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The consequences of inducing salt permeability in liposomes

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SUMMARY

When liposomes are made simultaneously permeable to both components of a salt, either as independent cations and anions or as ion pairs, a progressive increase in volume is observed. A hypothesis is proposed to account for this volume alteration. Normally, the electrostatic repulsion between adjacent phospholipid lamellae is balanced by an osmotic pressure difference due to these lamellae being permeable to water but not salt. When the salt is made permeable this osmotic pressure difference is dissipated and the electrostatic repulsion leads to an increase in liposomal volume. A new stable state is reached, when, at this increased volume, the repulsive force is balanced by a mechanical resistance of the liposome to further shape alteration. Experiments are described which support this hypothesis and a conclusion is reached that liposomes used in this way are a useful model for the study of the true permeability of charged species.

While investigating the effect of a variety of anions on cation permeability in liposomes a somewhat surprising but reproducible observation was noted with salicylate salts. Liposomes having potassium salicylate solutions of presumed identical composition both inside and outside increased in volume when exposed to valinomycin. This spontaneous swelling phenomenon proved not to be unique for potassium salicylate since it also occurred in the presence of sodium salicylate, KI, and under certain conditions potassium acetate. In this communication the observation will be described in more detail and some of the forces determining liposomal volume will be considered.

Phosphatidyl choline was extracted from egg yolks and purified by alumina and silicic acid chromatography[1]. Dicetyl phosphate was obtained from Sigma Chemical Co., St. Louis, Mo. and twice recrystallized from 2-ethoxyethanol. Valinomycin was purchased from Calbiochem, Calif. while dianemycin was a gift of A.R. Crofts, University of Bristol Medical School. ^{22}Na was obtained as the chloride salt from Amersham Radiochemicals. All other reagents were of analytical grade. Once distilled water was redistilled from $KMnO_4$ in glass apparatus.

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Preparation of liposomes. A description of the experimental method has appeared in previous communications[1—3]. Briefly, appropriate aliquots of stock chloroform solutions of phosphatidyl choline or phosphatidyl choline *plus* dicetyl phosphate were dried under vacuum in a glass tube. The required salt solution was pipetted into the tube and the mixture mechanically shaken on a rotamixer until all of the lipid was suspended.

Flux measurements were made in lipid dispersions originally prepared at a concentration of 30 mM in an aqueous phase containing 10 μC ^{22}NaCl. Excess tracer was removed by passing the lipid dispersion down a column of Sephadex G-50 coarse (Pharmacia, Uppsala, Sweden). 1-ml portions of the eluted lipid were pipetted into dialysis bags (1 cm, Visking) which were dropped into stoppered glass tubes containing 10 ml of aqueous solution. The tubes were placed in a constant temperature water bath (20°) and shaken throughout the experiment. At the end of each flux period the bags were transferred to a new set of identical tubes. At the termination of the experiment the contents of the dialysis bags were emptied into a further set of tubes. The ^{22}Na content of these various tubes was counted on a Packard Auto Gamma Spectrometer. Fluxes were expressed as the percentage of initial counts in the liposome lost over a given period of time.

Absorbance measurements. Bangham *et al.*[3] have correlated volume changes in liposomes with changes in absorbance readings at 450 nm. The absorbance of lipid dispersions was continuously recorded on a Perkin–Elmer model No. 402, dual beam spectrophotometer using quartz cuvettes of 1 cm path length. Liposomes were prepared at a concentration of 4 mM. A 200-μl aliquot of this suspension was added to 2.3 ml of appropriate aqueous solution in the cuvette.

Volume changes in the presence of salicylate. When valinomycin was added to liposomes (95% phosphatidyl choline, 5% dicetyl phosphate) formed and dispersed in 50 mM potassium salicylate a progressive fall in absorbance occurred (Fig. 1a). The addition of KCl at a final concentration of 8 mM reversed the absorbance change indicating that the liposomes were still intact and in fact impermeable to chloride. When the experiment was repeated with sodium salicylate, valinomycin again induced a volume increase (Fig. 1b) which was reversed by a small amount of KCl or glucose, added to the outside solution. Liposomes formed and suspended in either 100 mM KCl or NaCl showed no similar volume change.

