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LIPID COMPOSITION AND PERMEABILITY OF LIPOSOMES

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SUMMARY

1. Layered latticed liquid crystals (liposomes) in dilute electrolyte solutions have been prepared from a number of synthetic lecithins with variations in length and number of double bonds of the paraffin chains. Using the property of these structures to act as practical, ideal osmometers, their permeability behaviour towards glycerol and erythritol has been studied. The penetration of such non-electrolytes into the liposome appears to be strongly temperature dependent in a way similar to that demonstrated for glycerol permeation into erythrocytes.

2. Introduction of double bonds into the paraffin chains causes a marked enhancement of the permeability. The diffusion rate of glycerol into the liposomes of (distearoyl)lecithin, (1-stearoyl-2-oleoyl)lecithin, (dioleoyl)lecithin and (dilineoyl)-lecithin, increases in that order.

3. Decreasing chain length also increases the permeability which is demonstrated by comparing the swelling rate of liposomes prepared from (distearoyl)lecithin, (dipalmitoyl)lecithin and (dimyristoyl)lecithin and that of those prepared from (1-stearoyl-2-myristoyl)lecithin and (1-stearoyl-2-decanoyl)lecithin.

4. Comparison of the permeability of the liposomes from (1-stearoyl-2-decanoyl)-lecithin and (1-stearoyl-2-myristoyl)lecithin with those obtained from (dimyristoyl)-lecithin and (dipalmitoyl)lecithin, respectively, suggests that, certainly at lower temperatures, the model structures of phospholipids with asymmetric chains are much more permeable than those of lecithins with chains of equal length but with the same total number of paraffin carbon atoms.

5. Liposomes of mixtures of phospholipid and cholesterol normally demonstrate a decrease in permeability which is proportional to the concentration of cholesterol.

INTRODUCTION

Of the numerous biological interfaces occurring in nature, a considerable number have been isolated in a more or less pure form and characterised by analysis of the structural lipids. These analyses on the various isolated membranes demonstrate striking differences in the chemical nature and the quantitative distribution of the lipids which may be related to differences in special properties such as permeability behaviour of these interfaces[1]. This hypothesis is underlined by the fact that, for a given membrane, the composition is very constant and specific. Extensive dietary

changes can induce only limited variations in the fatty acids of the membrane phospholipids which are selected in such a way by the organism as not to alter the physical properties of the lipid mixture to a notable extent[2,3]. Attempts to correlate differences in permeability data for a number of membranes with varying lipid composition have been made[4], but conclusive information on this relation is still lacking because of the complex composition and architecture of the natural membrane.

In contrast to the possibilities with biological interfaces, well defined variations in the chemical composition can easily be obtained in artificial membranes. With respect to the relation between permeability properties and lipid composition, the model systems of the lipid bilayer type are of particular interest. BANGHAM, STANDISH AND WATKINS[5] have described the spontaneously formed liquid crystals of phospholipids in salt solutions (liposomes or Bangosomes) as concentric bimolecular lamellae separated from each other by water compartments. These structures appear to be suitable model systems for the study of permeability properties. Measurements on the rate of diffusional exchanges of trapped radioactive compounds with unlabelled molecules on the outside, have revealed useful information on the permeability of slowly penetrating compounds[5,13,14]. The property of the liposomes to act as almost ideal osmometers offers one the possibility of studying the permeability of faster-moving compounds by examining the osmotic swelling of the lipid aggregates in isotonic solutions of the penetrating solutes[8]. Using this method, this paper presents information on the permeability behaviour towards glycerol and erythritol of liposomes prepared from various synthetic lecithins, with or without cholesterol.

EXPERIMENTAL PROCEDURE

Materials

Egg-yolk lecithin was purified by 3 subsequent precipitations as $CdCl_2$ adducts and chromatography on silicic acid. Phosphatidic acid was obtained by enzymic hydrolysis of the egg lecithin with a phospholipase D preparation extracted from Savoy cabbage. Synthetic lecithins were prepared by deacylation of egg-yolk lecithin and reacylation of the glycerolphosphorylcholine as $CdCl_2$ adducts with fatty acid chlorides. Mixed acid lecithins were obtained by degradation of the synthetic lecithin with phospholipase A and acylation of the lysolecithin with a second fatty acid chloride[6]. Careful chromatography on silicic acid yielded pure lecithins, demonstrating a single spot on thin-layer chromatograms. Fully synthetic *rac.*-1-palmitoyl-2-oleoyl-3-glycerolphosphorylcholine* was generously supplied by Dr. A. J. SLOTBOOM. The lipids were stored at −20° in chloroform or pentane solutions.

