Biochimica et Biophysica Acta, 311 (1973) 1–5

BBA 76328

THE EFFECTS OF LYSOZYME ON ANION AND CATION DIFFUSION FROM PHOSPHOLIPID LIPOSOMES

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(Received January 8th, 1973)

SUMMARY

Lysozyme has been shown to increase the rate at which $^{22}Na^+$ diffuses out of liposomes composed of varying amounts of phosphatidylserine and phosphatidylcholine. As the proportion of phosphatidylserine is increased, the effectiveness with which the protein increases the $^{22}Na^+$-diffusion rate becomes greater. The increase in effect with increasing proportions of phosphatidylserine can be related to the amount of protein bound to the vesicles. The simultaneous rate of CrO_4^{2-} diffusion from the liposomes was not affected by the addition of lysozyme. A tentative model is proposed for this discrimination.

It has recently been shown that the addition of the basic protein lysozyme to sonicated dispersions of phospholipid vesicles can cause an increase in the efflux rate of CrO_4^{2-} across the bilayer membranes[1]. This increase was observed with liposomes composed of lecithin and dicetyl phosphate, but not with liposomes composed of lecithin alone nor of lecithin and stearylamine. It was concluded that the opposite charges of the negative liposomes and basic protein facilitated an electrostatic binding and that the binding of lysozyme to the liposome resulted in an increase in CrO_4^{2-} efflux rates, probably due to a localized change in bilayer structure.

This report describes experiments relating to the effect of lysozyme on the permeability of liposomes formed from mixtures of phosphatidylserine and lecithin. It is shown that simultaneous measurements of $^{22}Na^+$- and CrO_4^{2-}-efflux rates from phosphatidylserine–lecithin liposomes indicate that lysozyme exerts a differential effect on the efflux rates of the two species.

Lecithin was prepared[2] from hens' eggs and purified[3] by methods previously described; dicetyl phosphate was obtained from Sigma Ltd and the monosodium salt of phosphatidylserine from Lipid Products Ltd. Lysozyme was purchased from Sigma Ltd. Protein estimations were carried out using the method of Lowry *et al.*[4]. Sonicated phospholipid vesicles containing CrO_4^{2-} were prepared, as described previously[1], from 80 μmoles total lipid and the subsequent rates of efflux, at pH 7.7, measured by dialysis at 37 °C. In studies of simultaneous $^{22}Na^+$- and CrO_4^{2-}-

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diffusion rates 30 μl of ^{22}NaCl (100 μCi/ml) was added to the swelling medium.

The assay of CrO_4^{2-} was carried out spectrophotometrically[1] at 360 nm. $^{22}Na^+$ diffusion was measured as the cpm present in the dialysate at the end of each hour of dialysis (dialysis being continued for 4 h) and expressed as a percentage of the total cpm in the original dispersion. The rate equation derived in the earlier work[1] was applied to both $^{22}Na^+$ and CrO_4^{2-} effluxes and in all experiments three control samples (without protein) and three experimental samples (containing 10 mg protein) were used in parallel. Each sample contained 0.5 ml of the liposomes suspension (5–7 μM phospholipid) and 0.5 ml buffer (controls) or 0.5 ml protein solution.

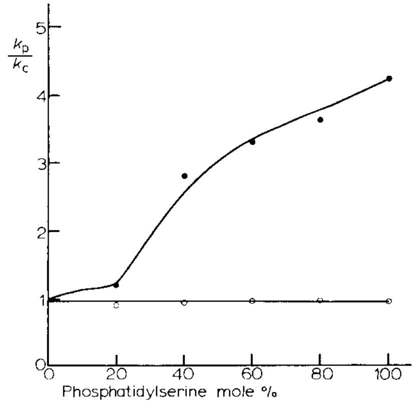
The rate equation

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

where T refers to the total numbers of moles of the diffusing ion and F refers to the number of moles of the ion diffused out of the liposomes at time t, yielded linear plots of $\ln (T-F)/T$ *vs* t. The rate constants k_p and k_c (for protein and control samples respectively) could be obtained and compared from the slopes of these plots.

As the proportion of phosphatidylserine, the negatively charged component of the liposomal membranes, was increased, the effect of lysozyme on the rate of efflux of $^{22}Na^+$ became greater (Fig. 1). However, k_p/k_c, the ratio of rate constants showed no change in this range for CrO_4^{2-} efflux. The ratio remained at unity, indicating no increase in the rate of efflux of CrO_4^{2-} from the liposomes.

