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MOVEMENT OF CALCIUM THROUGH ARTIFICIAL LIPID MEMBRANES AND THE EFFECTS OF IONOPHORES

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SUMMARY

The calcium efflux from multi-layered vesicles (liposomes) of different lipid composition has been studied. Liposomes composed of lipids extracted from cattle retinas are compared with liposomes which consist of phosphatidylcholine or a 1 : 1 phosphatidylcholine/phosphatidylserine mixture. The percentages of ^{45}Ca capture by these three types of liposomes are 10, 1 and 4 % respectively.

The efflux rates are $2.5 \cdot 10^{-6}$, $2 \cdot 10^{-6}$ and $4 \cdot 10^{-5}$ s^{-1} respectively. The semilogarithmic efflux curves for phosphatidylcholine and phosphatidylcholine/phosphatidylserine liposomes are linear with time, but those for the retinal lipid liposomes are discontinuous. The activation energy for the calcium efflux from the latter liposomes is about 10.5 kcal/mol, both before and after the discontinuity.

The ionophores X537A and A23187 enhance the calcium leakage from retinal lipid liposomes, the latter ionophore being much more effective than the former. At high concentrations both ionophores seem to transport calcium as a 1 : 2 Ca · ionophore complex. At low ionophore concentrations, however, X537A appears to transport calcium as a 1 : 1 complex, but A23187 as a 2 : 1 complex.

INTRODUCTION

Light reduces the sodium permeability of the outer membrane of the rod outer segment [1]. Hagins [2] has proposed that this permeability decrease is caused by calcium ions released upon illumination from the rod sacs which contain the bulk of the visual pigment. This hypothesis is based upon electrophysiological measurements in experiments in which the external calcium concentration is raised or lowered. Recently, supporting evidence has been reported by Hendriks et al. [3], who have demonstrated a light-induced calcium loss from a particulate rod outer segment fraction.

These findings have stimulated an interest both in calcium release from the rod sacs and in the effect of calcium ions on the sodium permeability of the outer

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membrane. In order to obtain more information about the first type of process we have studied calcium movements through model membranes. In order to study the calcium permeability of the basic phospholipid bilayer structure of biomembranes, the efflux of calcium from various types of liposomes has been determined. Since calcium transport through biomembranes is generally thought to involve specific protein or peptide channels and/or carriers, calcium-specific ionophores have been introduced into the liposomes in an attempt to approach more closely the situation in a calcium transporting biomembrane. Studies of this kind should also be of interest in view of the important role of calcium in muscle contraction. So far, relatively little is known about the movement of calcium ions through biological membranes.

We report here experiments with liposomes prepared from retinal lipids, phosphatidylcholine and phosphatidylcholine plus phosphatidylserine. These liposomes are charged with ^{45}Ca and different parameters of the leakage rate, such as time, temperature, calcium concentration and the effects of ionophores, have been examined.

MATERIALS AND METHODS

Lipid preparation

Retinal lipids are extracted from whole cattle retina. The retinas are homogenized in an equal volume of methanol in a Potter-Elvehjem homogenizer, the same volume of chloroform is added and the mixture is homogenized again. After centrifugation (10 min, $5900 \times g$) the chloroform layer is isolated and extracted with 0.2 vols of 0.1 M KCl. After evaporation of the chloroform the lipids are dissolved in benzene/ethanol (4 : 1) and stored at 4 °C under N_2. Phosphatidylcholine is isolated from egg yolk according to Pangborn [4] and phosphatidylserine from brain according to Sanders [5]. Both phospholipids are stored under N_2 in benzene/ethanol (4 : 1) at 4 °C.

Liposome preparation

Liposomes are prepared in the following way. Organic solvent is removed from 40 mg of lipid in a rotating evaporator. To the resulting thin film is added 4 ml of a salt solution containing: NaCl, 135 mM; $CaCl_2$, 0.15 mM (except in experiments where the internal Ca concentration is varied); Tris · HCl (pH 7.4), 10 mM; 0.1 mCi of ^{45}Ca. Suspension is achieved by vigorous mechanical shaking of the mixture under N_2 for 30 min. The suspension is then left for 2 h at room temperature.

