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PHOSPHOLIPID MODEL MEMBRANES

II. PERMEABILITY PROPERTIES OF HYDRATED LIQUID CRYSTALS

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SUMMARY

The rates of diffusional exchange of trapped ions through hydrated liquid crystals and related vesicular structures composed of various naturally occurring, purified phospholipids were determined. Correlations were observed between the permeability characteristics and structure of the particles as reported in the preceding article. The results were consistent with the view that the particles are 'closed', *i.e.* the internal aqueous compartments are completely separated from the bulk aqueous phase by bimolecular lipid lamellae. Apparently only phosphatidylethanolamine formed incompletely sealed particles. Admixture of other phospholipids with phosphatidylethanolamine resulted in the usual closed structures. Chloride diffusion through phosphatidylcholine, phosphatidylserine, and mixtures of phosphatidylcholine, phosphatidylethanolamine and cholesterol was considerably faster than that of K^+ or Na^+, while the cations diffused faster through phosphatidic acid and phosphatidylinositol. Explanation of the Cl^-/K^+ differential was sought in the possible orientation of permanent dipoles and of charged groups. The presence of Ca^{2+} was shown to have a marked effect on the permeability properties of the acidic phospholipids—phosphatidylserine, phosphatidylinositol, and phosphatidic acid. It was concluded, on the basis of the above observations, that this system constitutes a valid model for many biological membrane phenomena.

INTRODUCTION

Although most biological membranes are complex mixtures composed predominantly of lipids and proteins, it is conceivable that the lipid present within the native membrane is arranged in the form of a bimolecular leaflet as first proposed by DANIELLI and DAVSON[1]. It is quite probable however that biological membranes are diverse in structure (as in function) and that no general postulation about their con-

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figuration can be universally applied to all membranes. Whatever the case may be it is important to gain knowledge on the physicochemical properties of the individual components, before an assessment can be made as to their contribution to the overall behaviour of the parent biological membrane.

When phospholipids are equilibrated with excess water or salt solutions they form liquid crystals of the smectic mesophase type, composed of bimolecular lipid lamellae separated by aqueous layers[2-4]. The use of such liquid crystals as models for studying membrane permeability was described recently by BANGHAM, STANDISH AND WATKINS[4] in a study of the properties of structures composed mainly of phosphatidylcholine. Evidence was presented[4] suggesting that the aqueous compartments within the liquid crystals are completely enclosed by the bimolecular lipid lamellae. Thus, the diffusion of ions or other molecules from within the particles and into the external medium could be considered as occurring across rather than between the phospholipid lamellae, a supposition supported further by material presented in this paper. Several other characteristics of phosphatidylcholine liquid crystals have already been reported. These include the relatively faster rate of Cl^- diffusion compared with that of K^+ or Na^+ (see ref. 4), an increase in cation diffusion rate caused by certain steroids[5] and organic solvents[6], and a decrease in cation diffusion produced by local anaesthetics[6]. The only other phospholipid thus far investigated is phosphatidylserine. This substance exhibits a certain discrimination between Na^+ and K^+ which is reversed by low concentrations of Ca^{2+} (see ref. 7). It was also shown that local anaesthetics reduce the ability of Ca^{2+} to increase the diffusion of both Na^+ and K^+ through phosphatidylserine particles[7].

The structural characteristics of hydrated liquid crystals composed of a number of naturally occurring phospholipids have been presented in an accompanying article[8]. The work reported here is a comparison of the permeability properties of the above phospholipids, namely phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and phosphatidic acid. The results are discussed in terms of the chemistry of individual molecules and the general morphology of the liquid crystalline particles through which diffusion is taking place. In addition, a study was made of the effect of temperature, particle size, and the presence of bivalent metals and proteins.

MATERIALS AND METHODS

All reagents were A.R. grade and were used without further purification. The water was double-distilled over $KMnO_4$. Sephadex G-50, coarse, was obtained from Pharmacia Co; cytochrome *c* (horse heart, type III) from Sigma Chemical Co.; bovine plasma albumin ('crystallized') from Armour Pharmaceutical Co. The purification of phospholipids was described in the accompanying article[8].

Measurement of surface charge

The electrophoretic mobility of phospholipid particles[9], measured in 130 mM KCl containing Tris–HCl (15 mM, pH 7.4), was converted to ζ-potential using the formula:

$$\zeta = 12.9 \times \frac{V}{E_{app}}$$

where V = the electrophoretic mobility in $\mu \cdot sec^{-1}$, E_{app} = the applied voltage in $V \cdot cm^{-1}$ and ζ in mV (ref. 10).