All the other reagents were commercial and of Analytical Reagent Grade.

Preparation of liposomes

To prepare the phospholipid dispersions, amounts equivalent to 15 μmoles were pipetted into 25-ml round-bottomed flasks. The chloroform (or pentane) was removed *in vacuo* using a rotary evaporator. To the thin, dry lipid film, 1 glass bead and 1 ml of 50 mM KCl at 37° were added. Gentle agitation, during which the temperature was kept at 37°, normally caused complete dispersion of the lipids; this was followed

* All the other lecithins can be considered to be 1,2-diacyl-*sn*-glycerol-3-phosphorylcholines.

by a 30-sec shaking on a Vortex mixer. To all the lecithin solutions, 4 mole % phosphatidic acid was added to give the layers a useful charge. Using a microelectrophoresis apparatus, according to the method of BANGHAM *et al.*[7], the zeta potential of liposomes of the various lecithins was determined and found to vary between 15 and 20 mV. To the lecithin–cholesterol mixtures as much phosphatidic acid was added as required to keep the zeta potential of one series of mixed liposomes within a 1-mV variation. The liposomes of (dipalmitoyl)lecithin and (distearoyl)lecithin were prepared at 55° and 60°, respectively.

Optical measurements

The absorbance of the dispersions at 450 mμ was routinely measured at room temperature using a Unicam SP 500 spectrophotometer.

The swelling of the liposomes in isotonic solutions of glycerol and erythritol was determined by following the change in light transmission in a Vitatron UFD photometer connected to a recorder. The instrument is adapted with a double-walled cylindrical glass cuvette, the temperature of which was controlled with circulating water from a thermostat. With a fast-running stirrer in the cuvette, complete mixing of 50-μl samples of lipid emulsion with 5 ml of isotonic medium was obtained in 1 sec or less (compare Fig. 2).

RESULTS

Volume changes of liposomes can easily be followed by optical means. For osmotic swelling, it has been shown experimentally that, as for mitochondria, the increase in volume is linear with the reciprocal value of the absorbance ($1/A \sim V$) (ref. 8). For liposomes produced from egg-yolk lecithin, it has been already demonstrated that the osmotic swelling is very similar to the behaviour of an ideal osmometer[8,9]. That this is true for liposomes of synthetic lecithins is supported by the results given in Fig. 1, which show a linear relationship between the reciprocal value of the osmolarity and the volume ($1/A$) of liposomes originally prepared in 50 mM KCl and subsequently equilibrated in different salt concentrations. Such a relationship is in agreement with the law of Boyle–Van 't Hoff applied to an ideal osmometer.

In Table I, the percental osmotic-swelling changes are given for a number of different liposomes. Apparently these optical swelling changes are quite reasonably comparable. Microscopic observations have been made routinely on the dispersions. Although each preparation was heterogeneous, no obvious difference in size and shape of the liposome structures of the preparations has been observed. In the samples of liposomes of (dilineoyl)lecithin–cholesterol mixtures, small crystals, possibly of cholesterol, have sometimes been observed next to the liposome structures, suggesting that at least part of the sterol is not in the bilayer structure.

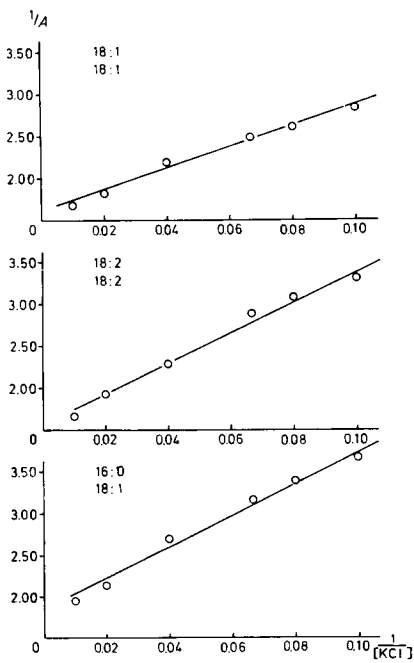
In Fig. 2, a typical tracing of an experiment is given in which the swelling rate is recorded when a sample of liposomes is suspended in isotonic glycerol. The initial change in light transmission, dT/dt, is recalculated as $d1/A/dt$ and considered to be representative of the rate of volume increase. Supposing the liposome acts as an ideal osmometer and assuming the water penetrates much faster than glycerol, the initial swelling in an isotonic solution is proportional to the permeability constant of the solute and the outer surface of the osmometer[10]. In as much as the microscopic