In order to remove radioactive ions which have not been trapped in the liposomes, a gel filtration method is used (Bangham et al. [6]). Two ml of the lipid suspension is passed down a column (2 cm × 23 cm) consisting of Sephadex G-50 equilibrated in a buffer containing 135 mM NaCl and 10 mM Tris · HCl (pH 7.4). The first and last lipid containing fractions, which contain the largest and the smallest liposomes respectively, are discarded in order to obtain a relatively uniform liposome population. The diluted suspension of liposomes thus obtained, free of untrapped ^{45}Ca, is used for the leakage experiments.

Leakage experiments

Samples of 1 ml of suspension are placed in small dialysis bags (Visking

tube, boiled in a solution containing 2 mM $NaHCO_3$ and 0.2 mM EDTA and thoroughly washed with double-distilled water) and the closed bags are placed in test tubes (1.3 cm × 13 cm) containing 9.5 ml of 135 mM NaCl in 10 mM Tris · HCl (pH 7.4). The stoppered tubes are attached to a vertically rotating disk (1 rev./min). Over a period of at least 2.5 h, samples for radioactive counting are taken every 20 min from the outer solution and at the end of the experiment the radioactivity remaining inside the dialysis bag is also measured.

All radioactive samples are dissolved in Aquasol (New England Nuclear) and counted in a Philips liquid scintillation analyser.

Ionophore experiments

The ionophore solution is added to the liposome suspension immediately before it is poured into the dialysis bag. The ionophores X537A, a gift from Hoffman-La Roche, and A23187, a gift from Eli Lilly and Company, are dissolved in ethanol. Care is taken that no more than 5 μl ethanol is added per ml lipid suspension, since higher ethanol concentrations affect the Ca leakage rate from the liposomes.

Calculations

A general equation describing diffusion from single-layered liposomes has been derived by Johnson and Bangham [7]. Fig. 1 shows a diagram of the system at a time t from the start of the leakage experiment. Liposomes with permeability p and volume v_c contain $N-n$ counts. The dialysis bag has a permeability p_1, a volume v_1 and contains $n-n_2$ counts. The external solution has a volume v_2 and contains n_2 counts. Ideally, at the start of the dialysis experiment, all N counts should be in the liposomes. However, there may be some free counts in the dialysis bag released by the liposomes after leaving the Sephadex column but before start of the dialysis. This number of counts is αN when $t = 0$. Johnson and Bangham [7] have derived the following general equation describing the leakage from the liposomes:

$$\frac{V_2}{V_0} - \frac{n_2}{N} = \frac{V_2}{V_0(K-L)}\{(1-\alpha)Ke^{-Lt} - (L-\alpha K)e^{-Kt}\} \quad (1)$$

where $V_0 = V_1 + V_2$, $L = p/V_c$ and $K = p_1 V_0/V_1 V_2$.

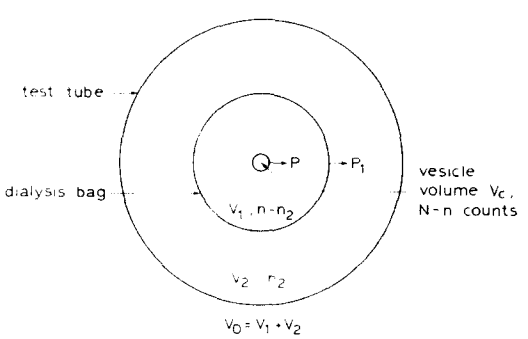

Fig. 1. Schematic presentation of the leakage experiments

When α is negligibly small, Eqn (1) becomes

$$\frac{V_2}{V_0} - \frac{n_2}{N} = \frac{V_2}{V_0(K-L)}\{Ke^{-Lt} - Le^{-Kt}\} \tag{2}$$

If the permeability of the dialysis bag is very much larger than that of the liposomes, then $K \gg L$ and Eqn (2) becomes

$$1 - \frac{n_2}{N}\frac{V_0}{V_2} = e^{-Lt} \tag{3}$$

If we now assume that $n_2 \cdot V_0/V_2 = n$, the following simple relation results

$$1 - \frac{n}{N} = e^{-Lt} \tag{4}$$

These relations have been derived for vesicles consisting of a single compartment surrounded by a single phospholipid bilayer, while our preparations consist of multi-layered liposomes. We can, however, apply these equations to our system by a simple change in the definition of L. In the appendix we show that by substituting $L = \lambda^2 P$ for $L = 1/V_c p$, our system can be described by the same equations as the single-layer system of Johnson and Bangham [7].

