readings usually did not exceed 10%. For example, in a typical set of kinetic runs employing the same liposome preparations and the same experimental conditions, slopes of voltage change against time were observed to 8, 9, 8, 8, 9, 8, 10, and 8 mV sec^{-1}. Typically, each data point for the rate of swelling was obtained as mean of 5 to 10 measurements on the same liposome preparation. The data scatter is indicated by vertical bars.

The accuracy of individual measurements is limited by the resolution permitted by gradations on the display screen of the oscilloscope employed to record the course of the swelling. Even with the precautions employed in liposome preparation, considerable differences were noted in the rate of swelling of different liposome preparations even though their initial transmittance was identical. These differences were sometimes as large as 25%. The ratio of the initial rate to the extent of change in transmittance ($\delta T/\Delta T$ in sec^{-1})

was, however, found to be fairly reproducible (within 10%). Both Bangham *et al.* (1967) and Bittman and Blau (1972), have used the term $\frac{1}{A^2} \cdot \frac{dA}{dt}$ to relate permeability of liposomes to the observed change in scattering (A is absorbance of liposomes). In our experience a scatter of about 25% in dA/dt and $\frac{1}{A^2} \cdot \frac{dA}{dt}$ terms is due to differences in the amount of "sealed" vesicles from preparation to preparation. This difference is, however, accommodated by relating permeability to the ratio of rate to extent of swelling at constant transmittance.

Results

The overall goals of this study include the measurement of the effect of cholesterol and simpler alcohols on the permeability of lecithin liposomes to water. Permeability is assessed by measurement of the time course of swelling of the liposomes following osmotic shock: some typical plots of such measurements are provided in Fig. 1. From data of this type two parameters were

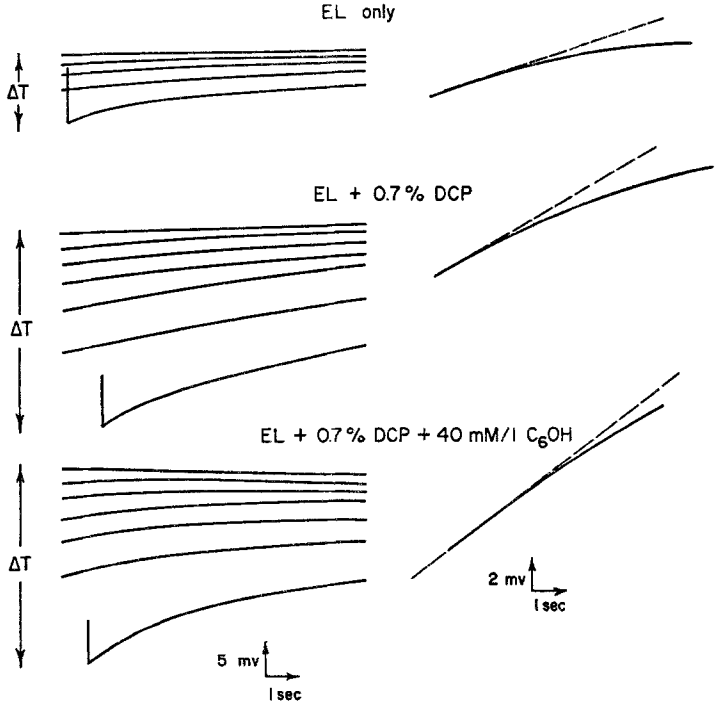

Fig. 1. Typical oscilloscope traces showing transmittance as a function of time following mixing of 0.2 ml of unsonicated liposomes of egg lecithin (4 mg/ml) in 0.05 M KCl with 2 ml of distilled water. On the left are shown complete traces for liposomes containing only egg lecithin (EL), egg lecithin plus dicetylphosphate (DCP), and egg lecithin plus dicetylphosphate plus *n*-hexanol (C_6OH). Values of ΔT shown on the ordinate measure the total transmittance change observed. On the right are shown plots of transmittance against time for the early stages of each run. Note that the transmittance change is linear in time for the first 2 to 3 sec following mixing. Values of the rate of swelling δT were measured from the slope of such plots

