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STUDIES ON MODEL MEMBRANES

I. EFFECTS OF Ca^{2+} AND ANTIBIOTICS ON PERMEABILITY OF CARDIOLIPIN LIQUID-CRYSTALLINE VESICLES

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SUMMARY

Pure cardiolipin forms closed liquid-crystalline vesicles when suspended in aqueous salt solutions. Electronmicrographs of unsonicated suspensions indicate multilamellar vesicles and after sonication unilamellar vesicles are demonstrated by OsO_4 fixation techniques. Both types of vesicles are relatively impermeable to sucrose. The self-diffusion rate of $^{22}Na^+$ efflux in sonicated vesicles in 0.037 μmole Na^+ per μmole cardiolipin per h and for $^{42}K^+$ is about 0.047 μmole K^+ per μmole cardiolipin per h. This rate of cation efflux by cardiolipin is typical of other vesicles prepared from acidic phospholipids. The efflux rate particularly of the sonicated particles is markedly increased in the presence of air, presumably because of the high percentage of unsaturated fatty acids which are susceptible to oxidation. Once prepared under N_2, the permeability of the particles does not increase with time. Ca^{2+} at low concentrations (1 mM or less) is not effective in increasing permeability of cardiolipin vesicles in contrast to other acidic phospholipids. Valinomycin increases $^{42}K^+$ rate 8-fold at 0.4 nM. Gramicidin increases $^{42}K^+$ rate 5-fold at 20 nM and also accelerates $^{22}Na^+$ rate 16-fold at 20 nM.

INTRODUCTION

Only recently has it been possible to isolate inner and outer membranes of mitochondria in a relatively pure form[1]. Analysis of the two layers indicates that cardiolipin is mainly present in inner membrane[2]. It is now well established that inner membrane is the site of oxidative phosphorylation[3]. Furthermore, a role has been suggested for phospholipids in oxidative phosphorylation and electron transport in terms of maintaining electron transport activity[4,5]. Since mitochondrial functions are intimately associated with ion movements and the recent "chemiosmotic hypothesis" of MITCHELL[6] emphasizes the role of the membrane in separating H^+ and other cations, it is essential to have a better understanding of the permeability properties of inner membrane constituents. FLEISCHER *et al.*[5] have shown that mitochondria can maintain their structural integrity after removal of lipids. This, of course, does not rule out the possibility that lipids might be necessary to maintain

a tight membrane. In fact, in analogy with model membrane studies[7], it would appear that lipids of mitochondria might be partly responsible for separating ions and maintaining a potential difference.

The discovery by BANGHAM *et al.*[8] that phospholipids (amphipathic molecules) can form closed vesicles when suspended in aqueous salt solutions, has led to the study of physico-chemical properties of many phospholipids except cardiolipin[9–11]. Permeability properties of cardiolipin liquid–crystalline vesicles to $^{22}Na^+$, $^{42}K^+$ and uniformly ^{14}C-labeled sucrose and the effects of gramicidin, valinomycin and Ca^{2+} were studied in order to find the role played by cardiolipin as an inner membrane constituent.

MATERIALS

All reagents were analytical grade. The water was glass distilled. Cardiolipin was obtained from Supelco, Bellefonte, Pa., in sealed ampules under N_2 and kept at $-10°$ when not in use. Microanalysis of ions present in cardiolipin was carried out by Referred Chemistry, North Hollywood, Calif., and Na^+ and K^+ contents were verified in our own laboratory. The amount of ions present in cardiolipin (expressed as μmoles per μmole of cardiolipin) are: $Na^+ = 2.3$, $K^+ = 0.05$, $Ca^{2+} = 0.1$. $^{22}Na^+$ was supplied by Amersham and Searle, DesPlanes, Ill., as $^{22}NaCl$ in aqueous solution (0.1 mC/ml). $^{42}K^+$ was obtained from Nuclear Science Division of ICN as ^{42}KCl in aqueous solution (17 mC/ml). Uniformly ^{14}C-labeled sucrose was obtained from Nuclear Chicago in crystalline form (0.5 mC per ampule). Gramicidin (a mixture of A and B) was obtained from Nutritional Biochemical Corp., and valinomycin was received from California Biochemical Corp. The dialysis bags (0.125 inch diameter) were a product of Union Carbide. Sephadex G-50 (coarse) was purchased from Pharmacia Fine Chemicals, Picataway, N.J. N_2 used to store cardiolipin after opening the ampule and for experiments is 99.996 % pure N_2 of Liquid Carbonic Division of General Dynamics. Tris was Trizma base of Sigma.