TABLE I

OSMOTIC SWELLING OF VARIOUS LIPOSOME PREPARATIONS IN DIFFERENT SALT SOLUTIONS

$1/A$ at 450 mμ is measured at room temperature 1 h after addition of 100 μl emulsion to 5 ml of salt solutions of different concentrations and is represented here as percentage of the value in a 50 mM KCl solution.

Liposome composition	$1/A$ (%)					
Concn. KCl soln. (mM):	*100*	*50*	*25*	*15*	*12.5*	*10*
Egg lecithin	91	100	119	139	148	163
(Dioleoyl)lecithin	91	100	120	137	142	157
(Dilineoyl)lecithin	86	100	118	150	159	170
(Palmitoyl)lecithin	91	100	126	136	157	171
(Dimyristoyl)lecithin	86.5	100	117	132	139	146
Egg lecithin with 10% cholesterol	88	100	119	138	142	156
Egg lecithin with 20% cholesterol	91	100	122	139	148	159
Egg lecithin with 30% cholesterol	90	100	117	134	138	153
Egg lecithin with 40% cholesterol	91	100	118	136	145	156
Egg lecithin with 50% cholesterol	93	100	118	130	138	148



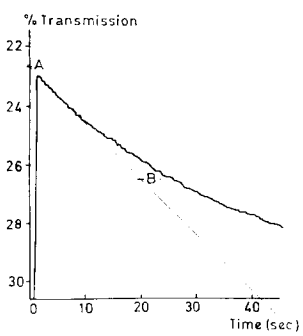


Fig. 1. Relationship between the reciprocal of the absorbance at 450 mμ ($\sim$ volumes) of liposome systems obtained from (dioleoyl)lecithin, (dilineoyl)lecithin and (1-palmitoyl-2-oleoyl)lecithin, originally prepared in 50 mM KCl, and the reciprocals of KCl concentration in which they are swollen until equilibrium.

Fig. 2. Typical tracing of a swelling experiment. A 50-μl sample of concentrated (1-stearoyl-2-myristoyl)lecithin liposome preparation is rapidly mixed with 5 ml isotonic glycerol at a temperature of 42°. The change in light transmission is recorded. The slope of the line AB is supposed to represent the initial change in transmission.

observations suggested no great variation in the outer surface, the swelling rate is considered to be a fair indication of permeability. In Figs. 3, 4 and 5, the swelling rate is recorded as a function of temperature. In all these experiments, a strong temperature dependence can be noted. In the direction of higher temperature, the line representing the swelling rate ended when the volume increase became so rapid that an accurate measurement of the initial swelling rate was impossible. Towards the lower temperature, the optical changes have been found to become very small and sometimes irreproducible. In this respect, it is relevant to mention that, for the formation of liposomes, a liquid crystalline structure of the phospholipids is supposed