RESULTS

Capture of ^{45}Ca by the liposomes

The sequestration of radioactivity by the liposomes, i.e. the percentage of the total amount of radioactivity remaining in the lipid fraction after column elution, varies with the lipid composition. Liposomes derived from retinal lipids capture about 10%, phosphatidylcholine/phosphatidylserine (1 : 1) liposomes about 4% and phosphatidylcholine liposomes about 1 % of the total ^{45}C. Although considerable variations between different preparations of the same lipid composition may occur, due to a fairly random discarding of large (and thus many ^{45}Ca containing) liposomes, a clear difference in ^{45}Ca capture between the three types of liposomes remains.

Calcium efflux from liposomes

The calcium efflux from the three types of liposomes, plotted as $\log(1-n/N)$ vs t, is given in Fig. 2. A control experiment without liposomes shows that the efflux rate from the dialysis bag is about $6 \cdot 10^{-4}\ s^{-1}$. The efflux rates from the liposomes are about $2 \cdot 10^{-6}\ s^{-1}$ for the phosphatidylcholine type, $2.5 \cdot 10^{-6}\ s^{-1}$ for retinal lipid liposomes and $4 \cdot 10^{-5}\ s^{-1}$ for the phosphatidylcholine/phosphatidylserine type. Since the efflux from the dialysis bag is much faster than that from the liposomes, Eqn. (4) will apply and result in a linear plot. Only in the case of the phosphatidylcholine plus phosphatidylserine liposomes, where the efflux rate for the liposomes is rather close to that for the dialysis bag, do the plotted curves deviate from the linear course expected on the basis of Eqn (4).

A very striking phenomenon, which is shown more prominently in Fig. 3, is the fact that the efflux curve for retinal lipid liposomes seems to be discontinuous.

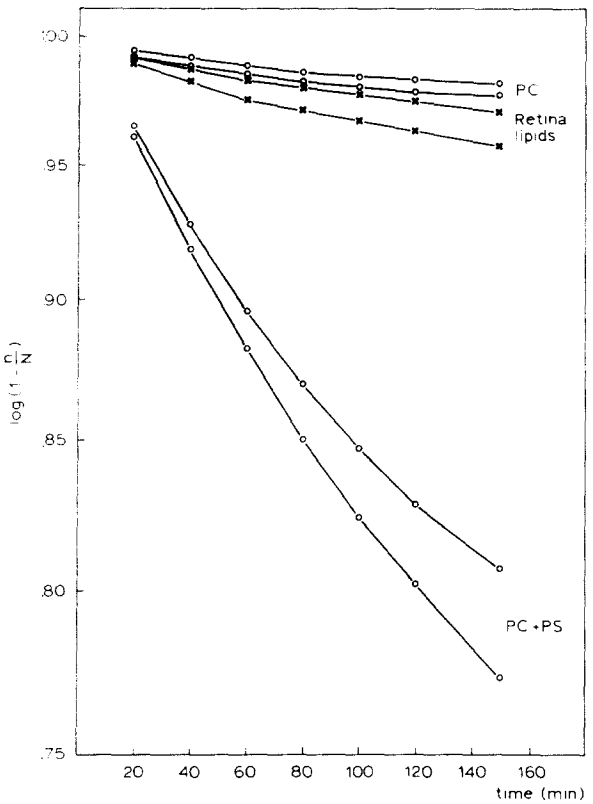

Fig. 2. ^{45}Ca efflux from different liposomes. Log $(1-n/N)$ is plotted against time (in min). PC, phosphatidylcholine; PS, phosphatidylserine.

While the curves for both the phosphatidylcholine and the phosphatidylcholine/phosphatidylserine liposomes are smooth lines, the curve for the retinal lipid liposomes is composed of two straight lines. The discontinuity in the curve usually occurs 60–100 min after the start of the dialysis experiment.

In most experiments the liposomes are prepared in a medium containing 0.15 mM Ca^{2+}. When this concentration is varied between 0.05 and 10 mM, which should presumably change the calcium concentration inside the liposomes, no significant effects upon efflux rates and capture percentage are noted.