Preparation of phospholipid dispersions

The isotopically labelled salt solutions contained ^{42}KCl (5 μC/ml), $^{22}NaCl$ (5 μC/ml), and $K^{36}Cl$ (3 μC/ml). For the preparation of dispersions, these salts were made to 145 mM, and buffered with 1/9 vol. of 145 mM Tris–HCl (pH 7.4) immediately prior to use. The phospholipid was thinly coated on the inner surface of a 50-ml round bottom flask (or 25-ml test tube, as appropriate) by rotary evaporation of a chloroform solution under vacuum. The isotopically labelled salt solution was then added to the extent of 1 ml per 5–15 μmoles of phospholipid. Dispersions were then prepared by either of the following methods: (a) after the addition of 2 or 3 small glass beads, the flask was gently agitated by hand until phospholipid could no longer be seen adhering to the walls of the vessel; (b) the flask, with added beads, was vigorously agitated for 15 min on a mechanical flask shaker ('Microid', Griffin and George, London); (c) hand-prepared suspensions in 25-ml stoppered test tubes were treated for 5–50 min in a bath-type sonicator (Ultrasonic Instr. Inc., Model G40C2H-T40Cl, 80 kcycles/sec). The water in the bath was changed frequently to minimize temperature increases during sonication. Finally, the suspension was left to equilibrate at room temperature for 1 h before starting the experiment.

An alternative method of preparing dispersions was as follows: The phospholipid was first dissolved in 1–2 ml of petroleum ether (60–80° fraction). The solution was layered onto an equal volume of the isotopically labelled salt solution in a 25-ml test tube, 2 or 3 small glass beads were added, and the interfacial level was marked. The tube was then agitated vigorously on a mechanical shaker while a rapid stream of O_2-free N_2 was passed through the emulsion by means of a thin polythene tube. To avoid cooling during evaporation, the bottom of the test tube was immersed in water kept slightly above room temperature. Progress of the evaporation was followed closely, and when the solvent was judged to have been completely removed, the N_2 flow was slowed to a very gentle stream and vigorous agitation continued for another 5 min. This process is referred to in the text as the 'solvent evaporation' method.

Diffusional exchange measurements

Bulk-phase tracer ions were separated from those contained in the lipid particles either by dialysis or by Sephadex filtration. For direct dialysis, 0.5-ml aliquots of a dispersion, each containing 2–10 μmoles of phosphorus, were pipetted into dialysis bags (0.9 cm $\times$ 6 cm, previously rinsed with the exchange medium) and dialysed against 5 30-min changes of 500 ml of the exchange fluid which was the same as the dispersing medium but without tracer. More rapid removal of the bulk tracer (5 min) was accomplished by the passage of the dispersion (1–2 ml) through a column of Sephadex (3 g, G-50, coarse grade), packed in the 'cold' exchange medium[11]. The column was washed with the exchange medium and the main lipid peak collected, (usually 5 ml, between 13 and 17 ml of eluted volume). Aliquots (0.5 ml) were then pipetted into rinsed dialysis bags. Where diffusional exchange was expected to be rapid, diffusion measurements were begun immediately. Otherwise precautions were taken to remove any small amounts of free tracer in the external medium by one

or two preliminary dialyses of 30 min duration against the non-isotopic medium.

Diffusional exchange measurements were carried out as follows. After removal of bulk-phase tracer, dialysis bags were transferred into duplicate stoppered tubes each containing 10 ml of the exchange medium. The tubes were rotated gently at a speed of 1 rev./min. At determined intervals, usually 1 h, the bags were transferred to fresh solutions. Finally, the tracer ions in each 10-ml dialysate, and those remaining inside the bags, were determined with the appropriate counting equipment. The amount of tracer appearing in the dialysate during the first hour after the removal of unincorporated ions was taken to represent the diffusion rate constant, for a concentration difference of 145 mM at zero time. It is referred to in the text as self-diffusion rate, and is expressed both as mequiv of ions/mole of lipid per h, and as a percentage of the ions present inside the particles at zero time. The total amount of ions present at zero time, referred to as capture, is expressed as equiv/mole of lipid. The amount of lipid present in each dialysis tube was estimated by phosphorus analysis[12]. Activation energies were calculated from the Arrhenius equation[4] by plotting the natural log of self-diffusion rates *vs.* the reciprocal of absolute temperature.

RESULTS AND DISCUSSION

General considerations

It is apparent from observations reported in the previous communication[8] that a wide variation in the structure of swollen phospholipid liquid crystals can be expected according to the method of preparation and the particular phospholipid system involved. Factors which must be taken into account in discussing ion capture and diffusional exchange include the following:

1. *The size of the lipid particles.* Assuming fairly uniformly spaced lamellae, the capture would be expected to be lower where the particles are smaller. This follows the ratio of the number of inside lamellae over those forming the external surface. Nevertheless, smaller particles would be expected to give faster diffusion rates due to increased total surface area.

2. *The inter-lamellar spacing.* Captures would be expected to be higher in those systems which favour greater separation of the lamellae.

3. *Vesicular structure.* Captures and exchange diffusion rates would depend on the size of any central aqueous compartments and on the number of surrounding lamellae; large central compartments and few lamellae would favour high capture and fast diffusional exchange.