Since in the above experiments valinomycin was always added to the solution outside the liposome, initially at least, there would be a large concentration gradient of valinomycin from outside to inside. Therefore in several experiments this gradient was reversed by adding the antibiotic to the lipid, prior to drying. The usual procedure of adding a 200-μl aliquot of the lipid dispersion to 2.3 ml of solution in the cuvette would now ensure a valinomycin concentration gradient from inside to outside. Despite the reversal of the gradient, the liposomes demonstrated a persistent fall in absorbance, indicating that swelling could still occur,

Na^+ permeability in the presence of salicylate and valinomycin. The volume change induced by valinomycin must have been accompanied by a net inflow of salt and water since the movement of water alone into the liposome seemed highly unlikely. Because valinomycin initiated the swelling of liposomes when either potassium or sodium salicylate was present, we measured the permeability of ^{22}Na in the presence of valinomycin and salicylate.

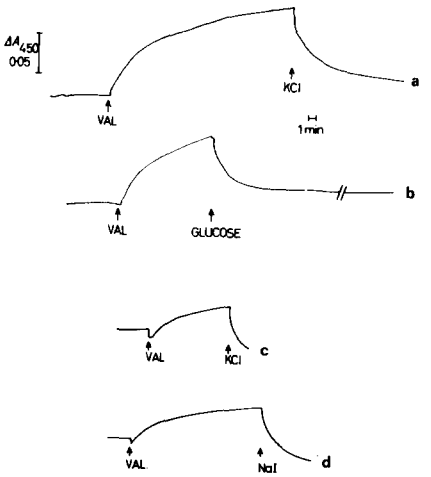

Fig. 1. Liposomes composed of 95% phosphatidyl choline, 5% dicetyl phosphate were formed and suspended in the following solutions: (a) 50 mM potassium salicylate; (b) 100 mM sodium salicylate, 5 mM sodium acetate (pH 6.0); (c) and (d) 100 mM KI. ΔA refers to a change in absorbance. An upward deflection indicates an increase in volume. The gap in Curve b represents a time interval of 3 h. Additions, with their final concentrations were: valinomycin (VAL.), 1 mole per 90 moles total lipid; KCl, 8 mM; glucose, 15 mM; NaI, 8 mM.

20 μmoles of lipid (95% phosphatidyl choline, 5% dicetyl phosphate) were dispersed in 0.5 ml of either 31 mM NaCl, 100 mM sodium salicylate, 10 μC ^{22}NaCl, *plus* 5 mM sodium acetate (pH 6.0) or 131 mM NaCl, 10 μC ^{22}NaCl, *plus* 5 mM sodium acetate (pH 6.0). For simplicity, these dispersions will be referred to as the sodium salicylate or NaCl liposomes. The NaCl liposomes and one portion of the sodium salicylate liposomes were passed down Sephadex columns and eluted with non-radioactive but otherwise identical solutions, as their respective dispersing solutions. The other portion of the sodium salicylate liposomes was also passed down a column, but eluted with a solution differing from its dispersing solution in that it also contained 25 mM glucose. The glucose was present to prevent the volume change attendant upon the subsequent addition of valinomycin to these liposomes (see Fig. 1b). 1-ml portions of the eluted lipid dispersions were pipetted into dialysis bags and the ^{22}Na effluxes measured as described under methods Following a control flux period, valinomycin was added to each bag. The results are illustrated in Fig. 2. Valinomycin increased the ^{22}Na efflux much more in sodium salicylate liposomes than in NaCl liposomes. Furthermore the presence of glucose in the outside solution did not prevent the antibiotic induced increased ^{22}Na efflux. The difference between the two upper lines in Fig. 2 is probably attributable to the different valinomycin concentrations used. Indeed, if the results are corrected by the ratio of valinomycin concentrations, the two upper curves become superimposable.

Volume changes in the presence of KI. Iodide has the ability to cross black lipid membranes as a charged polyiodide complex[4]. Since liposomes are probably equally permeable to iodide, it was reasoned that the addition of valinomycin to a lipid dispersion suspended in KI should permit both cation and anion to penetrate. As expected, when valinomycin was added to liposomes formed and suspended in 100 mM KI an increase in volume occurred which could be reversed with either KCl or NaI (Figs. 1c, 1d). No such