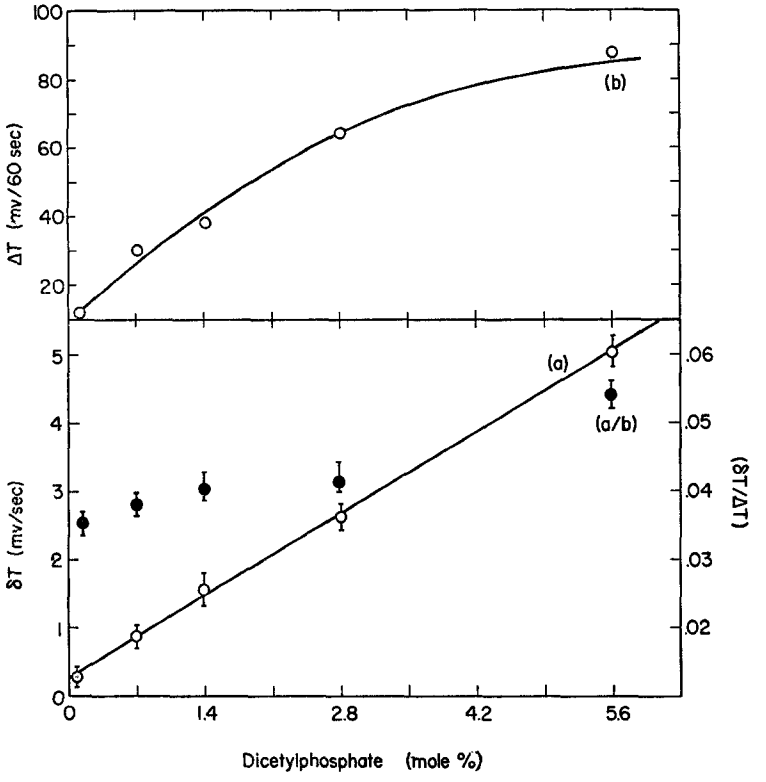

Fig. 2. Plots of rate of liposomal swelling δT (open circles, below), extent of swelling ΔT (open circles, above), and their ratio $\delta T/\Delta T$ (closed circles, below) following hypoosmotic shock plotted as a function of the mole fraction of dicetylphosphate incorporated into the egg lecithin liposomes. Osmotic shock was produced by mixing one volume of liposomes, lecithin concentration 4 mg/ml, in 0.05 M KCl with ten volumes of distilled water

routinely extracted: (*i*) the initial rate of liposomal swelling δT measured from the slope of transmittance change against time and (*ii*) the extent of liposomal swelling ΔT measured from the total transmittance change, which is usually complete within 60 sec (*see* Fig. 1).

Successful pursuit of these studies required a liposomal system for which reliable kinetic data could be routinely obtained. As noted above, certain experimental difficulties can be overcome by incorporation of small amounts of dicetylphosphate into the lecithin liposomes; this accords with the experience of Bittman and Blau (1972). As may be judged from the plots of transmittance against time in Fig. 1, dicetylphosphate affects both the initial rate and total extent of liposome swelling. Results of a systematic study of the effect of dicetylphosphate content on the rate and extent of liposome swelling are shown in Fig. 2. Both the rate and extent of swelling increase

monotonically with increasing dicetylphosphate content. However, the ratio of these, which is the most important measure of the effect of additives on the kinetics of swelling, is relatively insensitive to content of this anionic surfactant.

Effect of Cholesterol on the Water Permeability of Liposomes

The rate of swelling of liposomes containing a variable proportion of cholesterol is plotted as a function of cholesterol content in Fig. 3. Note that the ratio of initial rate to total extent of swelling first increases and then decreases with increasing mole fraction of cholesterol. The maximum in water permeability is reached at a cholesterol mole fraction near 0.1. The liposomes employed in this study did not contain added dicetylphosphate. The effect of cholesterol observed in this study is consistent with previous observations (Bittman & Blau, 1972) insofar as they are comparable. Specifically, it has been previously observed that addition of cholesterol to egg lecithin liposomes reduced the water permeability; a maximum of threefold