METHODS

The procedure used for the preparation of the cardiolipin vesicles and subsequent estimation of rates of diffusion was a minor modification of previous methods[8,11]. In most experiments, 4 μmoles of cardiolipin stored in ethanol were dried under vacuum at 30–35°. During evaporation, the tube was rotated by hand to make a thin layer at the bottom of the tube. Evaporation was continued for 10–15 min. Once the evaporation was complete, N_2 was allowed to flow over the dried cardiolipin layer. While N_2 was flushing over the thin shell of cardiolipin, an aqueous salt solution with radioactive solute was added to the test tube and mixed until all sides of the tube were clear. In cases where sonication was used, the tube after shaking on Vortex mixer was placed in a bath-type sonicator (Heat Systems Ultrasonic), so that the cardiolipin suspension was just below the water surface. N_2, after passing through a gas-washing bottle containing distilled water, was flushed continuously during mixing and also during sonication. Sonication was carried our for a total of 15 min, in three 5-min intervals, each followed by a 5-min rest period to prevent elevation of temperature. The temperature during this procedure was elevated only

slightly above room temperature (22–25°). The tube with unsonicated or sonicated vesicles was closed under N_2 and left at room temperature 1–2 h for equilibration. Removal of excess radioactive material outside the vesicles was accomplished by passing through Sephadex G-50 (coarse) as described by PAPAHADJOPOULOS AND WATKINS[11]. Dialysis was done at room temperature (22–25°) in stoppered tubes with 20 ml dialysate for each bag containing 0.5 ml of suspension (approx. 0.4 μmole of cardiolipin). The salt solution contained 135 mM NaCl or KCl and 10 mM Tris–HCl buffer adjusted to pH 7.4. For studies to determine the activation energy of $^{22}Na^+$ efflux, bags were first dialysed at room temperature for 30 min, then the bags were transferred to different tubes containing 20 ml salt solution preadjusted to desired temperature. Bags were then transferred to other tubes containing 20 ml medium equilibrated at the same temperature for additional time intervals (rates over a period of 2–3 h were similar). Gramicidin and valinomycin in ethanol solution were added to cardiolipin suspension before pipetting into dialysis bags in 10-μl quantities and controls had equivalent amount of ethanol. Ca^{2+} was added to the dialysis medium. The rate of diffusion was expressed as percent of radioactivity captured per h. In this paper the term "vesicles" is used for both unsonicated and sonicated cardiolipin particles. $^{22}Na^+$ and $^{42}K^+$ were counted in Packard γ-scintillation counter and [^{14}C]sucrose was counted in an ambient type liquid-scintillation counter (Nuclear Chicago). Cardiolipin was estimated from its phosphate content (1 mole of cardiolipin per 2 moles of phosphate). Organic phosphate was estimated according to the method of LOHMANN AND JENDRASSEK[12].

Electronmicrographs were prepared in the following way. Unsonicated cardiolipin vesicles were centrifuged at 100000 × *g* for 90 min. The sonicated vesicles were centrifuged at the same speed for 16 h. The pellets were then treated with OsO_4, dehydrated and the photographs of lead citrate-stained thin sections were taken.

RESULTS AND DISCUSSION

Physical characteristics of cardiolipin liquid–crystalline vesicles

Initially the electronmicrographs of cardiolipin vesicles were examined to correlate their physical characteristics to permeability properties. Fig. 1 (the electronmicrograph of unsonicated vesicles) shows heterogeneous multi-concentric, lamellar structures which vary greatly in size and shape. These vesicles are similar to other phospholipid particles as described by BANGHAM AND HORNE[13], and PAPAHADJOPOULOS AND MILLER[10]. After sonication (Fig. 2), the particles become smaller and most of them appear to be composed of one lamella of either spheroidal or crescent-like shape. The appearance of two lamellae is probably due to a cut through the infolded crescent-like particles.

The transverse dimension of a single lamella in both sonicated and unsonicated vesicles is in agreement with bimolecular leaflet arrangement as suggested by earlier work reviewed recently[14,15]. Nevertheless, it is not possible to establish from this study involving purified cardiolipin how cardiolipin or other phospholipids are arranged in mitochondrial membrane with large amount of proteins.