The efflux of calcium from retinal lipid liposomes is measured at three temperatures, 5.5 °C, 22.5 °C and 37.5 °C. Fig. 3 shows two typical efflux curves for each temperature. While experiments at the same temperature with different liposome preparations result in curves that do not completely coincide, due to differences in numbers of liposomes and capture of ^{45}Ca, there is a much larger difference between leakage rates at the various temperatures. Moreover, in all experiments the leakage curve seems to be discontinuous. Fig. 4 gives the Arrhenius plots for both parts of the efflux curve. The activation energies are 10.2 kcal/mol for the early part (0–80 min, Fig. 4-1) and 10.7 kcal/mol for the later part (80–150 min, Fig. 4-2), values which are not significantly different.

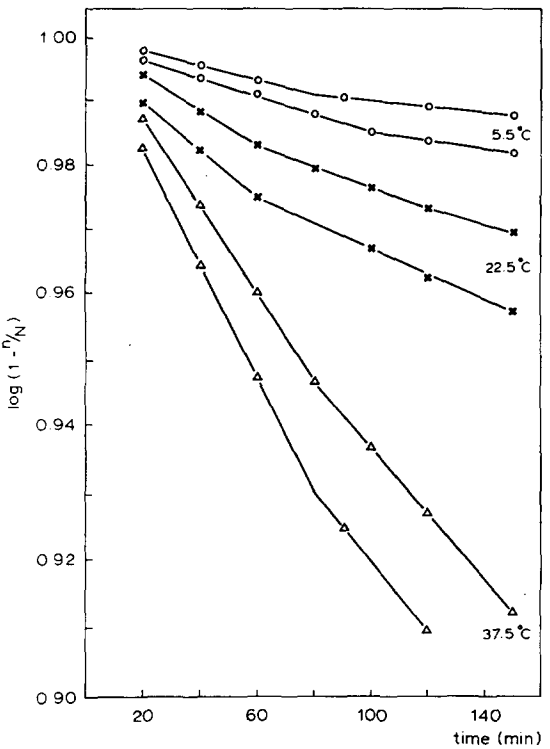

Fig. 3. ^{45}Ca efflux from retinal lipid liposomes. Log $(1-n/N)$ is plotted against time (in min) at three temperatures. Note that all lines are discontinuous.

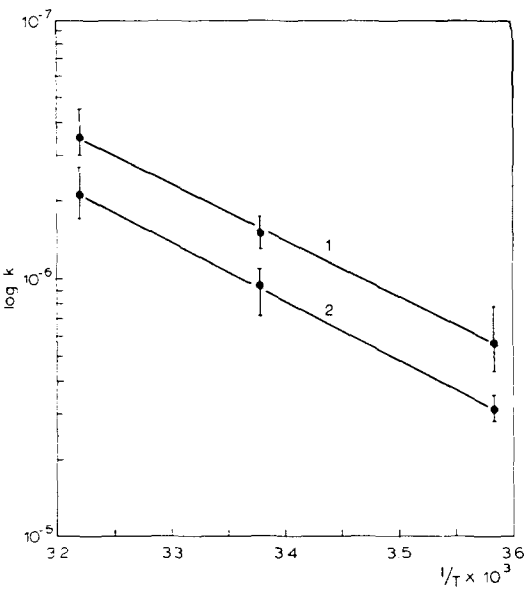

Fig. 4. Arrhenius plot for the ^{45}Ca efflux from retinal lipid liposomes. (1) Early efflux period, activation energy 10.2 kcal/mol, (2) later efflux period (after discontinuity in efflux curve), activation energy 10.7 kcal/mol.

Effects of ionophores on calcium efflux

Two ionophores have been used: A23187, which is specific for divalent cations, and X537A, which also transports monovalent cations. They have been added to retinal lipid liposomes. Both ionophores, especially when present in high concentration, increase the calcium efflux rate to such an extent that it is not permissible to apply Eqn (4) to describe the efflux. Assuming, however, that $\alpha = 0$, we may apply Eqn (2) and plot $\log(V_2/V_0 - n_2/N)$ against time. Typical results for experiments with

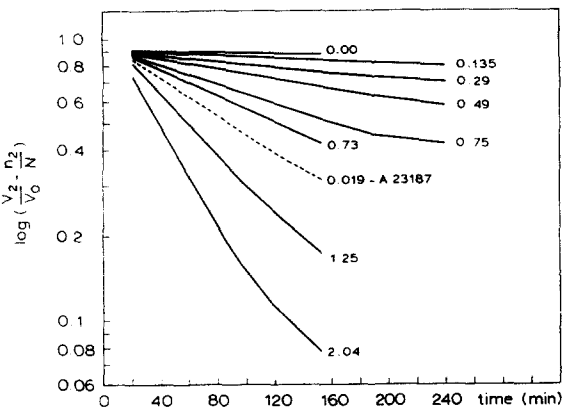

Fig. 5. ^{45}Ca efflux from retinal lipid liposomes in the presence of ionophore X537A. Log $(V_2/V_0 - n_2/N)$ is plotted against the time t (Eqn (2)). Note that the ordinate scale is much larger than in Figs 2 and 3. Seven different concentrations of X537A are used, while for comparison also one experiment with a relatively low concentration of A23187 is shown (broken line).

