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ANION DIFFUSION ACROSS ARTIFICIAL LIPID MEMBRANES:

THE EFFECTS OF LYSOZYME ON ANION DIFFUSION FROM PHOSPHOLIPID LIPOSOMES

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SUMMARY

1. By incorporating varying amounts of stearylamine or dicetylphosphate with phosphatidylcholine into vesicles, positively charged or negatively charged liposomes, respectively, were produced. Anion (CrO_4^{2-}) efflux rates were shown to be influenced by the prevailing charge on the liposome, the efflux rates being greater with positively charged vesicles.

2. The addition of the soluble basic protein, lysozyme, to suspensions of negatively charged liposomes produced an increase in the diffusion rate of CrO_4^{2-} across the bilayers. The diffusion rate of CrO_4^{2-} across positively charged bilayers was unaffected by the addition of lysozyme. A semi-empirical rate equation was derived in order to compare the effectiveness of the protein in altering the efflux rates under varying conditions.

3. The amount of protein bound to the liposomes was measured using an ultrafiltration technique, and was correlated to the increase in anion permeability caused by the addition of protein.

INTRODUCTION

Model membrane systems composed of phospholipids, as bilayer films or as vesicles (liposomes), exhibit many characteristics similar to those of biological membranes[1–3] It is of interest in the light of current work on cell membranes[4,5] to determine how the properties of these model systems are altered by the addition of proteins.

Recent work by Kimelberg and Papahadjopoulos[6] and by Calissano and Bangham[7] has shown that several proteins when added to sonicated dispersions of phospholipids increase the rate of efflux of sequestered cations. The effectiveness with which the protein elicited this response has been correlated to the ease with which the protein penetrated surface monolayers of phosphatidylserine at an air–water interface[6]. The only previously reported studies on the effects of added polypeptide or protein on the efflux rates of anions from liposomes involved the

highly lytic polypeptide melittin[8]. This polypeptide caused a breakdown of the bilayer structure of the liposomes and although this may be related to its effect upon biological membranes, it is not generally considered to be typical of other lipid bilayer–protein interactions.

The earlier work of Bangham *et al.*[9] on the diffusion of ions across the lamellae of swollen phospholipid vesicles showed that whilst the permeability behaviour of cations could be partly reconciled with the sign and magnitude of the charge prevailing at the membrane surface, the anions appeared to be relatively free to diffuse whether or not fixed charges of either sign were present.

It was decided to examine more closely the effect of charge in the membrane on the rate of efflux of a slowly moving anion, and to determine whether or not a protein which had been shown to increase cation permeability would also increase anion permeability. CrO_4^{2-} was selected as the anion since it is of the same size as the more biologically interesting HPO_4^{2-} (ref. 10) and is readily assayed spectrophotometrically.

By applying a semi-empirical rate equation (see Appendix) linear plots of the rates of efflux of CrO_4^{2-} were obtained. The effect of altering the reaction conditions could then be compared to give a more complete understanding of the nature of the phospholipid bilayer–protein interaction.

MATERIALS

Dicetylphosphate and stearylamine were obtained from K and K Ltd. Their purity was established by thin-layer chromatography on pre-coated silica gel plates (purchased from Merck) in two solvent systems, chloroform–methanol–water (130:50:6, by vol.) and chloroform–methanol–aqueous 7 M NH_3 (46:18:3, by vol.). Lecithin was isolated from hens' eggs according to the method of Vreeman[11] and purified by chromatography on alumina[12]. The purified phospholipid gave a single spot in the two thin-layer chromatography systems above. It was dissolved in chloroform–methanol (2:1, v/v) and aliquots containing 40 μmoles of lecithin in 5 ml were stored in sealed glass ampoules under N_2 at -5 °C.

Lysozyme, Grade 1, was obtained from Sigma Chemical Co. The buffer components, borax and boric acid, and all solvents, were Analar grade from B.D.H. Ltd and were used without further purification. Water used in these experiments was double distilled from glass apparatus.