4. *The integrity of the lamellae.* Low capture and high leakage rates with low activation energy should be observed if some of the particles are not completely 'closed' and have water channels of large diameter, relative to that of the diffusing ions, connecting the internal and external media. The leakage rates and activation energies should be similar for all ions and molecules present. The above situation could also arise when larger particles break into smaller ones during the experiment.

5. *The presence of fixed, uncompensated charged groups.* These would be expected to bind counter-ions throughout the particle, but on dialysis or gel filtration, those counter-ions bound to externally located groups would exchange rapidly with bulk-phase ions and would not be represented in the captures and diffusional ex-

changes later measured. Thus lower counter-ion capture would be expected for smaller particles, because of the relatively larger proportion of the fixed charges present on the outermost lamellae.

Capture and diffusion of ions

It can be seen in Table I that, in general, the amount of captured ions diminishes as the size of the particles decreases following sonication or use of the solvent-evaporation method. This is probably due to the expected decrease in the volume of the interior aqueous spaces (Item 1), as well as the number of counter-ions inside the particles (Item 5). In the case of phosphatidylcholine, where no counter-ions are thought to be involved, only the first argument applies, while both are operative for the capture of cations by the acidic phospholipids. Phosphatidylserine particles

TABLE I

SELF-DIFFUSION RATES OF UNIVALENT IONS OUT OF LIQUID CRYSTALS OF PURE PHOSPHOLIPIDS

Lipid	*Ion*	*Number of experiments**	*Dispersion method*	*Total capture (equiv/mole)*	*Self-diffusion rate*		*Activation energy (kcal/mole)*	$\left(\frac{Cl^-}{K^+}\right)$**
					mequiv/mole per h	*% per h*		
Phosphatidylcholine	^{42}K	3	Hand	0.05–0.1[b]	0.75–1.5	1.4–2.3	10	
		5	Mechanical	0.1–0.3[a,b]	1.0–4.0	1.1–1.3	—	
		3	Sonic. (20 min)	0.09–0.1[a,b]	0.4–0.5	0.4–0.5	15	
	^{36}Cl	2	Hand	0.08[b]	5	6	5.6	3–4
		1	Mechanical	0.13[a]	35	27	—	20–24
		2	Sonic. (20 min)	0.10–0.12[a]	25–27	17–19	4	35–47
Phosphatidylserine	^{42}K	3	Hand	1.0–1.6[a]	20–40	2–3	—	
		8	Mechanical	1.0–2.0[b]	10–20	0.5–1.0	8–10	
		3	Sonic. (5 min)	0.6–0.7[a,b]	2–4	0.5	15–18	
		2	Sonic. (40–80 min)	0.35–0.40[a]	2.5	0.5	—	
	^{22}Na	2	Sonic. (5 min)	0.5–0.8[a,b]	3–5	0.5–0.7	12–14	
	^{36}Cl	3	Mechanical	0.9–1.3[b]	80–100	6–9	—	9–12
		2	Sonic. (5 min)	0.2–0.3[a]	26–36	14	6	28
		1	Solvent evaporation	0.3[a]	100	33	—	
Phosphatidylinositol	^{42}K	2	Mechanical	0.8–1.3[a]	37–50	5.1–6.5	—	
		2	Sonic. (40 min)	0.13[a]	4.5	4	18	
	^{36}Cl	1	Mechanical	0.65[a]	37	3	—	0.5
Phosphatidic acid	^{42}K	2	Sonic. (5 min)	0.91[a]	39	4	15	
		1	Sonic. (40 min)	0.6[a]	16	3	—	
	^{36}Cl	1	Sonic. (5 min)	0.56[a]	14.5	2.6	8.6	0.6
Phosphatidylethanolamine			Did not form stable dispersions (see text)					

[a] Dispersions were passed through Sephadex.
[b] Dispersions were dialysed against 500 ml, changed 5 times at 30-min intervals.
* Each experiment involves 2–8 replicates.
** Only those values for Cl^- and K^+ diffusion obtained from the same phospholipid batches and dispersed by the same method have been included in this column.

prepared by simple shaking resulted in a capture of 1.5–2.0 equiv of K^+/mole of lipid, about half of which is present as physically trapped KCl (indicated by Cl^- capture) with the remainder bound as counter-ions to the negatively charged groups. Prolonged sonication reduces this capture to about 0.3–0.4 equiv/mole, with approximately one-half of this amount again being associated with the physically trapped Cl^-. For the ideal case of uni-lamellar vesicles, a theoretical minimum of 0.5 equiv K^+/mole lipid should be bound as counter-ions to the inside surface of the vesicles with any physically trapped KCl adding to the measured K^+ content. The observed K^+ content, being significantly lower than the theoretical minimum for closed lamellar particles, indicates that either some of the negative groups are not ionized or that some of the particles do not have an enclosed internal aqueous space. It should be noted in this connection that cations captured by small phosphatidylserine, phosphatidylinositol, or phosphatidic acid particles diffuse rather slowly and with an activation energy of 15–18 kcal/mole (Table I) indicating a high energy barrier which can probably be identified with a closed external membrane. ABRAMSON, KATZMAN AND GREGOR[13] reported a quantitative titration of all charged groups in sonicated suspension of phosphatidylserine. This result does not necessarily contradict the evidence for 'closed' particles presented here, because rupture of particles would be expected to occur following the changes in pH and osmolarity of the medium during titration.

