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CATION PERMEABILITY OF LIPOSOMES AS A FUNCTION OF THE CHEMICAL COMPOSITION OF THE LIPID BILAYERS

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SUMMARY

1. Comparable liposome preparations were obtained from lipids differing in degree of unsaturation and cholesterol content.

2. An exchange between alkali ions and protons through the bilayers was induced by replacing the alkali ions on the one side of the outer lipid membrane by impermeable choline ions.

3. The equilibration of the gradient was measured directly with ion selective electrodes or, indirectly as a volume change following a net uptake of osmotic material.

4. Individual permeabilities of H^+ and alkali ions were measured by making the membrane freely permeable to the counter ion using specific ion carriers; valinomycin for K^+ and FCCP (*p*-trifluoromethoxycarbonylcyanide phenylhydrazone) for H^+.

5. Lipid bilayers with different degrees of unsaturation or cholesterol content all demonstrated a very limited permeability to protons.

6. With increasing unsaturation, a significant increase in K^+ permeability could be measured.

7. The increase in Na^+ permeability with increasing unsaturation was much less than for K^+. This indicates a pertinent discrimination in permeability of K^+ with respect to Na^+ and H^+ in the very unsaturated membranes.

INTRODUCTION

Membrane model systems consisting of lipid bilayers are used to elucidate the extent to which the chemical identity of the structural lipids can explain the specific permeability properties of various interfaces. With respect to ion permeability, attention has been directed primarily to the importance of the charged polar groups. Both with the black film technique[2] and with the liposome system[3], the conclusion has been reached that a net charge on the membrane has a large effect; enhancing the permeation of ions of opposite sign and suppressing the penetration of ions of similar sign. The significance of the chemical composition of the apolar part of the lipid molecules for the permeability is less clear. Using black films, it has been found that the electrical resistances of films formed with (dioleoyl)-lecithin are less than

Abbreviation: FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone.

those of comparable films formed of egg yolk lecithins[4]. Furthermore, it has been observed that the presence of cholesterol gives an increase in resistance[5]. However, the conclusions that can be drawn from these results admit of some doubt as the bilayers in this system contain a considerable and perhaps variable amount of lipid solvent.

Liposomes are composed of pure lipid bilayers and therefore are preferable for studying the effect of changes in the paraffin core. Using this system, it has been claimed that the leak of Na^+ from vesicles prepared from lecithins isolated from essential fatty acid deficient rats is different from those prepared from normal rat lecithins[6]. It has also been shown that the valinomycin mediated $^{86}Rb^+$ permeation is strongly dependent on the number of double bonds in the fatty acid chains[7]. The experiments described in this paper add kinetic evidence to the view that the passive diffusion of ions through membranes is also determined by the composition of the paraffin layers and that, depending on the degree of unsaturation, there is an important discrimination in the permeability for the alkali ions.

EXPERIMENTAL

Materials

Egg lecithin was extracted from egg yolk and purified by acetone precipitation and subsequent chromatography over alumina oxide and silica gel. Essential phospholipid, a highly unsaturated lecithin containing about 70 % linoleate was a gift of Dr. H. Eikermann of Nattermann and Cie, Köln, Germany. (Dioleoyl)- and (dipalmitoyl)-lecithin were synthesized by reacylation of glycerylphosphorylcholine obtained by deacylation of egg lecithin[8]. Phosphatidic acid was prepared from egg lecithin by degradation with phospholipase D extracted from Savoy cabbage[9]. Valinomycin A grade was obtained from Calbiochem, Los Angeles, California, U.S.A. FCCP (*p*-trifluoromethoxycarbonylcyanide phenylhydrazone) was generously supplied by Dr. P. G. Heytler, Du Pont Co., Wilmington, Delaware, U.S.A. Both valinomycin and FCCP were added in ethanol solutions in a total volume of 1–5 μl. All other reagents were commercial and of Analytical Reagent Grade.