METHODS

Preparation of liposomes

A 5-ml sample of the lecithin solution was mixed with appropriate volumes of 1 mM stearylamine or 1 mM dicetylphosphate in chloroform and evaporated to dryness under reduced pressure at 30 °C in a round-bottomed flask. The dried lipid film was shaken with 3 ml of 0.1 M K_2CrO_4 in 50 mM borate buffer (pH 7.7)[13] containing several glass beads, until it had been dispersed into the aqueous solution. The dispersion was placed in a constant temperature bath at 37 °C for 2 h, and sonicated for 1 min at maximum amplitude in an MSE 100 W Ultrasonic Disintegrator, flushed with N_2 and left to stand overnight at 37 °C. The absence of hydrolytic

and oxidative breakdown products was established by thin-layer chromatography. This is an important point to establish, as Hauser[14] has recently shown that degradation of phospholipids may occur during sonication. The sonicated dispersion was layered on to the top of a Sephadex G-50 column (30 cm × 1 cm), previously equilibrated with the eluting buffer , and the vesicles eluted with a solution of 0.15 M KCl in 50 mM borate buffer (pH 7.7). Cro_4^{2-} which had not been sequestered by the vesicles was retarded by the column.

Permeability studies

Samples of the liposomes, 0.5 ml, from the column were transferred into lengths of 8/32 inch Visking tubing, previously boiled in dilute EDTA solution and in several successive volumes of distilled water for 4–5 h. For studies of the effect of added protein, the control samples were prepared by adding 0.5 ml of 0.15 M KCl in 50 mM borate buffer (pH 7.7) to the liposome suspension. The experimental samples were prepared in the same way, except that an appropriate amount of lysozyme was first dissolved in the buffered KCl solution. The sealed Visking tubes were placed in 5 ml of 0.15 M KCl in 50 mM borate buffer (pH 7.7) and dialyzed at 37 °C in capped bottles placed in a shaking water bath. The solution outside the Visking membrane was changed at hourly intervals. The diffusates obtained were made basic with 0.1 ml of 5 M NaOH and the CrO_4^{2-} present measured by its absorbance at 370 nm on a Perkin-Elmer 124 spectrophotometer. The solution was first made basic since previous experiments had shown that CrO_4^{2-} absorbance at 370 nm was maximal above pH 8. Slight disintegration of the Visking membrane was noted during most experiments. Cellophan particles were removed by centrifugation before assay of CrO_4^{2-}. In control experiments the diffusate was assayed for phospholipid by the method of Baginski *et al.*[15]. No phospholipid leakage was detected.

Determinations of the total CrO_4^{2-} present at the beginning of dialysis and of that which remained sequestered within the liposomes after dialysis, were made in essentially the same way. A 0.1-ml aliquot of the liposomes was diluted 10- or 20-fold with distilled water and sonicated for 3 min. The dilute dispersion was left to stand for 1 h and sonicated for a further 3 min. A 1-ml sample of the suspension was treated with 4 ml of 1.5 % sodium deoxycholate solution and the chromate present determined spectrophotometrically.

Having determined the number of moles, F, of CrO_4^{2-} diffused out of the liposomes, and the number of moles, T, originally sequestered within the liposomes, a plot of ln $(T-F)/T$ *vs* time gave a measure of the rate of efflux (see Appendix).

In all of the experiments the total recovery of CrO_4^{2-} from the diffusates and from the retentate remaining in the vesicles after 4 h dialysis was 97–104 % of the total CrO_4^{2-} determined at the beginning of the experiment by the osmotic shock (dilution)–sonication method. Treatment of the liposomes with sodium deoxycholate solution without first sonicating and diluting the suspension did not liberate all of the CrO_4^{2-} sequestered within the vesicles.

Thin-layer chromatography carried out on the liposomes at the end of dialysis procedures showed that no degradation of the lipids occurred during the course of the experiment.

Binding studies

In order to determine the amount of protein bound by the phospholipid vesicles it was necessary to separate the free protein remaining in solution from the bound protein. This was accomplished using a Millipore Swinnex-13 filter unit The unit consisted of a two-piece plastic diaphragm into which were fitted Millipore filters of pore size 0.22 $\pm$ 0.02 μm The unit was then fitted to a hypodermic syringe.

Liposomes were prepared as described above, except that after removal of solvents under reduced pressure, 3 ml of 0.15 M KCl in 50 mM borate buffer (pH 7.7) was added in place of K_2CrO_4 in buffer. The liposome suspension was left overnight in the constant temperature bath at 37 °C, the suspension was diluted to 5 ml with the buffered KCl and mixed with 5 ml of the protein solution in buffered KCl. The suspension was then placed in a shaking water bath at 37 °C. Using a syringe, 1-ml samples were taken from the liposome suspension to which protein had been added, the filter unit was fitted onto the syringe and the solution quickly filtered under a constant N_2 pressure of 1 kg/cm^2. The clear filtrate was sampled, 0.025 ml taken and the protein present assayed by the method of Lowry *et al.*[16]. Control experiments were carried out which established that lysozyme in solution was not retained by the Millipore filter and that by the method of Baginski *et al.*[15] phospholipid was not detectable in the filtrate