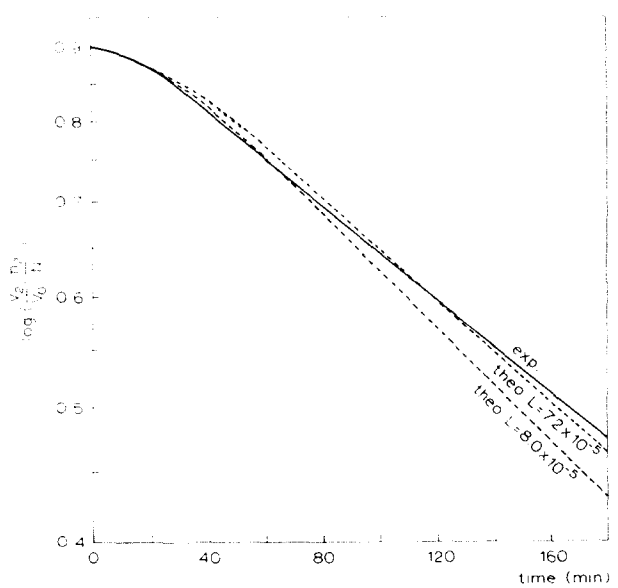

Fig. 6. Comparison between theoretical and experimental curves for the ^{45}Ca efflux from retinal lipid liposomes with X537A. Ionophore concentration: 0.75 μmol/l.

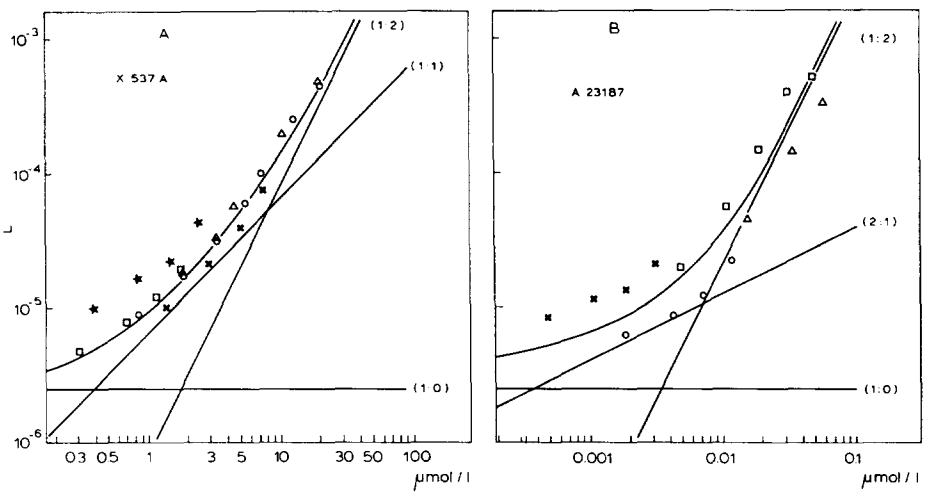

Fig. 7. Dependence of calcium permeability on ionophore concentration. Log L (L being proportional to the permeability) is plotted against log ionophore concentration (μmol/l). The different symbols represent separate experiments. (A) ionophore X537A, experimental points, theoretical lines for one calcium ion interacting with 2 (1 : 2), 1 (1 : 1) and 0 (1 : 0) ionophore molecules and the curve resulting from summation of these three processes are shown. (B) ionophore A23187, experimental points, theoretical lines for one calcium ion interacting with 2 (1 : 2), $\frac{1}{2}$ (2 : 1) and 0 (1 : 0) ionphore molecules and the curve resulting from summation are shown.

X537A and A23187 are shown in Fig. 5, which indicates that increasing the X537A concentration from 0.13 to 2.04 μmol/l greatly increases the efflux rate. Ionophore A23187 is about 50 times as effective as X537A, as can be seen from the dotted curve in this figure.