Diffusion of [^{14}C]sucrose

Normally sucrose is impermeable to most cell membranes and it is usually used to measure intercellular space. KLINGENBERG AND PFAFF[16] have shown that sucrose

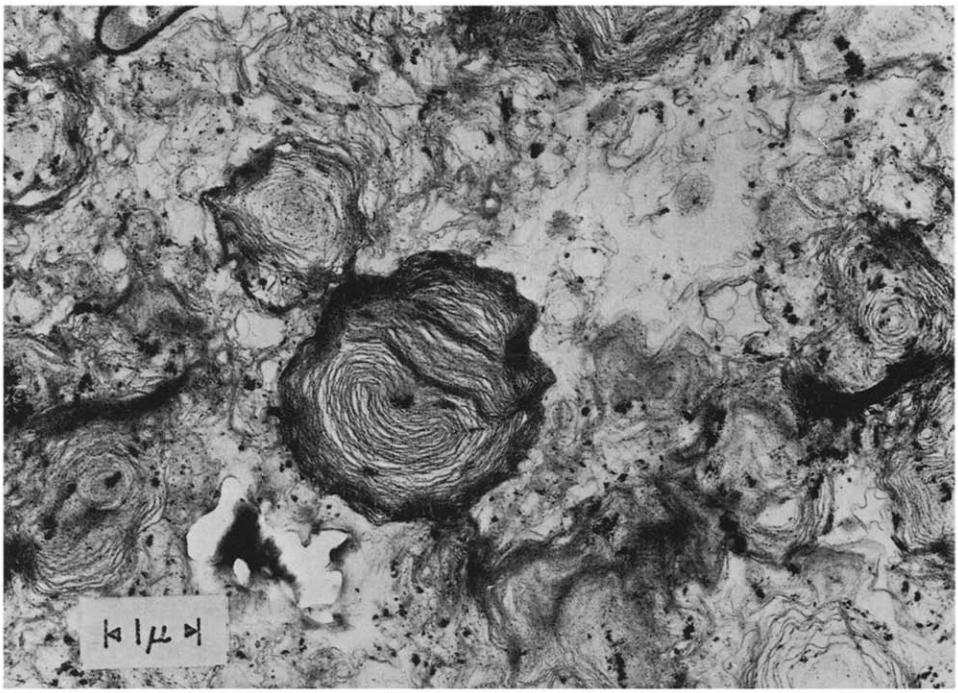

Fig. 1. Electronmicrograph of cardiolipin liquid–crystalline vesicles (unsonicated) after O_8O_4 fixation, acetone dehydration and lead citrate staining of Epon-embeded thin sections. Magnification 11 400 ×.

is not permeable to mitochondrial matrix space, but is permeable to outer membrane. When [^{14}C]sucrose was trapped inside vesicles at room temperature and dialysed in the cold room (5°), no detectable amount of [^{14}C]sucrose appeared in the medium. This indicates that cardiolipin vesicles are stable and particularly impermeable to sucrose at 5°. At room temperature (23–25°), the rate of efflux of sucrose is low. The average rate is found to be 0.45 %/h (0.08–0.75). With sonicated vesicles the rate was found to be 0.67 %/h (0.00–1.40). So even though the surface area increased with sonication, the rate did not increase. This is in agreement with earlier work with other phospholipids and the possible explanations were discussed previously[11]. The [^{14}C]sucrose associated with the vesicles seems to be captured inside the vesicles rather than being adsorbed, since changing the non-radioactive sucrose concentration 70 times did not decrease the [^{14}C]sucrose capture (about 0.5 % per μmole cardiolipin in sonicated vesicles). Furthermore, the capture of about 1 % [^{14}C]sucrose by the unsonicated vesicles is in agreement with 2 % Na^+ capture per μmole of cardiolipin. The high capture of Na^+ can be attributed in part to the presence of Na^+ as a counter ion in cardiolipin. Gramicidin up to a concentration of 0.5 μM had no effect on sucrose permeability.