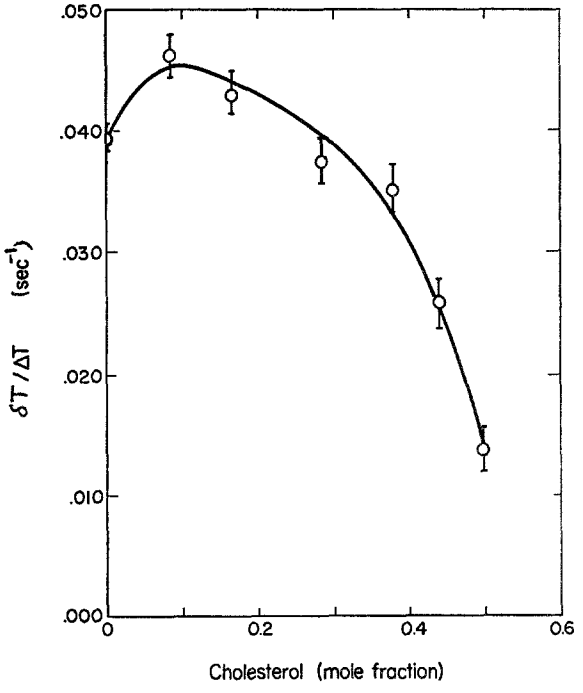

Fig. 3. Plot of the ratio of the rate of liposome swelling to the total extent of liposome swelling $\delta T/\Delta T$ following osmotic shock as a function of the mole fraction of cholesterol incorporated into egg lecithin liposomes. Lecithin concentration in the liposome preparation was 4 mg/ml

decrease is observed at a lecithin/cholesterol mole ratio of unity, similar to that observed in this study. Bittman and Blau did not detect the increase in permeability observed at low cholesterol content since they did not investigate systems containing less than a 0.2 mole fraction of cholesterol.

Effect of n-Hexanol on Water Permeability of Liposomes

Known amounts of *n*-hexanol were incorporated into lecithin liposomes containing small amounts of dicetylphosphate through direct addition of the alcohol to the reservoir syringe of the stopped-flow apparatus followed by magnetic stirring. Successful mixing of *n*-hexanol with liposomes is best accomplished within the reservoir syringe since this avoids complications (actually encountered) arising from lipid oxidation when mixing was carried out in contact with atmospheric oxygen. Control experiments established that the mixing procedure itself did not affect the liposomes structurally as reflected in their transmittance at 450 nm and their rate of swelling following osmotic shock. Moreover, liposomes with concentrations of added *n*-hexanol varying from zero to 0.05 M did not exhibit detectable swelling or shrinking when mixed with isotonic KCl containing *n*-hexanol over the same concentration range.

In Fig. 4, the total extent of swelling, the zero-order rate of swelling, and the ratio of the two, for lecithin liposomes subjected to osmotic shock are plotted as a function of the concentration of added *n*-hexanol. Except for the highest concentrations of alcohol employed, the extent of swelling ΔT is not detectably affected by *n*-hexanol content. Although addition of *n*-hexanol at these concentrations undoubtedly increases leakage of KCl to some extent, the change in leakage rate is simply too small to modify water permeability. At higher concentrations, the extent of swelling does decrease indicating that such leakiness has become important. At the highest concentrations of *n*-hexanol employed, it is possible that the method employed to follow the kinetics may yield equivocal results, although this possibility does not appear to be strong. Were high concentrations of alcohol to induce a structural change in the smectic mesophase, this would be revealed in the initial measurements of transmittance. However, it is possible that osmotic shock of alcohol-treated liposomes might induce a structural change (i.e., smectic mesophase to micelles) as well as a size change. Consequently kinetic data obtained at the highest concentrations of alcohol employed should be viewed with some caution.

The rate of liposomal swelling, as well as the ratio of rate to extent of swelling, first increases and then decreases with increasing concentration of