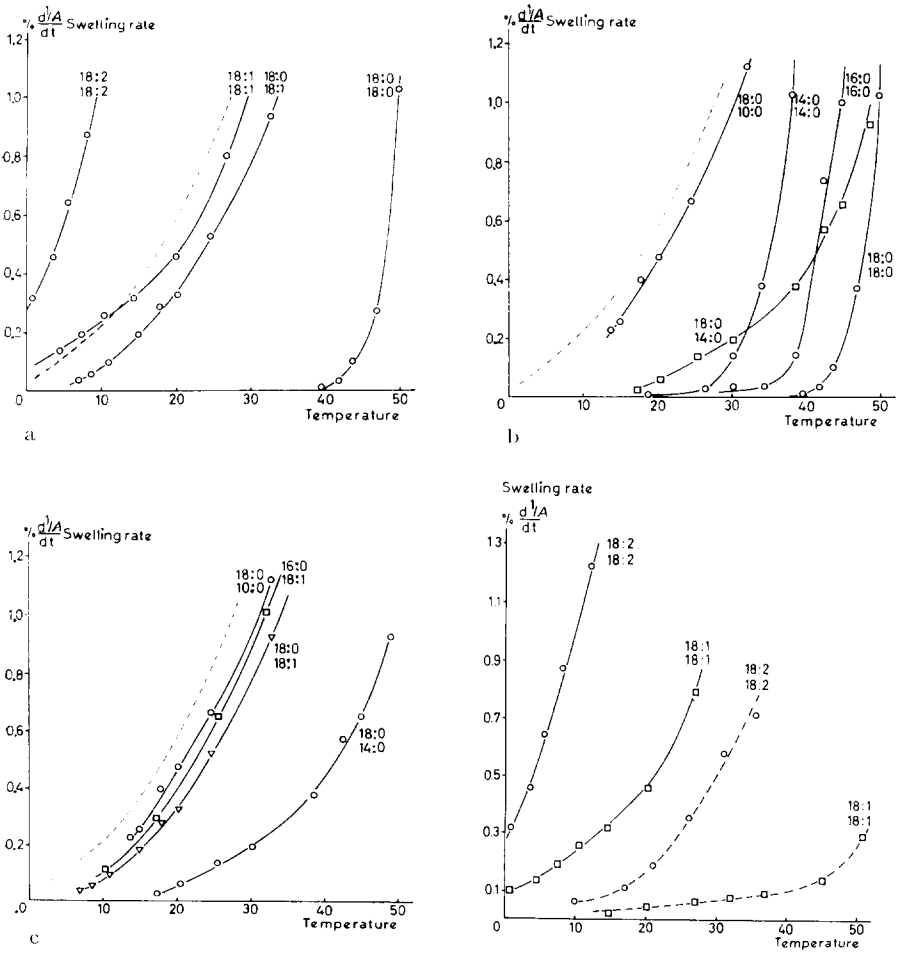


Fig. 3. Initial swelling rate in isotonic glycerol of liposomes of various lecithins as a function of temperature. a. Curves of (distearoyl)-, (1-stearoyl-2-oleoyl)-, (dioleoyl)- and (dilineoyl)lecithin. b. Curves of (distearoyl)-, (dipalmitoyl)-, (dimyristoyl)-, (1-stearoyl-2-myristoyl)- and (1-stearoyl-2-decanoyl)lecithin. c. Curves of (1-stearoyl-2-myristoyl)-, (1-stearoyl-2-oleoyl)-, (1-palmitoyl-2-oleoyl)- and (1-stearoyl-2-decanoyl)lecithin. The dotted line in the 3 figures represents the swelling curve of egg-yolk lecithin.

Fig. 4. Initial swelling rates of liposomes prepared from (dilineoyl)- and (dioleoyl)lecithin in isotonic glycerol (———) and erythritol solutions (– – – –) as a function of temperature.

to be necessary[11]. When the temperature is lowered, the chains may go into the solid state. Using differential thermal analysis techniques, these phase changes have been measured for saturated lecithin–water systems, and phase transition temperatures of 23, 41 and 60° are reported for (dimyristoyl)lecithin, (dipalmitoyl)lecithin and (distearoyl)lecithin, respectively[12]. Considering these temperatures and comparing them with the temperature effect on permeability, it must be realised that our systems contained 4 % of phosphatidic acid with a fatty acid composition of egg-yolk lecithin. However, the relatively, steep drop in the swelling curves (Fig. 3b) of the liposomes of these saturated phospholipids can be related to the liquid–solid transition of the paraffin chains.