RESULTS

The rates of efflux of CrO_4^{2-} across the phospholipid bilayers of the liposomes were altered by the sign of the prevailing charge on the liposome (Fig. 1). The efflux rates from positively charged liposomes containing up to 20 mole% stearyl-

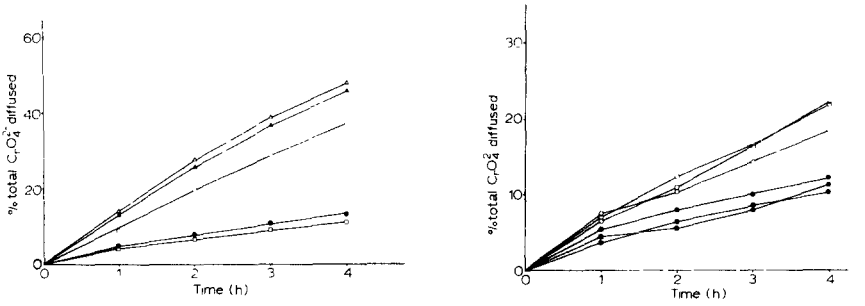

Fig 1 Effect of lipid composition of the liposomes on the rate of CrO_4^{2-} efflux. Liposomes were prepared from 40 μmoles lecithin and appropriate amounts of dicetylphosphate or stearylamine The liposomes were prepared in 0 1 M K_2CrO_4 in 50 mM borate buffer (pH 7 7) and dialysed at 37 °C against 0 15 M KCl in the same buffer △—△, liposomes composed of 20 mole% stearylamine; ▲—▲, 10 mole% stearylamine; ○—○, liposomes composed of lecithin alone, ●—●, liposomes composed of 10 mole% dicetylphosphate; □—□, 20 mole% dicetylphosphate

Fig 2 Effect of addition of lysozyme to a suspension of liposomes on the efflux rate of CrO_4^{2-} from the liposomes The liposomes were prepared from 40 μmoles lecithin and 10 μmoles dicetylphosphate, *i e* 20 mole% dicetylphosphate ●—●, controls, 0 5 ml of liposome suspension to which had been added 0 5 ml of 0 15 M KCl in borate buffer (pH 7 7), ○—○, liposome suspension to which had been added 10 mg lysozyme in 0 5 ml of 0 15 M KCl in borate buffer (pH 7 7) The dialysis was carried out at 37 °C against 0 15 M KCl in 50 mM borate buffer (pH 7 7)

amine were higher than the rates of efflux from liposomes composed only of zwitterionic lecithin. The efflux rates of CrO_4^{2-} from liposomes incorporating up to 20 mole% dicetylphosphate were lower than the rates of efflux from lecithin liposomes. Since the rate constants obtained from the rate equation (see Appendix) varied from one preparation of liposomes to another, this effect can be shown only as a qualitative comparison.

The addition of lysozyme to the negatively charged liposomes resulted in an increase in the diffusion rate of CrO_4^{2-} from the liposomes. The results of a typical experiment are shown in Fig. 2. The increase in permeability was not observed in liposomes which did not bear a fixed negative charge (Fig. 3). The change in

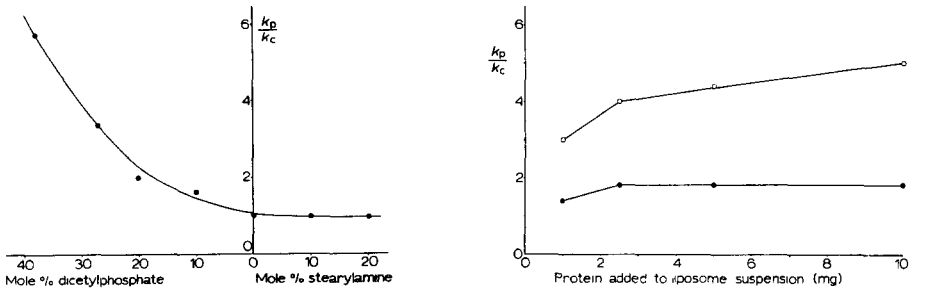

Fig 3. Effect of the addition of lysozyme to liposomes of differing lipid composition on the CrO_4^{2-} diffusion rate constant ratio, k_p/k_c. Liposomes having lipid compositions shown to the left of the vertical axis had a net negative charge, to the right were positively charged Dialysis was carried out under the same conditions as Fig 2

Fig 4 Effect of the addition of increasing concentrations of lysozyme to negatively charged liposomes on the CrO_4^{2-} diffusion rate constant ratio k_p/k_c Dialysis was carried out under the same conditions as for Fig 2 ○—○, liposomes composed of 40 mole% dicetylphosphate, ●—●, liposomes composed of 20 mole% dicetylphosphate

permeability is expressed quantitatively as the ratio of the rate constants (k_p/k_c) for the samples containing protein (k_p) compared with the control samples (k_c) in the same experiment.