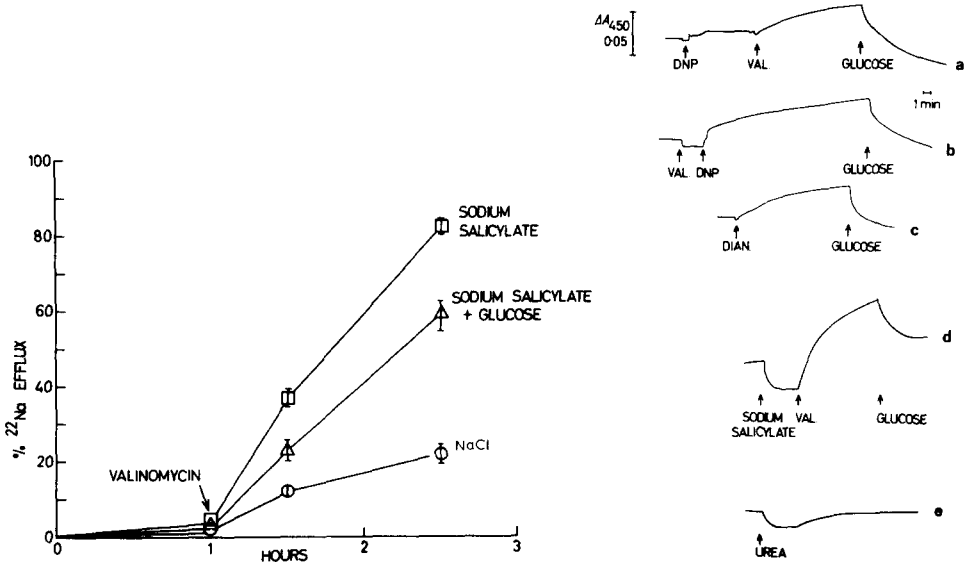

Fig. 2. The effect of salicylate and valinomycin on ^{22}Na efflux. NaCl and sodium salicylate liposomes were prepared as described in the text. To the outside solution of one portion of the sodium salicylate liposomes 25 mM glucose was added. Valinomycin was added at the time indicated, in the following final concentrations: sodium salicylate liposomes: 1 mole per 18 moles total lipid; sodium salicylate liposomes exposed to glucose: 1 mole per 28 moles total lipid; NaCl liposomes: 1 mole per 23 moles total lipid. The experimental points represent means ± S.E.

Fig. 3. In the upper three curves liposomes composed of 90% phosphatidyl choline, 10% dicetyl phosphate were formed and suspended in either 100 mM potassium acetate (pH 5.0) (Curves a and b) or 100 mM potassium acetate (pH 8.5) (Curve c). In the lower two curves liposomes of pure phosphatidyl choline were dispersed in distilled water. ΔA refers to a change in absorbance. An upper deflection indicates an increase in volume. Additions with their final contractions were: valinomycin (VAL.), 1 mole per 90 moles total lipid; 2,4-dinitrophenol (DNP), 10^{-5} M; glucose, 15 mM; dianemycin (DIAN.), 1 mole per 90 moles total lipid; sodium salicylate, 40 mM; urea, 80 mM.

swelling occurred when liposomes made up in 100 mM NaI were exposed to valinomycin.

For swelling to occur it appears that the liposome must be permeable to both the cation and anion present. These may cross as an ion pair or as separate charged species. When salicylate is the anion, valinomycin is equally effective with either the potassium or sodium salt. In the presence of iodide, Na^+ cannot be substituted for K^+.

Volume changes in presence of potassium and ammonium acetate. Liposomes are normally impermeable to potassium acetate[3]. However, under conditions in which K^+ for H^+ ion exchange is promoted this salt becomes a permeable species[5]. When phospholipid dispersions were formed and suspended in 100 mM potassium acetate an increase in volume could be induced with either valinomycin *plus* dinitrophenol or dianemycin alone (Figs. 3a, 3b, 3c). Under both sets of conditions K^+ for H^+ ion exchange is facilitated making the liposomes permeable to potassium acetate. The mechanism of penetration of this salt is shown schematically in Fig. 4.

On the other hand, liposomes are extremely permeable to ammonium acetate[3], the transported species presumably being ammonia and acetic acid. Lipid dispersions formed in ammonium acetate begin to increase in volume immediately making it difficult

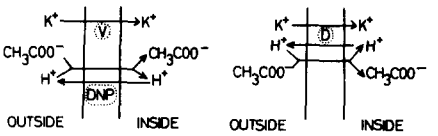

Fig. 4. Mechanism of penetration of liposomes by potassium acetate. The abbreviations used are: V, valinomycin; D, dianemycin; DNP, 2,4-dinitrophenol.

to examine the initial phase of swelling due to the finite time required to add the lipid dispersion to the cuvette. To obviate this problem, we compared the relative volumes of liposomes of identical composition but formed in either 100 mM ammonium or potassium acetate. As expected, ammonium acetate liposomes were more swollen, as indicated by their lower absorbance reading and their marked shrinking when a small amount of glucose was added to the outside solution. This same concentration of glucose caused no detectable volume change in potassium acetate liposomes.