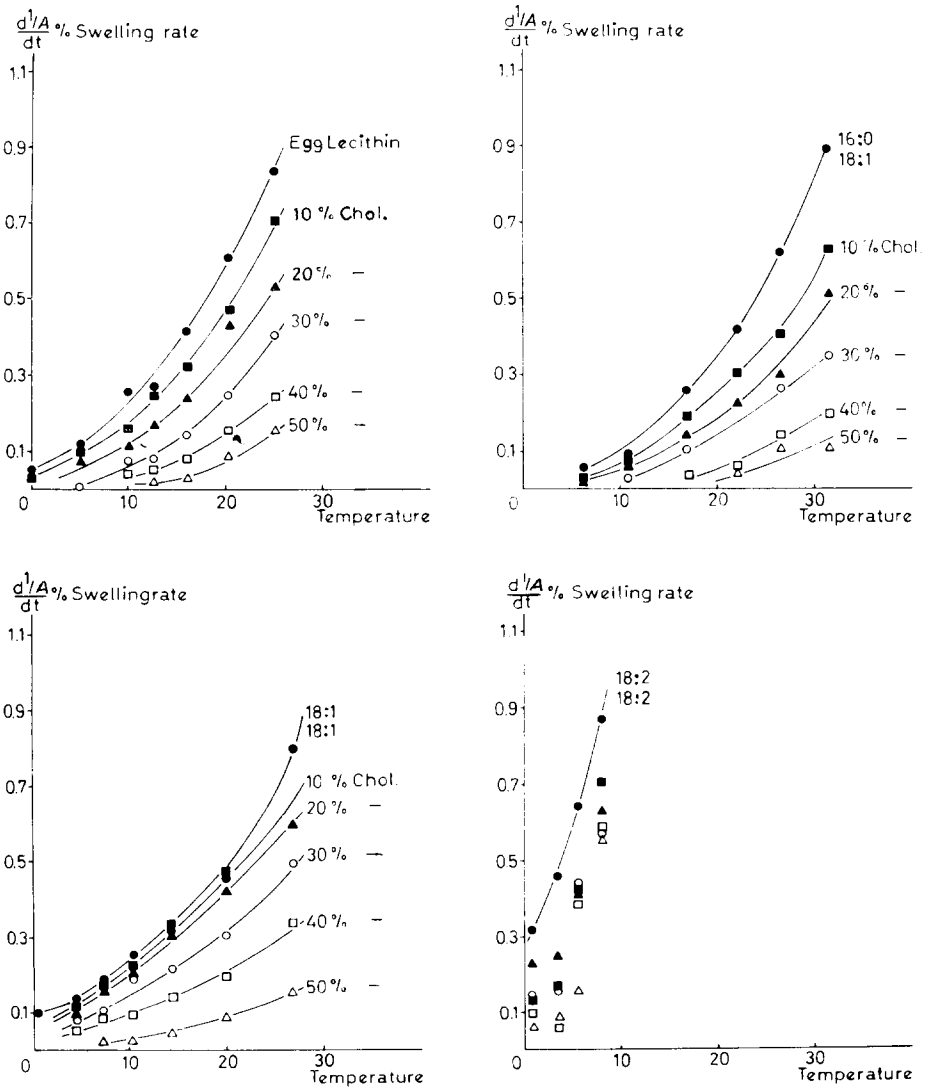


Fig. 5. Initial swelling rates of liposome systems prepared from lipid mixtures of cholesterol with: a, egg-yolk lecithin; b, (1-palmitoyl-2-oleoyl)lecithin; c, (dioleoyl)lecithin; d, (dilineoyl)lecithin.

The influence of the chemical nature on the permeability of the liposome is apparent. Comparison of the swelling rates of (distearoyl)lecithin, (1-stearoyl-2-oleoyl)lecithin, (dioleoyl)lecithin and (dilineoyl)lecithin (Fig. 3a), clearly demonstrate that introduction of double bonds causes an important increase in permeability.

From the swelling of liposomes prepared from (distearoyl)lecithin, (dipalmitoyl)-lecithin and (dimyristoyl)lecithin (Fig. 3b), and also from those of (1-stearoyl-2-oleoyl)lecithin and (1-palmitoyl-2-oleoyl)lecithin (Fig. 3c), it can be concluded that increasing chain length decreases the ability of glycerol to penetrate the lipid barrier.

Previously, the influence of the molecular size of the solute penetrating into liposomes of egg-yolk lecithin has been demonstrated[8]. In Fig. 4, the swelling curves of (dilineoyl)lecithin and (dioleoyl)lecithin, both in isotonic solutions of glycerol and erythritol, are given, clearly showing a much slower penetration of the bigger molecules. Also, the permeability of erythritol appears to be strongly temperature dependent and the difference between the liposomes from (dioleoyl)lecithin and (dilineoyl)lecithin suggests that for the erythritol permeability–chain composition relationship, the same rules are valid as found for glycerol.