The diffusion rates were also influenced by particle geometry (Tables I and II). It can be seen that large particles produced by simple shaking, generally gave higher diffusion rates for cations compared with the smaller particles produced by sonication or by the solvent-evaporation method. The difference is diminished when diffusion rates are expressed as percentages of the amount captured, but it is still the opposite result to that which would be expected following an increase in surface area available for diffusion. The diffusion rates of Cl^- (Table I), usually much higher than that of K^+, show an increase after sonication probably due to the increase of surface area. Thus when the Cl^-/K^+ ratio of a particular phospholipid is examined as a function of particle size, it is apparent that smaller particles show a higher degree of discrimination. This suggests that large particles are exchanging a certain proportion of their trapped salt through large channels which do not discriminate between Cl^- and the cations. Such non-selective leakage could take place through rupture of some of the large particles. This interpretation is supported by the lower activation energies for K^+ diffusion associated with hand-shaken dispersions. Presumably small sonicated particles require greater energy before fracturing and thus remain relatively stable during the experiment. It is relevant that synaptic vesicles (about 500 Å in diameter) are resistant to sonication in contrast to the larger (1 μ) synaptosomes[14].

Of all the phospholipids studied, phosphatidylethanolamine was the only species showing high diffusion rates for both K^+ and Cl^- associated with very small captures (Table I). The conclusion drawn from these results was that pure phosphatidylethanolamine, when swollen in 145 mM salt solution, formed well organized but incompletely 'closed' structures with ions diffusing through large openings between lamellae rather than across well formed bilayers. This view is corroborated by the structural characteristics of phosphatidylethanolamine particles already dealt with[8]. However, when phosphatidylcholine is mixed with phosphatidylethanolamine in

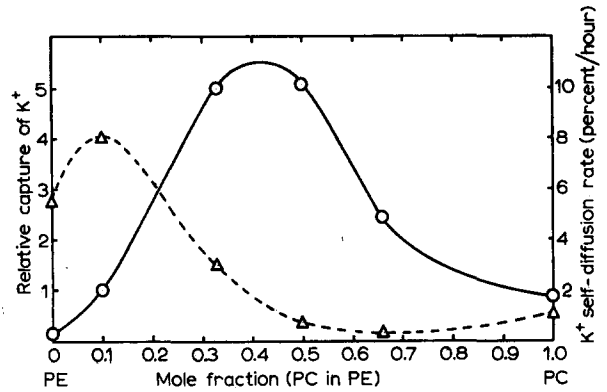

Fig. 1. Capture and self-diffusion rate of K^+ through phosphatidylcholine–phosphatidylethanolamine liquid crystals. △, self-diffusion rate of K^+ expressed as percent of capture per h; ○, capture of K^+ by phosphatidylcholine–phosphatidylethanolamine mixtures relative to that by phosphatidylcholine taken as unity. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

molar ratios greater than 0.33, the resulting particles exhibit higher captures and lower K^+ diffusion rates than either of the pure compounds (Fig. 1).

Fig. 2 gives a comparison of the permeability properties of the different phospholipids and various mixtures (see also Table II) by showing the relative diffusion rates of Cl^- and K^+ as well as the Cl^-/K^+ ratio and the ζ-potential of the individual particles. Most of the dispersions included in Fig. 2 were prepared by sonication and

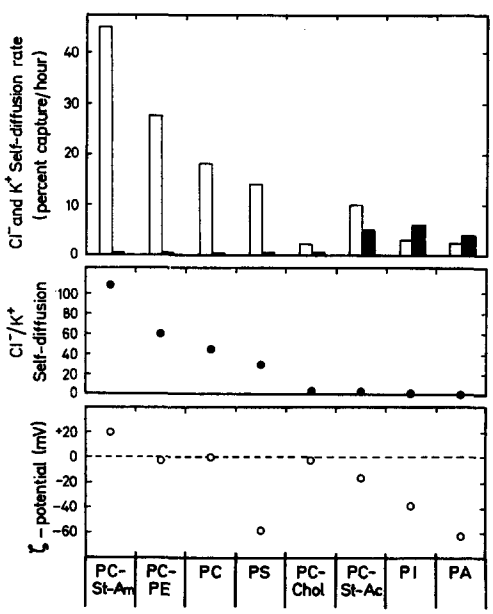

Fig. 2. Self-diffusion rates of Cl^- and K^+, and ζ-potential of various phospholipid liquid crystals. Open bars, Cl^- self-diffusion rate; solid bars, K^+ self-diffusion rate; ●, Cl^-/K^+ diffusion ratio; ○, ζ-potential in mV. Details are given in text. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; St-Am, stearylamine; St-Ac, stearic acid; Chol, cholesterol.