Methods

Multilayered liposomes were prepared from isolated and synthetic lecithins and of mixtures of these lipids with cholesterol after addition of about 4 mole % phosphatidic acid as described earlier[10]. We obtained comparable dispersions of osmotic sensitive particles. The concentration of the dispersion was always 75 mM lipid. When the liposomes were formed in buffer solutions, the outside buffer was removed by dialysis against isotonic ice cold KCl or choline–chloride solutions. Changes in H^+ activity were monitored by a Schott and Gen. 9259/8 combination electrode (Jena Glasswerk, Mainz, Germany) connected with a Radiometer type PHM 26 pH meter (Radiometer, Copenhagen, Denmark) and a recorder.

Changes in K^+ activity were monitored by a Philips liquid membrane K^+ electrode type IS 560-K connected to the Radiometer pH meter. This new electrode made with an ion selective membrane of valinomycin dissolved in diphenylether demonstrated a much higher selectivity and also a better reproducibility and stability than the commercially available glass electrodes. Leak of valinomycin from the electrode into the medium, which might be a limit of the electrode, could not be deter-

mined in our experiments. The calibration of the electrode was made in each experiment by adding, in a separate sample and under identical experimental conditions, the amounts of a standard solution of KCl necessary to obtain the same potential changes as observed in the experiments with liposomes.

Absorbances of the liposome dispersions were measured with a Hitachi–Perkin Elmer double beam spectrophotometer model 356 using a single wavelength (550 nm). The cuvette of 1-cm light path was equipped with a mechanical stirrer and thermostated as indicated for each experiment.

RESULTS

pH equilibrations

The experiments demonstrated in Figs. 1 and 2 deal with changes in pH which can be observed when liposomes are suspended in media different from that in which they are formed. In the first experiment, liposomes with buffered KCl inside were added to a medium of 50 mM choline chloride. The concentration gradient of K^+ across the membrane will tend to equilibrate only when there is a compensation of charge. The compensation can be found in a counterflow of outside positive ions into or a concurrent leak of anions out of the liposome. As the choline ions may be considered to be very impermeable[11] and the penetration of negatively charged anions will be restricted because of the negative charge on the bilayers, the most important process will be a K^+ (inside) $\rightleftharpoons$ H^+ (outside) exchange. In agreement with this analysis an alkalinization of the external medium could be measured when KCl containing liposomes were dispersed in isotonic choline chloride. In the experiment demonstrated

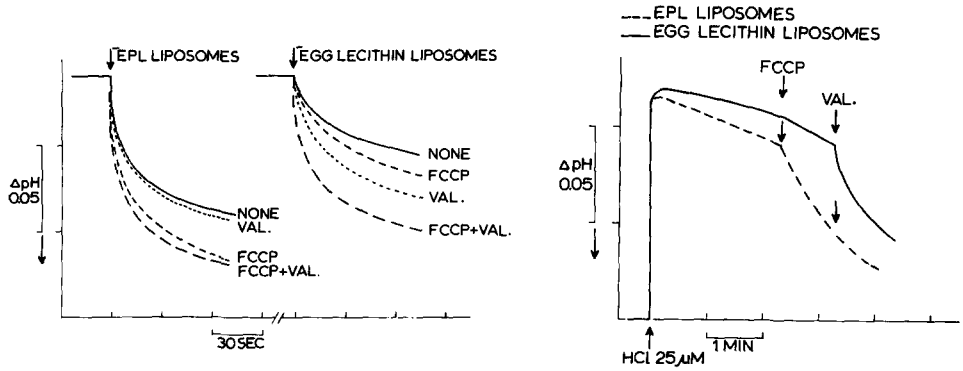

Fig. 1. Alkalinisation of choline media following the addition of KCl containing liposomes. The liposomes were prepared in 40 mM KCl buffered at pH 5.8 with 10 mM citrate and dialysed extensively against ice cold 50 mM KCl. 0.2-ml samples of the dispersions were added to 3.5 ml 50 mM choline chloride supplemented with 0.5 mM choline citrate (pH 5.8). Where indicated, valinomycin (VAL.) was added to the medium before the addition of the liposomes in an amount of 0.25 μg/ml and FCCP in a final concentration of c.3 μM. Temperature 30°. Abbreviation for all figures: EPL = essential phospholipid(s).