The swelling rate for a number of liposomes from lecithins mixed with increasing concentrations of cholesterol are shown in Fig. 5. Considering the fact that they are quite comparable osmometers (Table I), the results demonstrate that the presence of cholesterol in the liposome structures of egg-yolk lecithin, (1-palmitoyl-2-oleoyl)-lecithin and (dioleoyl)lecithin, reduces the permeability considerably and that the reduction in swelling rates is more or less proportional to the amount of cholesterol. The effect of low concentrations is most pronounced in the liposomes of the mixed acid lecithins and in those of egg yolk lecithin, whilst in the (dioleoyl)lecithin liposomes, it is less so, but still significant. Higher concentrations in the latter system also cause a considerable reduction in the permeability. The addition of cholesterol to the (dilineoyl)lec thin system normally results in reduced values of the swelling rate, but the results are variable and irreproducible. This could be in agreement with the suggestion made from the light microscopic observation that cholesterol, in this case, is easily excluded from the liposome structure.

DISCUSSION

Based on light and electron microscopic evidence coupled with X-ray diffraction and birefringent properties, together with the ability of the liposomes to trap the solutes of the medium in which they are formed, BANGHAM, STANDISH AND WATKINS[5] concluded that these aggregates of phospholipid molecules are to be considered as a system of concentric, discrete, bimolecular layers intercalated by aqueous compartments. This conclusion is supported by the osmotic swelling properties of the liposomes and the water permeability which was calculated for the outer lipid layer to be of the same order as has been found for "black membranes"[8].

In general, the permeability properties of the liposomes demonstrate a great deal of similarity to those of the natural membranes. From self-diffusion experiments, it has been shown that the permeation of cations and anions is remarkably similar for both model structures and the biological membrane[5]. In addition, the effects of narcotics[12] and the changes in ion permeability when liposomes are exposed to lytic or protective steroids[14] have been found to be qualitatively the same as for natural

interfaces. Also, the graded permeability of a number of non-electrolytes, as demonstrated by osmotic experiments, resembles that found for erythrocytes[8]. These close correlations support the concept that the barrier properties of the liposomes have much in common with those of a biological interface and that studies on these model systems can furnish useful information on the relation between the chemical composition of membrane lipids and the passive permeability of non-electrolytes.

An appealing result from the present findings is the strong temperature dependence of the permeation processes studied. Apparently an increased thermal mobility of the chains in the lipid barrier greatly enhances the penetration of non-electrolytes such as glycerol and erythritol. Also, the stimulating effects of introduction of double bonds and shortening the chain length can easily be explained as an increase in fluidity of the lipid barrier and consequently an increased permeability. Previously, we have studied the glycerol penetration into red cells of different mammals[15]. With respect to glycerol permeability, it appears that the erythrocytes from various species can be divided into two groups. In one group, the permeation is only slightly influenced by the temperature, while the second group exhibits a strong temperature dependence which is comparable to that found in the liposomes[15]. In addition to simple penetration through the lipid barrier, the glycerol molecules in the first group of erythrocytes probably utilise a facilitated transport system which can be inhibited by the presence of copper ions[16] or by lowering the pH. Under such conditions, these erythrocytes demonstrate the same temperature dependence as those of the second group[15,17], so it can be concluded that the surprisingly high temperature dependence is true for both the passive permeation through the biological membrane and the lipid barrier of the model system.

The liposomes demonstrate an important increase in permeability when the number of double bonds is increased. Some years ago we reported a possible correlation between data on glycerol permeability and differences in fatty acid composition of erythrocytes of a number of animal species[4]. For example, erythrocytes with low concentrations of arachidonate demonstrate a low permeability at room temperature and those in which high concentrations of this polyunsaturated acid are found, appear to be extremely permeable. Although we know from extension of our work that such a correlation for the erythrocytes is highly complicated by the presence of other analytical differences between the various membranes and the possible existence of facilitated transport systems in some of them, the idea of a relation between permeability and chain unsaturation coincides very well with conclusions that can be made from the present work on the model system. The result that an increasing number of double bonds enhances the permeability is also of interest with respect to the adaption of fatty acid composition of microorganisms towards the temperature of growth. For bacteria[18], it has been shown that, when this temperature decreases, the unsaturation increases and for marine ectotherms[19] it has been demonstrated that lower temperatures also lead to a loss of stearate and palmitate and to a big increase in palmitoleate.