TABLE II

SELF-DIFFUSION RATES OF UNIVALENT IONS OUT OF LIQUID CRYSTALS OF PHOSPHOLIPID MIXTURES

For footnotes see Table I.

Lipid	*Ion*	*Number of experiments**	*Dispersion method*	*Total capture (equiv/mole)*	*Self-diffusion rate*		*Activation energy (kcal/mole)*	$\left(\frac{Cl^-}{K^+}\right)^*$
					mequiv/mole per h	*% per h*		
Phosphatidylcholine–stearic acid (9:1)	^{42}K	2	Hand–mechanical	0.3–0.4[b]	2–4	0.6–1.5	15	
		2	Sonic. (20 min)	0.10–0.15[a,b]	4–8	4–6	15	
	^{22}Na	1	Sonic. (20 min)	0.15[a]	6	4	13.5	
	^{36}Cl	2	Sonic. (20 min)	0.15[a]	15	10	5	2.2
Phosphatidylcholine–stearylamine (9:1)	^{42}K	2	Hand shaken	0.3–0.4[b]	3–6	0.7–2.0	—	
		1	Sonic. (20 min)	0.15[b]	0.6	0.4	15	
	^{36}Cl	1	Sonic. (20 min)	0.15[b]	68	45	3	110
Phosphatidylcholine–phosphatidylethanolamine (1:1)	^{42}K	2	Mechanical	0.4[b]	4	1.0	—	
	^{42}K	1	Solvent evaporation	0.45[a]	2	0.5	—	
	^{42}K	2	Sonic. (20 min)	0.17[a]	0.7	0.4	—	
	^{36}Cl	1	Sonic. (20 min)	0.10[a]	20	20	—	57
	^{36}Cl	1	Solvent evaporation	0.40[a]	140	35	—	70
Phosphatidylcholine–cholesterol (1:1)	^{42}K	2	Sonic. (20 min)	0.13[a]	0.9	0.7	—	
	^{36}Cl	2	Sonic. (20 min)	0.11[a]	1.9	1.8	—	2.6
Phosphatidylserine–cytochrome *c*	^{42}K	2	Mechanical	1.2–1.4[b]	3–18	0.25–1.3	—	
	^{22}Na	1	Mechanical	0.94[b]	7	0.74	—	
	^{36}Cl	2	Mechanical	0.64–0.7[a]	40–80	6.2–11	—	9–25
Phosphatidylcholine–phosphatidylethanolamine (1:1) + cytochrome *c*	^{42}K	1	Mechanical	0.41[a]	4.5	1.1	—	
	^{22}Na	1	Mechanical	0.31[a]	1.7	0.6	—	
	^{36}Cl	2	Mechanical	0.3[a]	162	54	—	49
Phosphatidylcholine–phosphatidylethanolamine + albumin	^{42}K	2	Solvent evaporation	1.1[a]	6–17	0.5–1.5	—	
	^{22}Na	2	Solvent evaporation	1.1[a]	6–17	0.5–1.5	—	
	^{36}Cl	1	Solvent evaporation	1.1[a]	340	31	—	20–60

therefore contain particles of comparable size[8]. It can be seen that the surface charge has a marked effect on the relative diffusion rates between anion and cation. For instance, the Cl^-/K^+ ratio for phosphatidylcholine alone is approx. 40 which is to be compared with approx. 110 for phosphatidylcholine–stearylamine (9:1) and approx. 3 for phosphatidylcholine–stearic acid (9:1). Thus the introduction of a long-chain cation increased the ratio while a long-chain anion caused a reduction, as would be expected from charge considerations[4].

The presence of phosphatidylethanolamine (which is only slightly negative) in equimolar mixtures with phosphatidylcholine had only a small effect, but, in general, it increased the Cl^-/K^+ ratio. A very marked reduction of Cl^- diffusion rate was noted with phosphatidylcholine–cholesterol mixtures, an effect possibly associated with hydrocarbon interactions[15,16], rather than surface charge. It is of interest that particles composed of phosphatidylserine alone, although strongly negatively charged (ζ-potential: 60 mV, compared with only 16 mV for phosphatidylcholine–

stearic acid) still allowed a faster diffusional exchange of Cl^- than of K^+, the ratio being similar to that of phosphatidylcholine, rather than to the other acidic phospholipids.