As shown in Fig. 3, Ca^{2+} at low concentrations (up to 1 mM) has no significant effect on sucrose permeability. Only at higher concentrations (2–5 mM) does Ca^{2+} have an effect which results in an increase of the rate of sucrose efflux. This is in contrast to other acidic phospholipids[11], in which Ca^{2+} increased permeability to

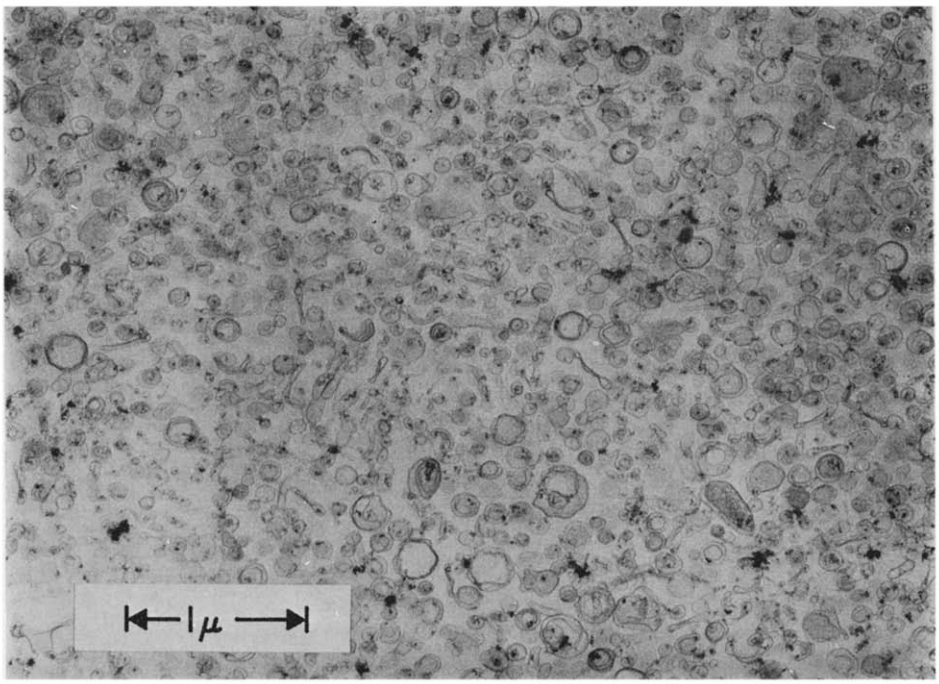

Fig. 2. Electronmicrograph of cardiolipin liquid-crystalline vesicles (sonicated) after OsO_4 fixation as in Fig. 1. Magnification 23 100 ×.

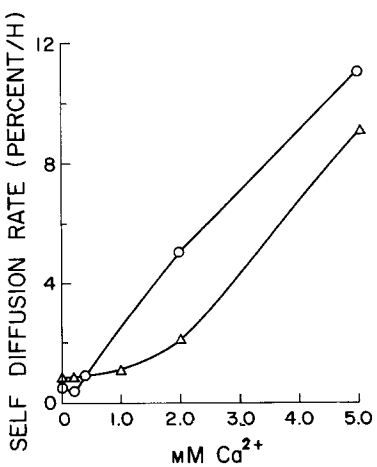

Fig. 3. Effect of Ca^{2+} on self-diffusion rate of uniformly ^{14}C-labeled sucrose through cardiolipin liquid crystals. ○, unsonicated vesicles; △, sonicated vesicles. All experiments were performed at room temperature, in 135 mM NaCl–10 mM Tris–HCl buffer (pH 7.4) with [^{14}C]sucrose as tracer.

$^{42}K^+$ at low concentrations (0.2–1 mM). It should be mentioned in this connection that a fine precipitation resulting from aggregation of cardiolipin vesicles was visible at 5 mM Ca^{2+} concentration. Large aggregates were observed with 10 mM Ca^{2+}.