Fig. 2. Recording traces of the external pH during pulsed acid titrations of dispersions of liposomes. The liposomes were prepared in a solution containing 40 mM choline chloride, 3 mM KCl and 10 mM choline citrate (pH 5.8) and subsequently dialysed against 50 mM choline chloride. 0.2-ml samples of the dispersions were added to 3.5 ml of 50 mM choline chloride at 20°. After the equilibration, the reaction was started with HCl. Valinomycin (VAL.) was added to 0.25 μg/ml and FCCP to 0.3 μM final concentration.

in Fig. 1 some citrate buffer was present both in the liposomes and in the external medium, which enabled a more stable recording of the phenomenon. However, the alkalinization could also be observed in the absence of buffer. Fig. 1 shows that in the presence of valinomycin and FCCP the gradient both in dispersions of essential phospholipids and egg lecithin liposomes equilibrated very quickly and at the same rate in both preparations. The differences in the extent of alkalinization can be accounted for by small variations in the amount of K^+ trapped inside. Addition of FCCP alone makes the membranes freely permeable to H^+ and consequently the rate of the exchange process will be dependent on the permeation of K^+. On the other hand, addition of valinomycin alone induces selectively high permeability for K^+ and in the presence of this ion carrier the rate of equilibration will be dependent on H^+ movements. The preaddition of FCCP alone had a marked effect on the equilibration in both dispersions, whereas the effect of valinomycin alone was much less on essential phospholipid liposomes than on those prepared with egg lecithin. In the system prepared with egg lecithin, the two ion carriers did have an additive effect, whereas FCCP alone is able to give a fast equilibration with essential phospholipid liposomes. These phenomena suggest that both types of lipid bilayers are impermeable to H^+ but that the very unsaturated essential phospholipid membranes are more permeable to K^+ than the more saturated ones formed of egg lecithin.

The same effects of the two carriers could be observed in pH jump experiments. Fig. 2 shows pulsed acid titrations of the systems of liposomes buffered inside and suspended in unbuffered media of 50 mM choline chloride. When a small amount of HCl was added to the system in equilibrium, the recording trace of pH changes showed two phases: a fast process leading to the acidification of the suspending medium and a slow process of external alkalinization which corresponds to H^+ penetration into and titration of the inner compartment enclosed by the lipid bilayers. A faster titration of the inner phase appeared to be strongly dependent on FCCP in the case of essential phospholipid and on both FCCP and valinomycin in the case of egg lecithin liposomes. Preaddition of both carriers before the jump (not shown)

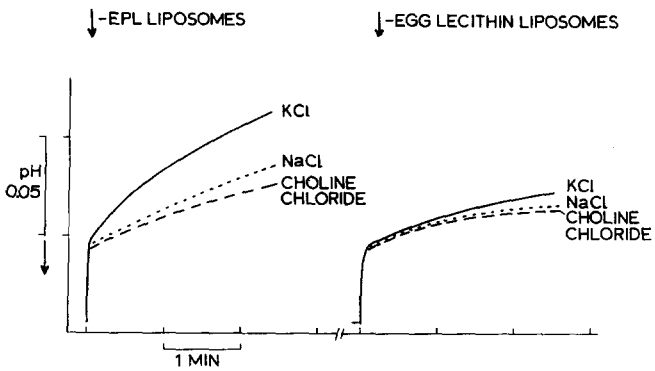

Fig. 3. pH Changes following the addition of liposomes to media containing different cations. Liposomes were prepared in a solution containing 40 mM choline chloride and 10 mM choline citrate (pH 5.8) and subsequently dialysed against 50 mM choline chloride. The experimental media contained 50 mM KCl, NaCl or choline chloride buffered at pH 8 with 1.5 mM glycylglycine and supplemented with 0.3 μM FCCP. Temperature 25°.

abolished the slow process and the titration of the inner compartment occurred instantly without any difference between the two liposome preparations.