Finally, phosphatidylinositol and phosphatidic acid, also shown in Fig. 2, were the only phospholipids that exhibited a Cl^-/K^+ ratio of less than unity. Compared with phosphatidylserine, phosphatidylinositol shows a 10-fold increase in K^+ and 5-fold decrease in Cl^- permeability. Obviously the spatial arrangement of specific head groups is of more importance in determining permeability properties than over-all charge per molecule, since both phosphatidylserine and phosphatidylinositol have one extra negative charge per molecule at pH 7.4 and a similar ζ-potential. In phosphatidylserine, it is possible that the PO_4^- (or COO^-) and NH_3^+ groups, compensating each other inter- or intra-molecularly confer an additional cohesiveness to the bimolecular leaflet, resulting in low permeability to cations. Phosphatidic acid and phosphatidylinositol of course, have no compensating positive charges, carrying respectively only mono- and diesterified phosphate groups. There was no evidence of discrimination between Na^+ and K^+ with most of the lipids or lipid mixtures used in the present work except for phosphatidylserine as previously reported[7].

Effect of temperature

The difference between the self-diffusion rates of Cl^- and K^+ is also reflected in the different activation energies (Tables I and II). There was a high activation energy (15–17 kcal/mole) for the diffusion of K^+ through both neutral (phosphatidylcholine) and negatively charged (phosphatidylcholine–stearic acid, phosphatidylserine, phosphatidylinositol, and phosphatidic acid) particles for temperatures between 37° and 50°. This value is similar to the reported activation energy for the diffusion through red blood cells[17], and much higher than that for Na^+ diffusion through highly cross-linked ion-exchangers[18]. The diffusion of Cl^- indicated a much smaller dependence on temperature, the activation energy being only 4–8 kcal/mole, the lower figure being not much higher than that for free diffusion[17]. The presence of uncompensated negative groups produced a substantial increase in the permeability barrier to Cl^- (activation energy: 5 kcal/mole for phosphatidylcholine, and 8 kcal/mole for phosphatidic acid), but it always remained lower than that found for the diffusion of K^+. The plot of the natural log of K^+ self-diffusion rate *vs.* the reciprocal absolute temperature reveals a discontinuity at approx. 35–37° for the rather slowly leaking particles composed of phosphatidylcholine, phosphatidylcholine–stearylamine and also phosphatidylserine[7]. It is possible that this observation could be the result of a minor phase change occurring at that temperature.

Effect of bivalent metals

One of the most interesting aspects of the permeability properties of the acidic phospholipids is the changes observed in the presence of various bivalent metals. Fig. 3 shows the effect of Ca^{2+} on the diffusion of K^+ through particles of phosphatidic acid, phosphatidylserine, and phosphatidylinositol. In general it can be seen that an increase in the diffusion rate of K^+ was produced when Ca^{2+} was present above a certain concentration which was markedly different for each particular phospholipid (0.1 mM for phosphatidic acid, 0.8 mM for phosphatidylserine, and 5 mM for phosphatidylinositol). Another interesting feature demonstrated by Fig. 3,

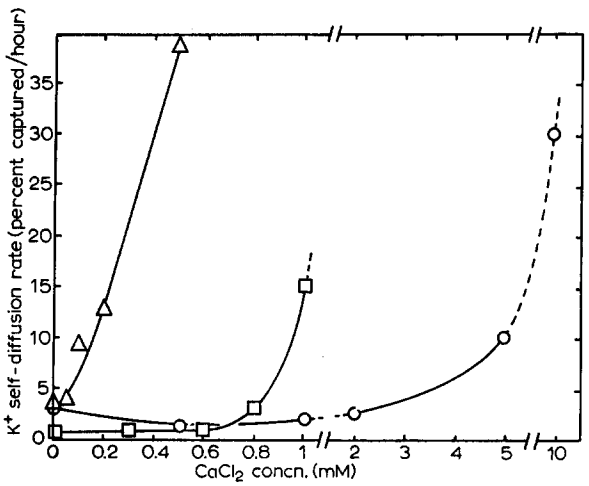

Fig. 3. Effect of Ca^{2+} on self-diffusion rate of K^+ through phosphatidic acid, phosphatidylserine, and phosphatidylinositol liquid crystals. △, phosphatidic acid; □, phosphatidylserine; ○, phosphatidylinositol. All experiments were performed at room temperature, in 145 mM KCl–Tris–HCl (pH 7.4).

is that low Ca^{2+} concentrations (0.5–1 mM) produced a decrease in the diffusion rate of K^+ through phosphatidylinositol. The same tendency for such a decrease at low Ca^{2+} concentrations was shown by phosphatidic acid, but the effect was smaller. The possible significance of the above results can be seen in terms of the well recognized effects of bivalent metals in increasing (*e.g.* red cells[19], nerve cell junctions[20]) or decreasing (*e.g.* squid axon[21], red cells[19]) the permeability of biological cell membranes.