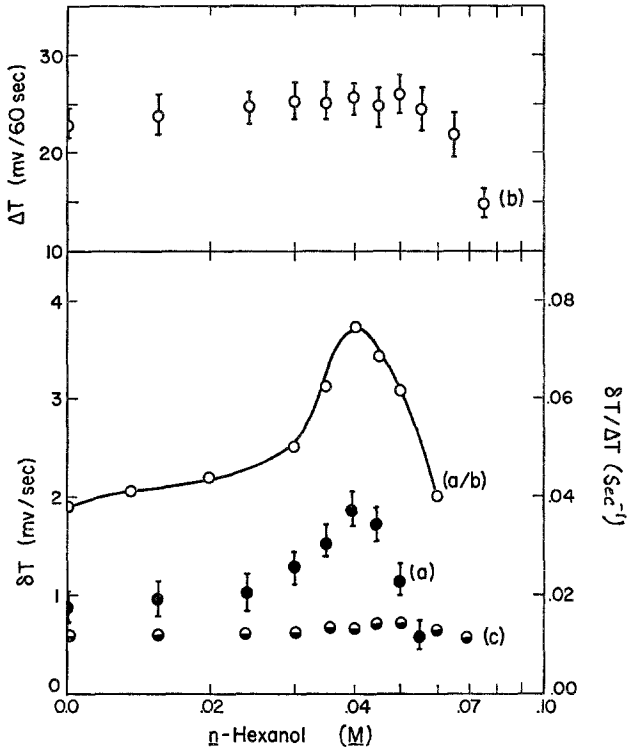

Fig. 4. Plots of rate of liposome swelling δT (filled circles, below), extent of swelling ΔT (open circles, above), and $\delta T/\Delta T$ (open circles, below) following osmotic shock as a function of the concentration of *n*-hexanol, which was added at the indicated concentrations to the liposomes prior to osmotic shock. Liposomes were prepared from egg lecithin (4 mg/ml) containing 0.7 mole per cent dicetylphosphate in 0.05 M KCl and were shocked by mixing with distilled water in a 1:10 ratio. Liposomes prepared from egg lecithin, cholesterol, and dicetylphosphate, 100:100:4 mole per cent, swell at a rate which is independent of the concentration of *n*-hexanol (half-filled circles, below) under the same experimental conditions

n-hexanol (Fig. 4). It is particularly interesting to note that this behavior is not observed for liposomes containing equimolar quantities of lecithin and cholesterol: in this case, the rate of swelling is independent of *n*-hexanol concentration (Fig. 4). Control experiments indicate that the content of dicetylphosphate did not change the response of the rate of liposomal swelling to the addition of *n*-hexanol.

Effect of Other Alcohols on the Water Permeability of Liposomes

In Fig. 5, the ratio of rate to extent of liposomal swelling under osmotic shock is plotted as a function of the concentration of several simple alcohols. In a qualitative sense, the results obtained are similar to those found for *n*-

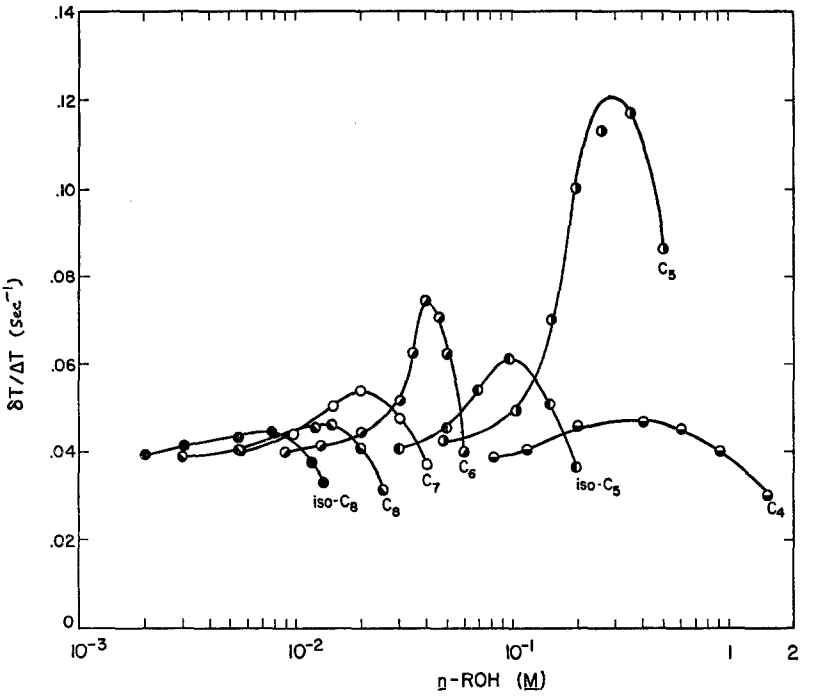

Fig. 5. Plots of the ratio of rate of liposome swelling to extent of swelling $\delta T/\Delta T$ as a function of the concentration of several alcohols. The liposome preparation contained 4 mg/ml lecithin and 0.7 mole per cent dicetylphosphate in 0.05 M KCl. Swelling was initiated by mixing one volume of liposomes with 10 volumes of distilled water. Alcohols were added to the liposome preparations at the indicated concentrations prior to osmotic shock. All data points are the mean of 5 to 7 individual measurements. C_4, *n*-butanol; C_5, *n*-pentanol; C_6, *n*-hexanol; C_7, *n*-heptanol; C_8, *n*-octanol; iso-C_5, 2-pentanol; iso-C_8, 2-octanol