Fig. 3 presents an experiment in which liposomes prepared in buffered choline chloride were suspended in only slightly buffered media of isosmolar choline chloride. Due to the opposite direction of the gradient compared to that of Fig. 1, in this case we could observe an acidification of the medium caused by the exchange H^+ (inside) $\rightleftharpoons$ cation (outside). Because our media were supplemented with the H^+ carrier FCCP, the rate of equilibration was dependent on choline$^+$, Na^+ or K^+ permeability only. The recording traces of the figure show that upon addition of liposomes of the two different preparations, the acidification of the media containing choline$^+$ or Na^+ is similar. On the other hand, the addition of liposomes to K^+-containing media did start a much faster acidification, but only in the case of the very unsaturated essential phospholipid membranes. The immediate acidification observed in Fig. 3 was due to the difference in pH between the suspension of liposomes and the medium. The small and slow acidification observed in the case of choline and Na^+ media after the fast initial pH changes may be influenced by the pH difference between the internal and external compartments.

Swelling of liposomes in alkali acetates

The experiments demonstrated in Fig. 4 refer to liposomes prepared in choline chloride and suspended in hypertonic sodium and potassium acetate, respectively. After an osmotic shrinking of the liposomes an equilibrium was reached as indicated by a constant absorbance of the dispersions. Then FCCP was added to make the membrane permeable to protons. As a consequence of this addition, a gradual decrease in the absorbance took place demonstrating an osmotic swelling of the liposomes[12]. This swelling is caused by an uptake of water in response to a net flow of the alkali acetate into the liposome. To explain this we assume that FCCP facilitates acetate penetration indirectly. Addition of FCCP initiates the exchange K^+ (outside) $\rightleftharpoons$ H^+ (inside). The decrease in pH in the outside medium causes the formation of undissociated acetic acid and this uncharged molecule can easily penetrate the membrane[12].

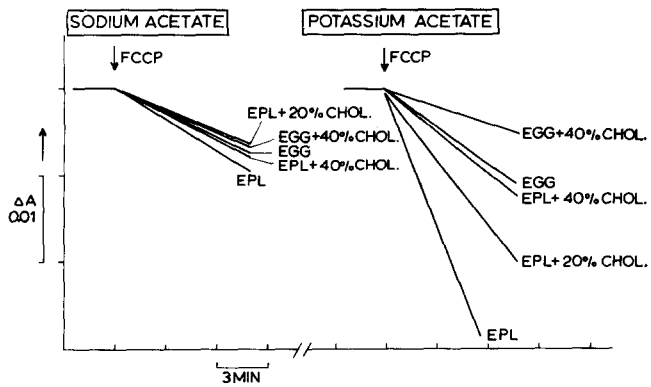

Fig. 4. Osmotic swelling of liposomes in alkali acetate. Liposomes of essential phospholipids, egg lecithin and of mixtures of these lipids with 20 or 40 mole % cholesterol (CHOL.) were prepared in 50 mM choline chloride. 0.2-ml samples were added to 5 ml of 100 mM sodium or potassium acetate. After equilibration 0.3 μM FCCP was added to initiate the change in absorbance which was measured at 550 nm. Temperature 28°.

The combined movement of K^+ (or Na^+) and acetic acid into the liposome is equivalent to the permeation of the complete alkali acetate molecule.

In sodium acetate the swelling is only limited and the small differences between the various liposome preparations may be explained by small differences in size and geometric structure of the particles in the various dispersions. Qualitatively similar results were obtained when the liposome preparations were added to media containing lithium acetate or choline acetate. However, when suspended in potassium acetate, the same liposome preparations did show much greater differences in their swelling behavior. The swelling rate of the essential phospholipid liposomes was much faster than that of the more saturated egg lecithin liposomes. Preparations of both types of lecithins mixed with cholesterol demonstrated that cholesterol is able to reduce the swelling rate. The tentative conclusion that can be drawn from these experiments is that all the lipid bilayers are relatively impermeable to choline, Na^+, and Li^+ but that with increasing unsaturation, there is an increasing permeability with respect to K^+, whereas cholesterol is able to restrict permeability.