It is difficult to envisage the actual structure of the phospholipid–Ca^{2+} complex which results in increased permeability. It has been suggested[22] that interaction between the phospholipid head-groups and polyvalent metals produces an 'inverted-micelle' type of structure, and the resulting instability of the bilayer causes an increased permeability. Indeed, ALLEN, CHAPMAN AND SALSBURY[23] recently interpreted changes in the electron paramagnetic resonance spectrum of Mn^{2+} after interaction with phosphatidylserine, in terms of a similar structure. Nevertheless, X-ray diffraction patterns of phosphatidylserine–Ca^{2+} complexes, produced under the conditions resulting in high cation diffusion rates, indicated a well-ordered lamellar structure with a repeating distance of 54 Å (see ref. 8). This structure does not appear to be compatible with an hexagonal arrangement of inverted-micelle type structures[24]. The contraction of the interlamellar spacing after addition of Ca^{2+} outside the particles (observed many years ago by PALMER AND SCHMITT[25] for brain cephalin mixtures) certainly indicates that Ca^{2+} first increases the permeability of the lamellae and subsequently enters into the interior of the particles. Under these conditions, it would be difficult to establish the extent to which the observed increases in leakage of K^+ represent an actual increase in exchange diffusion as distinct from extrusion of both water and salt out of the particles. Further experiments relating to this effect will be reported in a subsequent communication[26].

Effect of proteins

As previously discussed[8], the presence of cytochrome *c* and bovine plasma albumin tended to produce vesicular structures of small size (200–1000 Å). The protein concentrations used routinely, 0.4 mg/ml, represented only about 1–10% of the phospholipid on a weight/weight basis. It is not known with any certainty how much of the protein was incorporated into the particles, but estimates were made in the case of cytochrome *c* after high-speed (130 000 × *g*) centrifugation of the dispersion by measuring the absorbance of the supernatant (representing unincorporated protein) at 410 mμ. Phosphatidylcholine and phosphatidylcholine–phosphatidylethanolamine particles, absorbed only as much protein as would be expected on the basis of the physically trapped aqueous medium, while phosphatidylserine particles absorbed 80% or more of the cytochrome *c*. The presence of protein seemed generally to increase salt capture in the case of phosphatidylcholine and phosphatidylcholine–phosphatidylethanolamine particles (Table II), an effect which can presumably be attributed to the vesicular nature of the particles. Any trapped protein would, of course, be associated with counter-ions which would make an additional contribution to the measured ionic capture. The proteins alone did not capture any labelled ions under the conditions of the experiments, indicating a rapid exchange with the bulk-phase ions. Salt capture was decreased in the case of phosphatidylserine particles swollen in the presence of cytochrome *c*, probably because of complex formation between the basic protein and acidic lipid. This would result both in a diminution in the number of ionic groups available for counter-ion binding and in a general tightening of the structure due to charge neutralization, with consequent decrease in the size of the aqueous compartments. It is interesting to note that the diffusional exchange rates for the captured ions (Table II) were not markedly different from those observed in the absence of protein. The ratio of the diffusion rate of Cl^- to that of Na^+ or K^+ most closely resembled the ratio observed with lipid dispersions prepared by sonication, and this can also be correlated with the generally small size of the particles in each case[8]. The lack of an effect on diffusion rates can be taken as an indication that no appreciable penetration of protein chains into the phospholipid bilayer occurs under these conditions. This may be because of the close packing of the phospholipid molecules[27] or because of the very low protein/lipid molar ratio (less than 0.01) employed.

General conclusions

The main question arising from these and previous studies[4] is whether the exchange diffusion takes place across lamellae, or along aqueous channels between lamellae. Arguments in favour of translamellar diffusion have been presented[4]. Although the question still cannot be answered with complete certainty, it is relevant that phosphatidylcholine and phosphatidylethanolamine–phosphatidylcholine particles, which had the most vesicular appearance in the electron micrographs also exhibited very high Cl^-/K^+ discrimination. If the large, central, aqueous compartments demonstrated by the electron microscope were connected with the external medium in the wet state, a Cl^-/K^+ ratio of near unity would have been found. Moreover, captures would be expected to be low in such structures, but, in fact, captures were considerably higher in the vesicular particles than in those which presented a more myelinic appearance. The above argument is further strengthened by the finding

that sonication, which produced smaller particles, also resulted in a higher activation energy for the diffusion of the captured K^+. These findings obviously favour 'closed' structures and therefore a predominantly translamellar diffusion pathway.