hexanol (Fig. 4). Specifically, the alcohols tend to increase water permeability at low concentrations and to decrease it at higher ones. The following specific features of this data are important. First, the concentration of alcohol required to elicit maximal permeability to water decreases with increasing number of carbon atoms. These concentrations are plotted as a function of their *n*-octanol/water partition coefficients in Fig. 6; a good linear relationship is observed. Second, although an exact comparison of the maximal swelling rates elicited by the various alcohols is not meaningful since all of the data could not be obtained with a single liposome preparation, the data do suggest that the maximal swelling rates increase with decreasing length of the alcohol (with the exception of *n*-butanol). Third, significant differences between the effects of isomeric alcohols exist; compare the profiles for *n*-pentanol and 2-pentanol and those for *n*-octanol and 2-octanol.

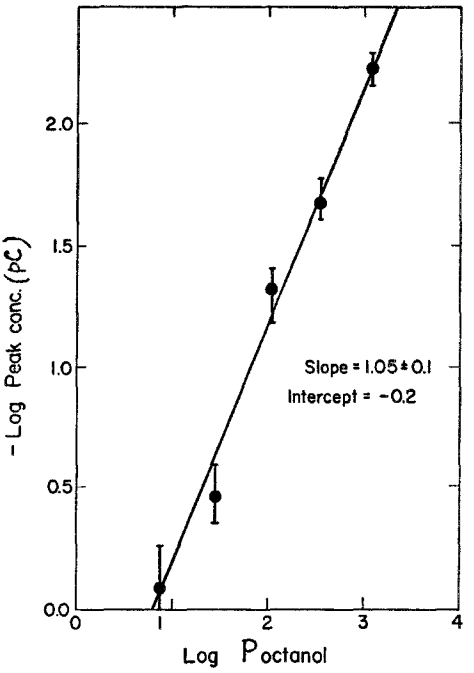

Fig. 6. Plot of the negative logarithm of the alcohol concentration required to elicit the maximal rate of liposomal swelling *pC* against the logarithm of the *n*-octanol/water partition coefficient $P_{octanol}$, for a series of *n*-alkanols

Discussion

Results presented above confirm the observations of Bittman and Blau (1972) concerning the suitability of stopped-flow methods for study of the kinetics of liposome swelling under osmotic shock. Due to the increased time resolution, stopped-flow methods are clearly superior to ordinary mechanical mixing for studies of this type (Bangham *et al.*, 1967). It should be emphasized that optimal use of the stopped-flow technique requires that conditions be carefully controlled. For example, Bittman and Blau (1972) consistently observed an artifact in the course of the reaction during the first 0.15 to 0.20 sec. With the use of 10:1 ratio drive syringes and by increasing the nozzle bore of the smaller syringe, we found it possible to eliminate this complication. Stopped-flow techniques have also been employed to follow the rate of swelling of red blood cells: a complicated but reproducible series of changes in light scattering is observed (Blum & Forster, 1970; Sha'afi *et al.*, 1967, 1970). In part, these changes appear to reflect artifacts resulting from stoppage of the net forward flow of the red blood cells in the apparatus. One can

attempt to minimize the importance of artifactual results through a series of control measurements which involve mixing of cells or liposomes but without change in osmolarity of the medium. Effects due to mixing alone can then be subtracted from the totality of changes observed, presumably leaving just those due to cellular shrinking or swelling. In our system such corrections proved unneccessary since the mixing process in the absence of a change in medium osmolarity did not result in a detectable change in light scattering.