Liposomes formed in an impermeable salt solution appear to behave as stable structures. However, it is now apparent that if the liposomes are made permeable to both components of the salt, either by the addition of valinomycin in the cases of KI, potassium salicylate or sodium salicylate or with valinomycin *plus* dinitrophenol in the case of potassium acetate, a rapid increase in volume occurs. Lipid dispersions formed in the presence of a permeable salt, such as ammonium acetate, have a much larger volume than comparable liposomes formed in an impermeable salt solution. Ammonium acetate liposomes are ultimately stable at a larger volume, probably because their swelling tendency is now balanced by a mechanical resistance to further volume changes. To explain these results we propose the following tentative model. Consider a cylindrical tube containing freely movable partitions. The partitions represent adjacent phospholipid lamellae and for simplicity the model will be limited to only two; the argument, however, could easily be extended to a system consisting of multiple partitions. The liposomes used in these experiments contained fixed negative charges arising either from the ionization of lipid head groups or through the selective adsorption of ions such as salicylate★. To the two partitions, then, fixed negative charges are added. A sodium salicylate solution of uniform concentration is present throughout the tube including the space between the partitions, which themselves are impermeable to at least the Na^+ of this salt, but freely permeable to water. The electrostatic repulsion between the two charged partitions moves them slightly apart and water is drawn in, thus diluting the sodium salicylate solution in the space they enclose. The dilution establishes an osmotic pressure difference across each partition and when a balance is reached between the electrostatic repulsive force and the osmotic pressure differences, the volume trapped by the two partitions will remain constant. This balanced condition represents the state of a liposome formed in an impermeable salt solution. If valinomycin is now added, making the partitions permeable to both Na^+ and salicylate, the osmotic pressure differences are dissipated and the now unbalanced electrostatic repulsive force drives the partitions further and further

★Liposomes composed of pure phosphatidyl choline had an electrophoretic mobility of $-1.85\ \mu sec^{-1} \cdot V^{-1} \cdot cm$ in the presence of 100 mM sodium salicylate indicating significant adsorption of salicylate ions. Electrophoresis was performed according to the method of Bangham *et al.*[6].

apart. Through this mechanism the liposome increases in volume until a new balance is reached between the repulsive force and a mechanical resistance of the liposome to further shape alteration.

Finally as a test of the hypothesis we examined experimentally one of its predictions. Consider a lipid dispersion of pure, uncharged phosphatidyl choline, formed and suspended in distilled water. The hypothesis predicts that such liposomes should decrease in volume when sodium salicylate is first placed in the outside aqueous phase. A subsequent addition of valinomycin will make the dispersion permeable to the salt and as the sodium salicylate equilibrates throughout the system the liposomes will increase in volume towards their previous value. However, since salicylate ions are selectively adsorbed, the phospholipid lamellae will become negatively charged. The presence of charged lamellae, bathed by a permeable salt solution represents a system similar to our cylindrical tube-partition model; the liposomes should therefore progressively swell over and above their previous base-line value. The results of such an experiment are shown in Fig. 3d, and conform to the predicted pattern. For comparison the effects of adding urea to the outside water phase of an identical system are illustrated in Fig. 3e. Urea is both uncharged and very permeable. In this case the liposomes initially shrink but soon return to their previous volume as the urea equilibrates. Since the liposomes remain uncharged there is no tendency for swelling to continue once the initial volume has been reached.

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REFERENCES

1 A.D. Bangham, M.M. Standish and J.C. Watkins, *J. Mol. Biol.*, 13 (1965) 238.
2 D. Papahadjopoulos and J.C. Watkins, *Biochim. Biophys. Acta*, 135 (1967) 639.
3 A.D. Bangham, J. de Gier and G.D. Greville, *Chem. Phys. Lipids*, 1 (1967) 225.
4 A. Finkelstein and A. Cass, *Proc. Symp. on Biological Interfaces: Flows and Exchanges, J. Gen. Physiol.*, 52 (1968) 145S.
5 J.D. McGivan, *The Movement of Ions Across Artificial Phospholipid Membranes*, a dissertation submitted to the University of Bristol for degree of Doctor of Philosophy, 1968, p. 144.
6 A.D. Bangham, R. Flemans, D.H. Heard and G.V.F. Seaman, *Nature*, 182 (1958) 642.