The marked anion/cation discrimination observed with most phospholipids indicates that the diffusion 'pores' are of comparable diameter to that of the ions themselves. It seems unlikely, however, that pore size alone is responsible for the selectivity since the hydrated radii of Cl^- and K^+ are similar[28] and one would have expected greater discrimination between K^+ and Na^+. The finding that Cl^-/K^+ diffusion ratios were lowest with phospholipids not possessing positive groups (phosphatidylinositol and phosphatidic acid), is compatible with the possibility that positively charged groups promote diffusion of anions by dominating the access to, or flow through, the diffusion channels[4]. Nevertheless, this supposition does not explain how negatively charged phospholipids maintain a Cl^- diffusion rate which is only slightly less than that of K^+. It seems that although the presence of extra positive or negative groups has some influence, it is perhaps not the overriding factor in determining anion–cation discrimination. DANIELLI[29] has pointed out that in considering the energy barriers for ion diffusion through a bimolecular leaflet, the alignment of the permanent dipoles of the interface could be more important than the formal charges. The alignment of the phospholipid permanent dipoles with the positive poles towards the hydrocarbon interior would be expected to favour anionic transport. This would explain the high Cl^-/K^+ diffusion ratio found with the neutral phospholipids (phosphatidylcholine and phosphatidylcholine–phosphatidylethanolamine). The introduction of a negative charge near to the hydrocarbon–water interface would be expected to diminish the effect of the oriented dipoles and thus result in a decrease of Cl^- diffusion rate. This was established experimentally for the case of phosphatidylinositol, phosphatidic acid, and phosphatidylcholine–stearic acid. Following the same argument, the relatively small influence of the extra carboxyl group of phosphatidylserine on Cl^- diffusion may be attributed to its localization further within the water phase. The observed effect of cholesterol in decreasing Cl^-/K^+ ratio could be seen as a reflection of the well recognized interaction of this compound with egg yolk phosphatidylcholine[15,16]. It should be emphasized that the Cl^- diffusion rates reported here for the neutral phospholipids (phosphatidylcholine, phosphatidylcholine–phosphatidylethanolamine, phosphatidylcholine–stearylamine) do not represent the initial rates and consequently the Cl^-/K^+ ratios given for these systems can only be an underestimation of the actual values.

The uncertainty regarding the surface area of the phospholipid makes it difficult to relate the diffusion rates given in this paper to reported fluxes of ions through biological membranes. Nevertheless, estimates of the average surface area per μmole of phospholipid have been made in two cases. A method based on the surface potential changes, during the titration of a phosphatidylcholine monomolecular film in the presence of phosphatidylcholine dispersion[30], gave an average surface area of 300 cm^2 per μmole of lipid. The other estimate was based on the electron micrographs of sonicated phosphatidylinositol dispersions[7] showing small vesicles containing one or two lamellae. An area of 900 cm^2 per μmole of lipid was calculated, assuming an average area per molecule of 50–60 Å (see refs. 27, 31) and an average of two lamellae per vesicle.

Fluxes for the diffusion of K^+, calculated on the basis of the areas mentioned,

are approx. $0.5 \cdot 10^{-15}$ equiv/cm^2 per sec for phosphatidylcholine, and approx. $2 \cdot 10^{-15}$ equiv/cm^2 per sec for phosphatidylinositol. These values are somewhat lower than the reported flux of K^+ through red cells[32] ($100 \cdot 10^{-15}$ equiv/cm^2 per sec). The comparison is more favorable if the rates are expressed as % per h. Thus phosphatidylcholine shows a value of 0.5%, phosphatidylinositol, 5%, and different species of red cells, 1–6% (see ref. 32). The fluxes of Cl^- through phosphatidylcholine liquid crystals can be converted to electrical resistance using the equation $G_i = F^2 z_i M_i / RT$ (see ref. 33) where G_i is the specific conductance in $\mu\Omega^{-1} \cdot cm^{-2}$, M_i the flux in pmole $\cdot$ $cm^{-2} \cdot sec^{-1}$, F the Faraday, R the gas constant, T the absolute temperature, and z_i the valency of the ion. Thus the flux for the faster-moving ion ($M_{Cl^-} = 3 \cdot 10^{-2}$ pmole $\cdot$ $cm^{-2} \cdot sec^{-1}$) gives a specific resistance of $1 \cdot 10^7\ \Omega \cdot cm^2$. This is certainly a value much higher than that of nerve axon[33], but comparable to the reported resistances of black films[34].

In a final assessment of the similarities between the behaviour of phospholipid bilayers and that of biological membranes, consideration should be given to the ability of bivalent metals to increase or decrease the permeability of the acidic phospholipids and possibly to additional effects due to specifically bound proteins. Extrapolating from the results with pure phospholipids presented here, it seems reasonable to suppose that membrane areas composed of mixtures of phosphatidylcholine, phosphatidylethanolamine, and cholesterol would represent the main permeability barrier to cations, while areas rich in phosphatidylserine, phosphatidylinositol, or phosphatidic acid could constitute loci exhibiting reversible permeability changes in response to small alterations in the ionic environment. In conclusion, it appears that the model system described here, consisting of lamellae of pure phospholipids, exhibits numerous properties similar to those of biological membranes and can therefore be taken as a valid model for studying passive diffusion of ions and metabolites.

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