Fig. 6 shows a comparison between an experimental curve and theoretical curves calculated from Eqn (2) for assumed values of L ($7.2 \cdot 10^{-5}$ min^{-1} and $8.0 \cdot 10^{-5}$ min^{-1}) and the value of K ($6.6 \cdot 10^{-4}$ min^{-1}) derived from measurements on the dialysis bag alone. The experimental curve deviates slightly upwards with time, which may be due to a non-uniform liposome size in the suspension. Eqn (2) assumes that the fraction of counts (α) leaked out at $t = 0$ is negligible, but in the presence of ionophore at $t = 0$ min already a substantial amount of ^{45}Ca has leaked out. This introduces an error upon using Eqn (2) which, however, becomes much smaller after considerable time has elapsed. Therefore we take the experimental values at $t = 120$ min to calculate the value of L for each ionophore concentration. Fig. 7 shows double-logarithmic plots of L (which is really a time constant, proportional to permeability) against the concentrations of X537A and A23187, respectively. The different symbols represent the various liposome preparations. Also shown are theoretical lines expected for the three most likely types of permeant complexes of calcium with ionophore, indicating also the corresponding relation between L and ionophore concentration, c:

1 Ca^{2+}+2 ionophore molecules: $L = Ac^2$ (1 : 2) (5)

1 Ca^{2+}+1 ionophore molecule: $L = Bc$ (1 : 1) (6)

2 Ca^{2+}+1 ionophore molecule: $L = Cc^{\frac{1}{2}}$ (2 : 1) (7)

where A, B and C are constant values. The 1 : 0 line represents the leakage of calcium in the absence of ionophore.

In the case of X537A (Fig. 7A) the calcium-ionophore complex apparently changes from a 1 : 1 ratio at low ionophore concentrations to a 1 : 2 ratio at high ionophore concentrations, since the curve that results from the summation of these two lines (together with the 1 : 0 line) gives the best fit with the experimental points. In the case of ionophore A23187 (Fig. 7B) combination of another set of lines, viz. 1 : 0, 2 : 1 and 1 : 2, gives the best-fitting curve. Thus it appears that A23187 transports at low concentrations two calcium ions per molecule, while this ratio changes at higher ionophore concentrations to one calcium ion per two ionophore molecules.

DISCUSSION

Most of our experiments have been done with liposomes made from retinal lipids in order to observe the behaviour of micelles with a lipid composition close to that of the natural rod sac membrane. A whole retina extract has been used, since it is easier to obtain than a rod sac extract, and the phospholipid composition of the retina [8] is rather similar to that of the rod outer segment [9]. It is somewhat surprising that the ^{45}Ca capture in these retinal lipid liposomes (10 %) is significantly higher than that in the phosphatidylcholine/phosphatidylserine type (4 %) and the phosphatidylcholine type (1 %). Since the phosphatidylcholine/phosphatidylserine ratio in retina is 4 : 1, it might be expected that retinal lipid liposomes are less negatively charged than the phosphatidylcholine/phosphatidylserine (1 : 1) type and thus capture less ^{45}Ca. However, as the opposite seems to be the case, it appears that the negatively charged serine residues of phosphatidylserine are not the main factor determining the calcium capture.

It is possible to calculate the partition of calcium ions between liposomes and medium. The lipid fraction after column elution contains about 10 mg lipid. If we assume that half of the liposome volume is water and the other half lipid, the total liposome volume should be about 20 μl. For retinal lipids this 20 μl contains about 10 % of the total ^{45}Ca added. Before column separation of extravesicular ^{45}Ca the volume is 2 ml. If all calcium is equally distributed in the suspension, i.e. if no binding to lipids occurs, 20 μl should be expected to contain 1 % of the radioactivity. This is indeed the case for phosphatidylcholine, which has no net negative charge and hence offers no binding site for Ca^{2+}. The fact that retinal lipid liposomes capture 10 % of the calcium means that a large part of the sequestered Ca^{2+} ions must be bound to the lipid bilayer. This means that the partition coefficient for Ca^{2+} between liposome and medium is 10.