Direct measurement of the K^+ leak from the liposomes

In order to obtain quantitative data, the leak of K^+ from liposomes prepared with 50 mM potassium acetate inside was followed directly with the K^+ electrode as described in the methods. After addition of FCCP, making the K^+ (inside) $\rightleftharpoons$ H^+ (outside) exchange independent of the H^+ penetration, important differences in the K^+ leak could be measured. In agreement with the foregoing experiments, the very unsaturated essential phospholipid membranes were found to be much more permeable than the bilayers of egg lecithins (see Fig. 5). This relation between unsaturation and K^+ permeability could also be demonstrated with liposomes prepared from synthetic (dioleoyl)-lecithin and mixtures of this lipid with (dipalmitoyl)-lecithin. When the relative amount of the saturated lecithins was increased, the leak of K^+ was reduced. On the other hand, all the liposome preparations demonstrated a large K^+ release upon addition of FCCP when they had been pretreated with valinomycin (2 μg/ml).

To exclude the possibility that the differences in release of K^+ might be due to

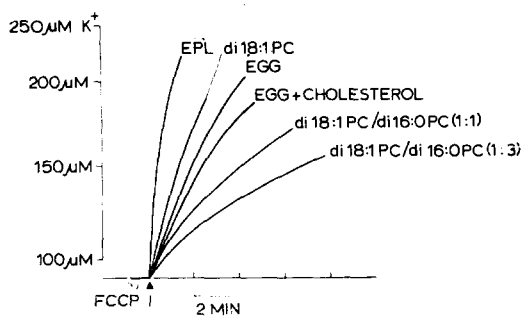

Fig. 5. Leak of K^+ from liposomes. The liposomes were prepared in 50 mM potassium acetate using essential phospholipids, egg lecithin, egg lecithin with 40 mole % of cholesterol, (dioleoyl)-lecithin and mixtures of this synthetic lipid with 50 and 75 mole % of (dipalmitoyl)-lecithin, respectively. The outside K^+ was removed by dialysis against ice cold 50 mM choline chloride and then 0.2-ml samples of the dispersion were added to 4 ml of medium containing 50 mM choline chloride and 0.3 M KCl thermostated at 22°. The leak of K^+ after the addition of FCCP was followed with the potassium electrode as described in *Methods*.

differences in the total amounts of K^+ trapped inside the various preparations, these values were determined. The results of these qualitative determinations are given in Table I. The total amount of K^+ was calculated as follows: in parallel experiments, samples of the dialysed liposomes were lysed by addition of alcohol up to a concentration of 80 % and then known amounts of this solution were added to the same choline chloride medium and changes in K^+ activity were recorded. The external K^+ concentration values are expressions of the instant K^+ changes following the addition of liposomes to the medium. The results show that the biggest variation in the K^+ trap is only a factor of 1.4. Furthermore, this variation was not related to the unsaturation of the liposome preparations.

TABLE I

COMPARATIVE DATA ON THE K^+ CAPTURE OF VARIOUS LIPOSOME SYSTEMS AND THE RELEASE OF THESE IONS INTO A CHOLINE CHLORIDE MEDIUM

For experimental details, see the legend to Fig. 5 and the text.

Liposome preparations lecithins	*K^+ present in liposomes (mmoles/mole of phospholipid)*			*K^+ release from liposomes (percentage/min)*	
	Total	*Outside*	*Inside*	*Before FCCP*	*After FCCP*
Essential phospholipids	229.3	16.6	212.6	0.9	25.7
di-$C_{18:1}$	155.2	6.6	148.6	0.6	13.8
Egg	194.6	6.0	188.6	0.7	7.0
Egg + 40% cholesterol	173.3	3.3	170.0	0.7	5.9
$C_{18:1}$ + $C_{16:0}$ (1:1)	190.6	12.6	178.0	0.9	4.3
$C_{18:1}$ + $C_{16:0}$ (1:3)	161.2	6.6	154.6	0.8	3.2

The quantitative data on the release of K^+ from liposomes in Table I, expressed as percentage of the total amount of K^+ enclosed, show that before the addition of FCCP, there were no significant variations between the different preparations. However, after the addition, the leak varies by a factor of 8, increasing parallel with the unsaturation of the lipid bilayers.