Diffusion of $^{22}Na^+$

The self-diffusion rate of $^{22}Na^+$ from unsonicated vesicles was found to be 6.23 %/h (3.21–9.96). In terms of μmoles, the rate is 0.085 μmole Na^+ per μmole cardiolipin per h. In a preliminary report[17], we have described the diffusion rate of $^{22}Na^+$ as 10–12 %/h. The high rate was probably due to the fact that we did not use N_2 during the initial phase of our experiments. All the experiments (except two experiments of temperature effect) reported here were carried out under N_2. The rate of $^{22}Na^+$ through sonicated vesicles is 4.61 %/h (3.15–6.86), and in terms of μmoles the rate is 0.037 μmole Na^+ per μmole cardiolipin per h. In order to compare the diffusion rates given in this paper to reported fluxes of ions through biological membranes, a theoretical area occupied by 1 μmole cardiolipin was calculated. Assuming an average area per molecule of 120 $Å^2$ (see ref. 18) and an average of one lamella per vesicle, an area of 3600 cm^2 per μmole cardiolipin was calculated. Fluxes for diffusion of Na^+, calculated on the basis of the area mentioned is approx. $6.6 \cdot 10^{-15}$ equiv/cm^2 per sec. This value is somewhat lower than the reported flux of K^+ through red cells[19] ($100 \cdot 10^{-15}$ equiv/cm^2 per sec). The low rate in sonicated vesicles indicates that the vesicles offer considerable resistance to cation movements and are very stable. PAPAHADJOPOULOS AND WATKINS[11] in their earlier studies with sonicated vesicles obtained similar diffusion rates using the acidic phospholipids, phosphatidic acid and phosphatidyl inositol. It should be noted that the earlier studies[11] were carried out in the presence of air. We have found that sonication in the presence of air tends to increase the leakage rate of cardiolipin vesicles from three to four-fold in the 1st h and the rate was observed to increase with time. These findings suggest that cardiolipin is susceptible to autooxidation.

In the sonicated vesicles, the amount of ions captured (0.80 μmole Na^+ per μmole cardiolipin) is about half of the unsonicated preparation (1.38 μmoles Na^+ per μmole cardiolipin), but the surface area increased many-fold. Of the 0.8 μmole Na^+ capture per μmole of cardiolipin in sonicated vesicles, about half could be present as physically trapped NaCl (due to Cl^- capture)[11]. For the ideal case of unilamellar vesicles, a theoretical minimum of 1 equiv Na^+ per μmole cardiolipin should be bound as counter ions to the inside surface of the vesicles with any physically trapped NaCl adding to the measured Na^+ content. The observed Na^+ content, being significantly lower than the theoretical minimum for closed lamellar particles, indicates that some of the particles may not have an enclosed internal aqueous space. In any case, sonication, by producing smaller unilamellar particles would be expected to reduce the volume of the internal aqueous space as well as the number of counter ions inside the particles for a given amount of phospholipid. Another explanation for the observed low capture could be based on a slow rate of exchange of the $^{22}Na^+$ from the aqueous medium with the Na^+ already present as a counter ion in the cardiolipin samples. The extent of exchange is not definitely known.

The $^{22}Na^+$ efflux rate is relatively high in comparison to lecithin vesicles where permeability to Na^+ and K^+ is very low[8,11]. We have carried out two experiments at temperature range of 5–40° for calculating the energy of activation. In one experiment carried out at pH 7.4 with 135 mM NaCl and 10 mM Tris–HCl buffer, the activation energy was found to be 13.25 kcal/mole. Another experiment was done at pH 5.2 with 130 mM NaCl and 15 mM Tris–maleate buffer; the activation energy was found to be 12.05 kcal/mole. Both these experiments were done in the presence

of air. While these experiments were carried out in the presence of air, the low rates of diffusion observed were indicative that extensive oxidation of fatty acids chains of cardiolipin did not occur. Further, the average value of an energy of activation was 12–14 kcal/mole which is in agreement with that observed with similar phospholipids[11]. This value indicates that Na^+ do not diffuse freely through aqueous channels; otherwise, the energy of activation would be very low[20] and efflux rate would be even higher. Consequently, we may conclude that $^{22}Na^+$ is moving across the lamellae as was found with other phospholipids[8,11].

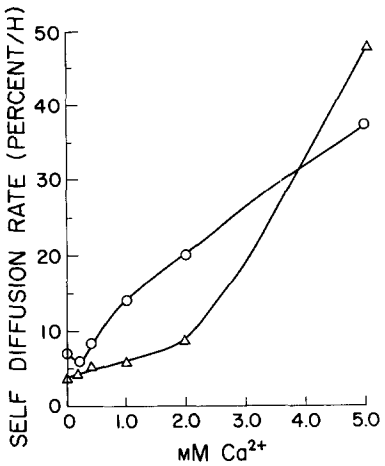

Fig. 4. Effect of Ca^{2+} on self-diffusion rate of $^{22}Na^+$ through cardiolipin liquid crystals. ○, unsonicated vesicles; △, sonicated vesicles. All experiments were performed at room temperature, in 135 mM NaCl–10 mM Tris–HCl buffer (pH 7.4).