It is also possible to calculate the free energy for capture of calcium. The loss in free energy upon capture of calcium is given by [10]:

$$\Delta G = -2.303\, RT \log K_D \tag{8}$$

where K_D is the partition coefficient. Inserting a value of 10 for K_D we obtain for ΔG a value of -1.4 kcal/mol. This low value becomes somewhat larger, if we assume that the calcium ions are located on the polar head groups of the phospholipids. If these groups represent about $\frac{1}{4}$ of the total liposome volume, the free energy difference is increased to -2.2 kcal/mol. This value is still too low for a direct

chemical binding between a calcium ion and a negatively charged site, which would be ~ 100 kcal/mol. Thus it appears that Ca^{2+} is loosely and exchangeably bound to the phospholipid head group.

Fig. 2 indicates that the efflux rate varies with the lipid composition of the liposomes. Clearly phosphatidylcholine liposomes are rather impermeable for calcium ions, but the incorporation of phosphatidylserine in these liposomes enhances the leakage rate considerably. This is a well-known phenomenon, generally attributed to the net negative surface charge of phosphatidylserine. The relatively slow leakage rate from retinal lipid liposomes is more difficult to explain, since phosphatidylserine comprises 10–15 % of these lipids. In addition, the degree of unsaturation of retinal lipids is considerably higher than in egg phosphatidylcholine. Both factors should be expected to enhance the leakage rate from retinal lipid liposomes as compared to that from pure phosphatidylcholine liposomes. The presence of a significant amount of cholesterol in retinal lipids might explain the discrepancy, but we have no quantitative data on the cholesterol content of our retinal lipid preparation.

The most prominent feature of the calcium leakage curve from retinal lipid liposomes is its biphasic character: each curve seems to be discontinuous and composed of two straight lines. Arrhenius plots of both parts of the curve give straight lines with nearly the same activation energies, 10.2 and 10.7 kcal/mol respectively. This activation energy is somewhat lower than that for the leakage of univalent cations from phosphatidylcholine and phosphatidylcholine/phosphatidylserine liposomes, which amounts to about 15 kcal/mol [11]. Since the hydration energies of Na^+ and K^+ are about 100 kcal/mol, while that of Ca^{2+} is about 350 kcal/mol [12], this suggests that Ca^{2+} ions would permeate through the bilayer in a (partly) hydrated form.

Since the Arrhenius plot shows a straight line, the retinal lipid liposomes do not undergo a phase transition between 5 and 37 °C. The leakage curves, however, show a biphasic character. A tentative explanation can be offered along the following lines. Iizuka [13] has shown that electric fields induce a high degree of orientation of liquid crystals of poly-γ-benzyl-L-glutamate. Hence it could be that gel filtration of the liposomes on the Sephadex column yields an electric field as the result of a special distribution of Ca^{2+} ions in the liposomes. The liposomes would thus be forced into a certain conformation during the Sephadex filtration. This conformation could revert back to the original one during the leakage experiment, which transition would cause the discontinuity in the leakage curve.

It seems unlikely that the calcium efflux in our experiments would be caused wholly or in large part by mechanical rupture of the liposomes. The following arguments against this possibility can be cited. Firstly, varying the rotation rate of the tubes during incubation over a 100-fold range (from 0.1 to 10 rev./min) has no effect upon the efflux rates. Secondly, parallel experiments show that the efflux rate for sodium ions is consistently about three times higher than for calcium ions, while in case of mechanical rupture all ions should leak out at the same rate. Finally, the fact that both ionophores enhance the efflux of calcium is also an argument for the existence of closed liposomes. The possibility that the ionophores exert a lytic effect upon the liposomes is ruled out by the fact that they do not enhance the sodium efflux rate.

Both ionophore X537A and A23187 enhance calcium leakage from retinal

lipid liposomes. However, X537A is much less effective than A23187. At relatively high ionophore concentrations, giving a 30-fold increase in calcium efflux rate, a 50-fold higher concentration of X537A is needed, while at low ionophore concentrations, sufficient to double the calcium efflux rate, a 125-fold higher concentration of X537A is required. We also find indications for a difference in the medium by which the two ionophores transport calcium ions. In the presence of high ionophore concentrations a Ca^{2+} · ionophore complex appears to exist for both ionophores. This is in agreement with recent crystallographic studies of the ionophores. Johnson et al. [14] find in a study of the barium salt of X537A that one barium ion, together with one molecule of water, is surrounded by two ionophore molecules in the unit cell, while Chaney et al. [15] have shown that the unit cell of cation-free A23187 also contains two ionophore molecules. However, at low ionophore concentration we obtain evidence for the existence of two different complexes, Ca-X537A and Ca_2-A23187. This finding may possibly be explained from the chemical structures of the two ionophores. X537A and A23187 have each one carboxyl and one carbonyl group, whereas A23187 has in addition two ether-oxygen atoms, which might serve as ligands [15]. Hence X537A might bind at most one Ca^{2+} ion and A23187 two Ca^{2+} ions.