During the dilution procedure to determine the amount of CrO_4^{2-} retained in the liposomes after dialysis, a precipitate was formed in all experiments which showed the increase in permeability. This precipitate was not observed in those experiments which did not show the increased permeability in the presence of protein. Neither the liposomes alone nor the protein solution under the same conditions produced such a precipitate and preliminary studies suggest that it is a lipo-protein complex.

In a further series of experiments the amount of lysozyme added to the liposomes was altered to investigate the effect of protein concentration. Fig. 4 shows the results of these experiments which were carried out using liposomes containing 20 mole% and 40 mole% dicetylphosphate. In both cases protein concentrations above 1 mg/ml increased the diffusion rate constant ratio. In the case of the liposomes containing 20 mole% dicetylphosphate, the effect reaches a maximum between 3 mg protein/ml and 5 mg protein/ml, whereas the effect with the liposomes containing 40 mole% dicetylphosphate is still increasing at 10 mg protein/ml.

Studies on the amount of protein bound to the liposomes were carried out

as described in Methods, using a final concentration of 2.5 mg lysozyme/ml on the various lipid compositions used in the permeability studies. The free protein present in solution was measured 2 h after addition of lysozyme, as preliminary experiments had shown that by this time constant values were obtained. These experiments show (Fig. 5) that more lysozyme is bound to negatively charged liposomes com-

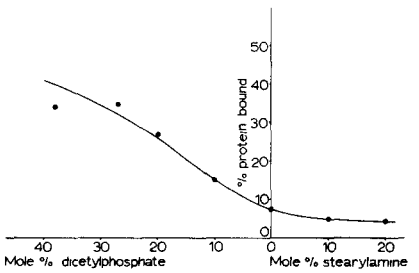

Fig. 5. Effect of the lipid composition of the liposomes on the amount of protein bound. Lysozyme in 0.15 M KCl in 50 mM borate buffer (pH 7.7) was added, at a final concentration of 2.5 mg/ml, to the liposome suspensions Incubation was carried out at 37 °C. Liposomes having lipid compositions shown to the left of the vertical axis had a net negative charge, to the right were positively charged.

posed of lecithin and dicetylphosphate than to liposomes composed entirely of lecithin or of lecithin and stearylamine. Only a very small percentage, of the order of 5 %, of the total protein (25 mg) was bound to lecithin and lecithin *plus* stearylamine liposomes. The shape of the curve (Fig. 5) for the most part parallels the change in permeability increase shown in Fig. 3. The deviation between the two curves which occured using liposomes containing greater than 30 mole% dicetylphosphate is the subject of experiments now under way in this laboratory.

DISCUSSION

The first studies by Bangham *et al.*[9] on the diffusion of ions across the bilayers of phospholipid vesicles led to the conclusion that there is a high degree of selectivity of diffusion between cations and anions. It was found that the permeabilities of cations could be partly reconciled with the size and magnitude of the charge prevailing at the membrane surface, but anions appeared to be relatively free to diffuse whether fixed charges of either sign were present or not. However, the experiments on the effect of surface charge upon the rate of anion efflux were carried out using the very highly permeable Cl^-. When the more slowly diffusing CrO_4^{2-} is studied, its rate of diffusion across the bilayers is influenced to some extent by the prevailing charge on the liposome. The rate of efflux from negative liposomes is lower than that from liposomes with either zero charge or those which are positively charged. Positively charged liposomes are totally impermeable to cations, possibly because of charge repulsion effects. In the case of anions, negatively charged liposomes are still permeable but to a lesser extent than are positively charged liposomes. This is not unexpected since anion diffusion rates in membranes are generally several orders of magnitude greater than cation diffusion rates[17].