The effect of Ca^{2+} on the diffusion rate of $^{22}Na^+$ is shown in Fig. 4. It is clear that up to a concentration of 0.4 mM there is no effect on $^{22}Na^+$ efflux. There is some effect at 1 and 2 mM concentrations, and there is an appreciable effect at 5 mM Ca^{2+} concentration. This behaviour is similar to the effect of Ca^{2+} on phosphatidylinositol and phosphatidylserine permeability to $^{42}K^+$, but different from phosphatidic acid where $^{42}K^+$ permeability greatly increases at low Ca^{2+} concentration (<0.4 mM)[11]. It has been suggested by SHAH AND SCHULMAN[18] that Ca^{2+} interacts with two phosphate groups of the same cardiolipin molecule as shown in Fig. 5a. This type of binding is more probable at low concentrations of Ca^{2+}, when the permeability is not affected. It has also been suggested that Ca^{2+} can interact with more than one different molecules forming linear polymeric arrangement[21]. This idea is in agreement with the fact that we obtained large aggregates at 10 mM Ca^{2+} concentration. It has recently been proposed that the increase in permeability of acidic phospholipids in the presence of Ca^{2+} results from an asymmetric distribution of Ca^{2+} on two sides of the bilayer membrane[22]. X-ray diffraction studies[10,23] indicate that Ca^{2+} brings out the water with other ions from phospholipid suspension, but still retains bimolecular leaflet structure. There is no doubt Ca^{2+} interacts with cardiolipin vesicles at 2–5 mM concentrations, but there is no evidence from this study that Ca^{2+} is effective at low concentration (<0.2 mM), at which it stimulates respiration in mitochondria. The reason for inactivity of Ca^{2+} at low concentration in

contrast to its effect on phosphatidic acid might be due to the fact that here only one phosphate oxygen is free instead of two and consequently the polymeric arrangement involving coordination bonds between each Ca^{2+} and four phospholipid molecules as proposed by PAPAHADJOPOULOS[21] is not possible.

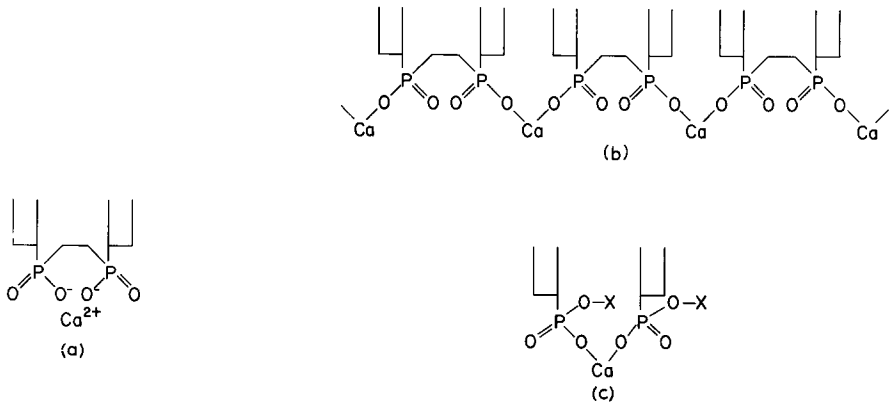

Fig. 5. a. Interaction of Ca^{2+} and two phosphate groups of a single cardiolipin molecule as visualized by SHAH AND SCHULMAN[18]. b. Polymeric arrangement by Ca^{2+} linking different cardiolipin molecules. c. Dimer formation by Ca^{2+} between two molecules of phosphatidylglycerol, phosphatidylinositol and phosphatidic acid at low pH. X represents either hydrogen or a glycerol or an inositol moiety.

In regard to the effect of Ca^{2+} in changing the permeability of acidic phospholipids, cardiolipin is intermediate between phospholipids as phosphatidic acid (at high pH) or phosphatidylserine, and other acidic phospholipids as phosphatidylinositol (see ref. 11) and phosphatidylglycerol or phosphatidic acid at low pH (see ref. 24). The former group is sensitive at Ca^{2+} concentrations of 0.2–1.0 mM. It is, therefore, tempting to speculate that cardiolipin can form polymeric arrangements with Ca^{2+} linking different molecules (see Fig. 5b) because of the special molecular configuration of cardiolipin[25] which would not be possible with other acidic phospholipids such as phosphatidylglycerol, phosphatidylinositol and phosphatidic acid at low pH. The latter phospholipids would only be capable of dimer formation (see Fig. 5c).