APPENDIX

We assume that the lipid vesicles all have the same radius r_0 and that each vesicle is composed of many concentric spheres of lipid bilayers (Fig. 8). The distance between two bilayers is always Δr. The radius of the ith sphere is r_i, its surface area S_i, the volume between S_i and S_{i-1} is v_i. This volume contains n_i cpm ^{45}Ca and the concentration of ^{45}Ca is c_i cpm/ml ($c_i = n_i/V_i$). Then:

$$S_i = 4\pi r_i^2 \tag{A.1}$$

$$V_i = 4\pi r_i^2 \Delta r \tag{A.2}$$

The calcium efflux across the surface S_i is proportional to the concentration gradient $(c_i - c_{i+1})/\Delta r$ over this surface, and P is the permeability constant. Thus:

$$\frac{dn_i}{dt} = -PS_i \frac{c_i - c_{i+1}}{\Delta} + PS_{i-1} \frac{c_{i-1} - c_i}{\Delta r} \tag{A.3}$$

which, with $\Delta r \to dr$ and $dc_i = c_{i+1} - c_i$, becomes

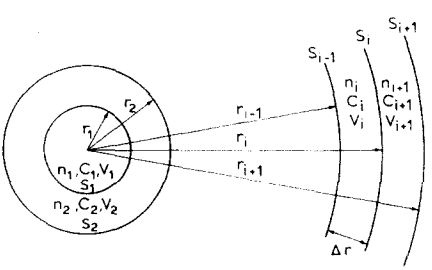

Fig. 8. Schematic presentation of leakage through multi-layered liposomes.

$$\frac{dn_i}{dt} = PS_i \frac{dc_i}{dr} - PS_{i-1} \frac{dc_{i-1}}{dr} \tag{A.4}$$

Now if,

$$\frac{dc_i}{dr} = \frac{dc_{i-1}}{dr} + dr \frac{d^2c_{i-1}}{dr^2},$$

then

$$\frac{dn_i}{dt} = P4\pi(r_{i-1}+dr)^2 \left\{\frac{dc_{i-1}}{dr} + dr \frac{d^2c_{i-1}}{dr^2}\right\} - P4\pi r_{i-1}^2 \frac{dc_{i-1}}{dr} \tag{A.5}$$

If we neglect the higher order terms of dr, substitute Eqn (A2) and leave out the suffixes:

$$\frac{dc}{dt} = \frac{2P}{r} \frac{dc}{dr} + P \frac{d^2c}{dr^2} \tag{A.6}$$

which is a diffusion equation. Its solution contains a Bessel function [16] and its approximate solution is given by

$$c = \frac{A}{\lambda r} e^{-\lambda^2 Pt} \sin(\lambda r) \tag{A.7}$$

where A and λ are constant values. When $\lambda r = \pi = \lambda r'$, $c = 0$. Thus

$$\lambda = \frac{\pi}{r'} \tag{A.8}$$

The total counts in the vesicle are

$$\begin{aligned} 1 &= j\int_0^{r_0} \frac{Ae^{-\lambda^2 Pt}}{\lambda r} \sin(\lambda r) 4\pi r^2 dr \\ &= \frac{4\pi jA}{\lambda^3} \{\sin(\lambda r_0) - \lambda r_0 \cos(\lambda r_0)\} e^{-\lambda^2 Pt} \end{aligned} \tag{A.9}$$

where j is the number of vesicles. If $1 = 1_0$ ($\equiv$ N) at t $= 0$, then

$$1 = 1_0 e^{-\lambda^2 Pt} \tag{A.10}$$

When we use the notation of Johnson and Bangham [7], Eqn (A10) becomes

$$N - n = Ne^{-\lambda^2 Pt}$$

or

$$1 - \frac{n}{N} = e^{-\lambda^2 Pt} \tag{A.11}$$

This is the same equation as (4), only here $L = (1/V_c)\ p$ is substituted by $L = \lambda^2 P$.

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