Discussing the effect of chain length, it is of interest to point out not only the finding that the permeability increases with decreasing chain length, but also the difference between (dimyristoyl)lecithin and (1-stearoyl-2-decanoyl)lecithin (Fig. 3b). Both lecithins contain the same number of carbon atoms, but the mixed acid system is much more permeable. In addition, the penetration of glycerol into (1-stearoyl-

2-myristoyl)lecithin liposomes is higher at lower temperatures than those of (dipalmitoyl)lecithin. Also with respect to the chain-length effect, interesting parallels can be found in nature. MEYER AND BLOCH[20] have shown that when yeast is grown anaerobically, the cells are incapable of synthesizing fatty acids. Under these conditions, an enhanced production of decanoate, laurate and myristate is apparent and these acids are found predominantly on the 2 position of the phospholipid molecule which is normally reserved for the unsaturated ones. Monolayer experiments[21] have shown already that the pressure–area curves of (1-stearoyl-2-oleoyl)lecithin and (1-stearoyl-2-lauroyl)lecithin are very similar. Fig. 3c clearly demonstrates also that the liposomes of the combinations of long chain unsaturated chain and long chain–short chain can be very close in their permeability behaviours.

Cholesterol occurs in biological interfaces in highly variable concentrations. In the membrane structures of the mitochondria and nuclei, the concentration is low, but in the outer cell membranes, the concentration is normally high and molar ratios of phospholipid:cholesterol are close to 1, although in bacterial membranes the sterol is lacking[1]. The importance of cholesterol is normally visualized in an association with the other membrane lipids and in theoretical considerations, both hydrogen bonding and London–Van der Waals interactions are thought to be of importance. The interaction between cholesterol and different lecithins has been tested extensively in monolayer studies[21,22]. On the basis of surface potential measurements, SHAH AND SCHULMAN[22] deny the importance of any ion–dipole interactions between lecithin and cholesterol. They also demonstrate that penetration of cholesterol into a very closely packed monolayer of (dipalmitoyl)lecithin reduces the cohesion forces in the hydrocarbon areas which is apparent from a decrease in the surface viscosity. However, from reduction in average area per molecule[21] in more expanded films of cholesterol and phospholipids, particularly those containing mono-unsaturated acids, an increase in cohesion forces is most likely when cholesterol enters the monolayers. Switching from monolayers to lipid emulsions, such an increased apolar interaction in this system is likely because of the reduced glycerol permeability in the presence of cholesterol. The irreproducible result that was shown when this sterol was added to the (dilineoyl)lecithin system, may be related to the results of monolayer studies which demonstrated no reduction in the mean area of phospholipids containing polyunsaturated fatty acids after the addition of cholesterol. A decreased permeability due to cholesterol is not limited to glycerol. PAPAHADJOPOULOS AND WATKINS[26] reported a decreased chloride permeability for liposomes from a mixture of egg-yolk lecithin and cholesterol, compared with those from the pure lecithin. In a study on the leakage of glucose from various liposomes, carried out simultaneously with this work, DEMEL, KINSKY AND VAN DEENEN have made identical observations with respect to the influence of chain length and unsaturation on the permeability, and therefore the effect of cholesterol was found to be in agreement with the observations made on the glycerol permeability. The conclusion that cholesterol normally increases the apolar interaction is also supported by NMR studies. CHAPMAN AND PENKETT[23] have shown an important broadening of the CH_2 signal of aqueous egg-yolk lecithin dispersions in the presence of cholesterol which suggests that the molecular motion of the chains is inhibited. In summarizing, the role of cholesterol in the liposome model system can be interpreted as reducing the chain mobility and consequently the permeability of the structure. It is tempting to extend the validity of this inter-

pretation to the biological interface. In doing so, it must be realized that, in the natural membrane, the fluidity of the chains can be influenced not only by interaction with cholesterol, but also by other membrane constituents. Recent NMR studies[24] on human erythrocyte ghosts suggest that structural proteins have an important contribution to make in this respect.

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