DISCUSSION

Lecithins supplemented with a suitable amount of a charge carrier, *e. g.*, 4 % phosphatidic acid, do form osmotically sensitive liposomes spontaneously when they are dispersed in diluted electrolyte solutions. Earlier reports from our laboratory described that microscopic examinations of miscellaneous preparations formed from lecithins differing in the composition of the fatty acid chains or from mixtures of these phospholipids with variable amounts of cholesterol did not reveal apparent differences in particle size[10]. Also the increases in osmotic volume of the various preparations in hypotonic solutions appeared to be very much the same. These observations together with the results of Table I show that the amount of K^+ trapped inside the structures varied only slightly and permit the conclusion that the various preparations consisted of comparable membrane model systems formed by concentric sheaths of lipid bilayers intercalated by water layers. However, the packing of the

chains in the paraffin core will be different as a consequence of variations in the degree of unsaturation or of the presence or absence of cholesterol.

In the present experiments, ion fluxes through the bilayers were measured in order to elucidate the effect of this variation in the packing of the hydrophobic barrier on the permeability behavior. The ion fluxes were induced by a gradient across the outer lipid bilayer brought about by replacing the alkali ions on the outside of the bilayers by choline ions. As discussed in the results, we assume that this gradient causes primarily an exchange of alkali ions against protons. Such an exchange is dependent on both the penetration rate of the alkali ions and the protons. In order to be able to study the individual cation permeabilities, we made use of valinomycin and FCCP as specific cation carriers. Valinomycin, because of specific complexing properties for K^+ is well known to act as a specific carrier for this ion[11]. The action of FCCP and other weak acid uncouplers on lipid bilayers has recently been described in several papers[13,14]. In a dissociation equilibrium they are considered to form uncharged molecules next to negative ions with a delocalised charge, both of which are well able to solubilize in the hydrophobic barrier. Because of this equilibrium in which protons are involved, FCCP is able to act as an effective and very selective carrier for H^+ permeation, a phenomenon which has been demonstrated beautifully for lipid bilayers in experiments with black films[15].

In all our experiments the addition of both ion carriers caused a rapid equilibration of the gradient and, in the cases where acetate was also present, resulted in a net uptake or release of osmotic material. The incubation with valinomycin alone produced a limited increase in the exchange rate. Even at high concentrations of valinomycin this stimulation was incomplete, as demonstrated by a strong complementary effect of the FCCP in all the systems we used. These results lead us to conclude that the liposomes are highly impermeable to H^+ irrespective of the degree of unsaturation. On the other hand, the addition of FCCP alone makes the membrane freely permeable to H^+ and equilibration will proceed with a rate dependent on the permeability of the alkali ion. Under these conditions, all the experiments demonstrated that the ion equilibration increased considerably with the degree of unsaturation, indicating an increase in the K^+ permeability. A comparable relationship between unsaturation and membrane penetration has been demonstrated for the permeation of small non-electrolyte molecules[10] and for the valinomycin mediated exchange of $^{86}Rb^+$ (ref. 7). The reducing effect of cholesterol on the K^+ permeation also appeared to be comparable with that found for non-electrolyte permeability.

The unsaturation-dependent cation discrimination measured in the presence of FCCP is interesting. Both the much slower pH equilibration in NaCl than in KCl and the more limited swelling in sodium acetate compared to that in potassium acetate solutions, especially in the more unsaturated systems, support the view that the increase in permeability with increasing unsaturation is less for Na^+ than for K^+. This means that the more unsaturated lipid bilayers exhibit a significant discrimination in permeability not only between H^+ and K^+, but also between Na^+ and K^+. From the present experimental approach it cannot be decided whether this selective higher K^+ permeability is purely related to the unsaturation or whether perhaps FCCP is mediating in the K^+ permeability as well. A weak valinomycin like activity of the FCCP combined with the high degree of unsaturation could explain the selectivity[7]. On the other hand, it is relevant to mention that the cation transference numbers[16,17]

for Na^+ and K^+ that have been calculated from membrane potential measurements on black films of egg lecithin in NaCl and KCl solutions showed only slight differences, but that $^tK^+$ was found slightly higher than $^tNa^+$. Furthermore, it has been claimed that the selectivity for alkali metal ions exhibited by black membranes of brain lipids[18], which probably are more unsaturated than those formed with egg lecithin, followed the Hoffmeister series: $Li < Na^+ < K^+ < Cs^+$.

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