In accord with the previous observations (Bangham *et al.*, 1967; Bittman & Blau, 1972), liposomes bearing a net negative charge, which increases the innate volume of the liposomes by a factor of 2 to 3 (Bangham *et al.*, 1967), are more suitable structures for measurements of water permeability than are those which bear no net charge. The presence of a net negative charge not only yields more stable liposomes but also increases both the initial rate and total extent of swelling to approximately equal extents (Fig. 2).

As cholesterol content is increased in the lecithin liposomes, there is an increase in swelling rate which reaches a maximum at about 10 mole per cent cholesterol (Fig. 3). The reason for this increase in rate of swelling is not known. However, it may be pertinent to point out that there is a large increase in long spacing from 70 Å (at zero cholesterol content) to 81 Å (at 7.5 mole per cent cholesterol) in dipalmitoyl-lecithin (Ladbrook, Williams & Chapman, 1968). On addition of more cholesterol, the long spacing eventually decreases to a constant value of 64 Å at 50 mole per cent. The initial increase in long spacing has been attributed to a change in configuration of hydrocarbon chains from a tilted to a perpendicular position and an increase in the thickness of the water layer. How these changes are expected to affect water permeability must await clarification of the mechanism of water permeation. It is, however, interesting to note that the effect of cholesterol on water permeability across black lipid membranes (BLM) is different than that observed for liposomes. In BLM, the permeability drops monotonically and almost linearly as cholesterol mole fraction increases from 0 to 0.8 (assuming that the composition of the BLM reflects that of the lipid mixture from which they were formed) (Cass & Finkelstein, 1967). This difference is consistent with the view that the state of cholesterol in BLM is different than in the bilayer of liposomes. Other components of BLM may modify the mode of interaction and organization of cholesterol and lecithin, as compared to that of cholesterol + lecithin alone[1]. The decrease in water permeability of lecithin liposomes observed when cholesterol content is increased beyond 10 mole

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per cent, Fig. 3, has been attributed to decreased fluidity of the liposomal bilayer on the basis of a solubility-diffusion mechanism for water permeation (Bittman & Blau, 1972).

A variety of alcohols are incorporated into liposomes prepared from egg lecithin. Liposomes so modified exhibit complex permeability characteristics (*cf.* Figs. 4 and 5). As the concentration of alcohol increases in the medium, there is an increase in the initial rate of swelling following hypoosmotic shock, indicative of an increased water permeability. At low alcohol concentrations, the modified membranes do not appear to become permeable to KCl. However, a decreased rate and extent of swelling was observed at higher concentrations of alcohol which may be indicative of an increased permeability of the membrane to KCl.

The significance of these results can be best grasped in terms of a suggestion made by Hansch and Dunn (1972). According to these authors the membrane/buffer partition coefficient of neutral drugs correlates very well with the octanol/buffer partition coefficients of these drugs. However, the measurement of membrane/buffer partition coefficient is experimentally difficult. If it is assumed that *optimal response* means equivalent numbers of modifier molecules in the membrane phase, one can relate the applied concentration (C_a) to *n*-octanol/water partition coefficient as follows:

$$\log 1/C_a = a \log P_{\text{oct}} + b$$

where a and b are constants.

Alcohols are known to induce a variety of effects on BLM and biomembranes (*see* Hansch & Dunn, 1972; Seeman, 1972). The values of a obtained in these various systems are 0.9 to 1.2. This suggests that these systems are most sensitive to hydrophobic effects, and that the environment in which alcohol molecules are located, while inducing their effect, is almost the same in both BLM and biomembranes. The values of b (intercept) have a much wider range of values. This indicates that the concentrations of alcohols needed to induce a particular effect have a much wider range than a (*see* Skou, 1958; Roth & Seeman, 1971; Jain & Cordes, 1973*a*, *b*). Probably the most meaningful comparison is between the effect of *n*-alkanols on the rate of hypoosmotic swelling reported in this paper and on the electrical conductance of BLM (Gutknecht & Tosteson, 1970). The presence of aliphatic alcohols in the aqueous solutions bathing BLM (made from red cell lipids containing cholesterol and *n*-decane) produce reversible changes in ionic permeability but not osmotic permeability. These changes in BLM are observed at much higher concentrations (smaller values for the intercept actually observed in Hansch plots) of *n*-alkanols than the concentrations at which