Lysozyme has been shown, at pH 7.4, to increase the diffusion rate of Na^+

from sonicated vesicles of phosphatidylserine. Under these conditions phosphatidylserine liposomes bear a negative charge. It is apparent from the present work that the diffusion rates of anions through phospholipid bilayers bearing a negative charge are also increased by lysozyme. As the isoelectric point of lysozyme is at pH 11.0[18], it carries a net positive charge under the experimental conditions used in these studies. It has been shown here that the diffusion rate of CrO_4^{2-} is increased by the addition of lysozyme only when the lipid bilayers have a net negative charge, and that the binding of the protein can be correlated with the change in permeability. This suggests that coulombic forces play a major role in the interaction of lysozyme with phospholipid bilayers. This conclusion is supported by the previous finding that lysozyme has no effect upon the rate of Na^+ efflux from lecithin vesicles[19], which have been shown to bear zero net charge[20]. Kimelberg and Papahadjopoulos[19] have shown that as the amount of negative phospholipid (phosphatidylserine) was decreased and the amount of phosphatidylcholine increased the permeability changes for Na^+ produced by lysozyme were decreased. This effect is paralleled in the present work where dilution of the negative lipid (dicetylphosphate) by increasing amounts of phosphatidylcholine decreases the effect of lysozyme on the permeability of the bilayers to CrO_4^{2-}.

Calissano and Bangham[7] have found that two brain-specific proteins interact with positively charged liposomes and with negatively charged liposomes to produce an increase in permeability of cations (without affecting glucose permeability). Also, using phosphatidylserine liposomes, it has been shown that human serum albumin interacts to produce increases in cation efflux rates in pH ranges above and below its isoelectric point[6], *i.e.* when it is negatively charged and positively charged, respectively.

It seems that both coulombic and hydrophobic interactions are involved in protein–phospholipid bilayer interactions. The extent to which each of these contributes to the overall interaction probably varies with the nature of the protein, its net charge and degree of hydrophobicity and with the phospholipid composition of the bilayer.

The findings reported in this paper suggest that the interaction of lysozyme with phospholipid bilayers involves a coulombic attraction between the vesicles and lysozyme, resulting in binding of the protein to the phospholipid vesicle. A consequence of this binding is an increased permeability to anions, the amount of protein bound being related to the extent of the increase in permeability. The increased permeability to anions can be envisaged as being due to a localized change in the orientation of the phospholipid bilayer, following penetration of part of the protein into the hydrocarbon core of the bilayer where hydrophobic interactions may occur.

It is hoped that studies now under way on the effect of altering further the lipid composition of the membrane as well as modifying the protein will further elucidate the mechanism of interaction of proteins with lipid membranes.

APPENDIX

Suppose that in a sample of n liposomes, in which each liposome has a volume w ml, there are L moles of ions sequestered. During dialysis suppose that F moles

of the sequestered ion diffused out into v ml of external solution Then if C is the concentration of ions within the liposomes and if D is the concentration of ions diffused

$$D = \frac{F}{v} \text{ and } C = \frac{L}{nw} \tag{1}$$

If in total there are T moles of ions present in the system,

$$T = L + F = nwC_0 \tag{2}$$

where C_0 is the initial concentration

Suppose that the efflux rate is directly proportional to the difference of ionic concentration inside and outside the liposomes,

$$i.e. \quad \frac{-dC}{dt} = k'(C - D) \tag{3}$$

where k' is a diffusion constant.
From Eqns 1, 2 and 3

$$\frac{dC}{dt} + k'\left(1 + \frac{nw}{v}\right)C = \frac{k'T}{v}$$

Put $k = k'(1 + nw/v)$ and $p = k'T/v$ where k is proportional to the diffusion constant of ions across the liposome membrane. We obtain

$$\frac{dC}{dt} + kC = p$$

$$i.e. \quad \frac{d(Ce^{kt})}{dt} = pe^{kt}.$$

Integrating with respect to time, where at time $t = 0$, $C = C_0$, we obtain

$$\frac{C - p/k}{C_0 - p/k} = e^{-kt}.$$

In this experimental system p/k is very small in comparison with C and C_0 so that

$$\frac{C}{C_0} = e^{-kt}. \tag{4}$$

From Eqns 1, 2 and 4,

$$\frac{T - F}{T} = e^{-kt},$$

$$\ln\left(\frac{T - F}{T}\right) = -kt.$$

The slope of the linear plot of $\ln (T-F)/T$ *vs* t gives a rate constant closely related to the permeability constant of the bilayer. In one experiment if k_p and k_c refer to the rate constants for the samples with and without protein, respectively, a value for k_p/k_c may be obtained, since the unmeasured constants n and w are the

same. The individual rate constants k_c and k_p from different experiments cannot quantitatively be compared since n and w vary from one liposome preparation to another.

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