Effect of antibiotics on diffusion of Na^+ and K^+

The effect of gramicidin on the self-diffusion rate of Na^+ efflux is shown in Fig. 6. There is an increase in the rate of Na^+ efflux as the concentration of gramicidin increases, with the slope of the curve (efflux *vs.* gramicidin concentration) rising steeply at higher concentrations. With equivalent concentrations of gramicidin, this antibiotic is less effective in increasing the $^{42}K^+$ efflux in cardiolipin vesicles in comparison to its effect on $^{22}Na^+$ efflux. Sonicated vesicles required a higher concentration for the same change observed with unsonicated vesicles.

Valinomycin is more effective than gramicidin in accelerating the rate of K^+ efflux at low concentrations (Fig. 6). Furthermore, as with gramicidin, higher concentrations of valinomycin were required to induce leakage in sonicated vesicles as compared to those which were unsonicated. This indicates that the action of antibiotics is dependent on the surface area.

At least for valinomycin and nonactin, it was proposed[26] that the antibiotics in cyclic configuration act as carrier for K^+. Gramicidins A and B are linear peptides[27] and no definitive evidence has yet been presented that gramicidin can form a cyclic conformation in solution or in the membrane, although some suggestive evidence has

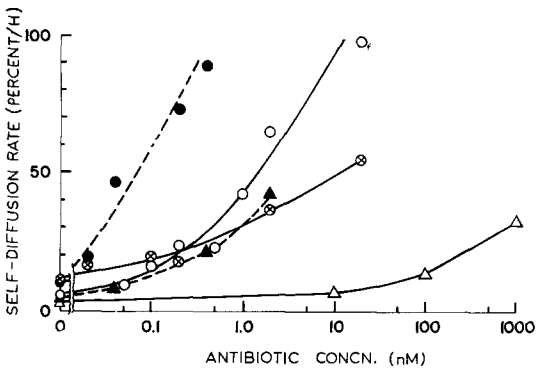

Fig. 6. Effect of antibiotics on self-diffusion rate of $^{22}Na^+$ or $^{42}K^+$ through cardiolipin liquid crystals. Symbols representing antibiotic-induced efflux of cations are as follows: ●, valinomycin, $^{42}K^+$, unsonicated; ▲, valiomycin, $^{42}K^+$, sonicated; ○, gramicidin, $^{22}Na^+$, unsonicated; △, gramicidin, $^{22}Na^+$, sonicated; ⊗, gramicidin, $^{42}K^+$, unsonicated. All experiments were performed at room temperature, in 135 mM NaCl–10 mM Tris–HCl buffer or in 135 mM KCl–10 mM Tris–HCl buffer (pH 7.4). Each bag contains 0.5 ml suspension and about 0.4 μmole cardiolipin.

been reported[28]. Since most of the radioactive ions came out from unsonicated vesicles (see Fig. 6) within about 1 h, one would be led to think that all the lamellae in each vesicle came in contact with antibiotics. That means each lamella received a sufficient quantity of antibiotic molecules to increase the permeability of the membrane. However, with sonication, the number of vesicles increased by at least 1000-fold. When the concentration of antibiotic was 10 nM which was the highest concentration of antibiotic used for unsonicated vesicles (ratio cardiolipin:antibiotic = 10^5:1), the amount of antibiotics received by each small sonicated vesicle was not enough to increase the permeability of the membrane. This follows the argument that the aqueous phase per lamella in the unsonicated particles has a greater volume in relation to that of the sonicated particles. So for one unsonicated particle, one needs fewer "holes" or "carrier" to empty each bag. In terms of existing hypothesis, this could be interpreted by assuming that one needs more than one antibiotic molecules either to form a "pore"[29] or to act as a "shuttle-carrier"[30]. The present data does not distinguish between these mechanisms, and further kinetic analysis is necessary to establish whether the efflux rate reaches a plateau as the concentration of antibiotic is raised in accord with the shuttle-carrier hypothesis. Recent NMR studies[30] indicate inability of the valinomycin–KCNS complex to undergo exchange in a low dielectric constant medium, a property which was explained in support of a shuttle-carrier mechanism for this antibiotic. However, it should be noted that gramicidin does not partition in media of low dielectric constant (J. SAHA, D. SHEPARD, K. JACOBSON AND C. E. WENNER, unpublished observation), a prerequisite property for a simple shuttle-carrier. The possibility that gramicidin alters the surface properties providing favorable energy condition for ion translocation by means other than previously mentioned hypothesis is not excluded.

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