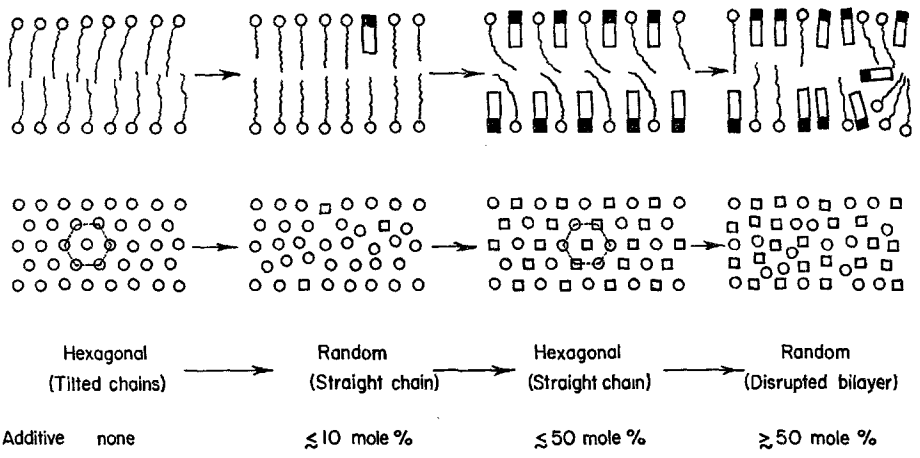

Fig. 7. A hypothetical model to illustrate changes induced by additives in the phospholipid bilayer. Unmodified bilayer is characterized by hexagonal arrangement and the hydrocarbon chains are tilted from the plane of the bilayer. Insertion of the additives (up to 10 mole per cent) causes straightening of the chains and disruption of the hexagonal lattice. Certain additives (such as cholesterol) can be incorporated to 0.33 and 0.50 mole proportion without causing disruption of the bilayer. In these cases the hexagonal lattice is restored. However, generally speaking, relatively high mole fraction of an additive would disrupt a bilayer causing lysis. *See* text for further details

maximal swelling rates are observed for liposomes. The difference is comparable to that observed in red cells. At lower concentrations *n*-alkanols (like several other drugs) not only protect red cells against lysis (Roth & Seeman, 1971) but also increase their rate of swelling in hypotonic media (Seeman *et al.*, 1970). At higher concentrations, however, these same agents cause lysis (Netsky & Jacobs, 1939; Jacobs & Willis, 1947; Ponder, 1947). The latter effects are comparable to the conductance increases across BLM.

A speculative model which can account for the observations made in this study is provided in Fig. 7. In terms of this model, the increase in rate of liposomal swelling elicited by low concentrations of cholesterol and *n*-alkanols is considered to be the consequence of the loosening of interchain packing through diminishing the tilt of the hydrocarbon chains. The subsequent decrease in water permeability with further increases in cholesterol content is consistent with the view that 1 : 1complexes of lecithin and cholesterol have better packing characteristics than lecithin alone. In contrast, high concentrations of *n*-alkanols as well as cholesterol induce leakiness into liposomes and other vesicular structures through disruption of the hexagonal lattice structure. This change in organization has been described as membrane

expansion, "fluidization," and disordering in the literature (Johnson & Bangham, 1969; Seeman *et al.*, 1971; Patterson *et al.*, 1972; Talkowski & Hamilton, *unpublished observations*). Our proposal (Fig. 7) implies that alcohols and other anesthetics induce their action by diminishing the tilt of hydrocarbon chains in a bilayer. Such a change in orientation of lipid molecules in the bilayer could also alter the bahavior of membrane-bound proteins (Fourcans & Jain, 1973) including Na-K-ATPase (Jain & Cordes, *unpublished observations*) and galactose permease[2], both of which show rate enchancement at low concentrations of *n*-alkanols. Such an effect could be induced only when the void volume of the bilayer has been "filled". Indeed, Seeman and co-workers (1971) have demonstrated that, irrespective of their structure, a large variety of drugs exert their effect when their concentration in the membrane reaches about 0.03 M/kg of membrane. The hypothesis we have proposed also implies that microviscosity and mobility in the "membrane phase" as a whole would undergo a characteristic change under the influence of various agents. It is conceivable that this change in microviscosity and mobility triggers activation and inactivation of membrane-bound functional molecules.

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