Binding studies were carried out using vesicles of the same composition as in the permeability studies described above. The free protein was separated from the vesicles and bound protein by an ultra-filtration technique using Millipore filters[1]. Increased proportions of phosphatidylserine produced an increase in protein binding



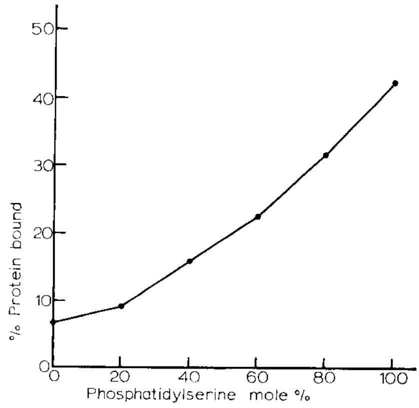


Fig. 1. Effect of addition of lysozyme to liposomes of differing lipid composition on the CrO_4^{2-} and $^{22}Na^+$ efflux rate constant ratios k_p/k_c. The liposomes were prepared in 0.1 M K_2CrO_4 in 50 mM borate buffer (pH 7.7) containing ^{22}NaCl and dialysed at 37 °C against 0.15 M KCl in the same buffer. ●—●, $^{22}Na^+$; ○—○, CrO_4^{2-}.

Fig. 2. 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.

(Fig. 2), and the curve shows a dependence on liposome composition similar to that shown in Fig. 1.

In the previous studies on CrO_4^{2-} efflux alone, using dicetyl phosphate as the negative lipid, increases in CrO_4^{2-} efflux rates were produced by lysozyme[1]. In experiments carried out under the same conditions but also simultaneously monitoring $^{22}Na^+$ efflux, 33 mole % dicetyl phosphate liposomes (67 mole % lecithin) gave values of $k_p/k_c = 2.6$ for $^{22}Na^+$ and $k_p/k_c = 2.0$ for CrO_4^{2-}. Thus, using liposomes formed from dicetyl phosphate and lecithin, both $^{22}Na^+$- and CrO_4^{2-}-efflux rates were increased by the addition of lysozyme. The permeabilities of both Na^+ and K^+ have been reported to be higher in liposomal membranes containing dicetyl phosphate than in membranes containing phosphatidylserine[5]. Since passive cation permeability through phospholipid membranes is generally envisaged as occurring *via* pores or charged clusters[6] it is not unlikely that the intrinsic 'leakiness' of membranes composed of mixtures of dicetyl phosphate and lecithin is greater than that of mixtures of phosphatidylserine and lecithin membranes. The stereochemistry of the dicetyl phosphate head group is sufficiently different from that of the head groups of phosphatidylserine and other naturally occurring membrane phospholipids to suggest that differences in permeability may well reside in the degree of close-packing allowed by the various phospholipid mixtures in a two-dimensional array.

Kimelberg and Papahadjopoulos[7], using unilamellar phosphatidylserine liposomes have reported similar increases of $^{22}Na^+$ efflux in the presence of lysozyme under comparable conditions of ionic strength to those reported here. They suggested that the initial interaction of basic proteins with phosphatidylserine vesicles is electrostatic. Other data[1] suggest that this situation also exists in the interaction of lysozyme with vesicles composed of dicetyl phosphate–lecithin mixtures.

From studies using mixtures of phosphatidylserine and lecithin it is clear that the interaction with lysozyme depends on electrostatic forces of attraction but the resultant effects on the bilayer membranes distinguish between $^{22}Na^+$ and CrO_4^{2-}. Bangham *et al.*[6] have shown that in liposomes formed from varying mixtures of phospholipids, as the membrane is made less negatively charged the cation permeability falls. Considerations of the resultant charge on the phospholipid membrane with adsorbed basic protein do not seem to offer plausible reason for the discrimination between $^{22}Na^+$ and CrO_4^{2-} described in the present studies. Binding of a positive protein to the negatively charged membrane should lower the negative charge on the membrane. Thus the binding of lysozyme to phosphatidylserine liposomes might be expected to lower the rate of $^{22}Na^+$ efflux on charge considerations alone, but this is clearly not the observed effect.

Perturbation of the hydrocarbon region of the bilayers by proteins has been postulated as a model to account for increases in cation permeability of phospholipid bilayer membranes[8]. In this model, the protein penetrates into and deforms the phospholipid array resulting in localized perturbations of the hydrocarbon regions of the phospholipids. These perturbations, it was postulated, were responsible for increases in cation permeability of phospholipid bilayer membranes in the presence of some proteins. If this model is applicable to increases in rates of diffusion of slowly moving anions (CrO_4^{2-}, for example), the discrimination between $^{22}Na^+$ and CrO_4^{2-} reported here may reflect the extent of perturbation, caused by lysozyme in the phospholipid bilayers.

Ito *et al.*[9] studied the passive permeabilities of various anions and cations through biological membranes and computed that the hydrated diameter of CrO_4^{2-} is 20% larger than the hydrated diameter of $^{22}Na^+$.

The extent of perturbation of the lecithin–phosphatidylserine bilayers caused by lysozyme could be sufficient to increase the rate of diffusion of the smaller $^{22}Na^+$ ion but insufficient to allow an increase in the rate of diffusion of the larger CrO_4^{2-} ion. This model predicts that in a situation where both $^{22}Na^+$- and CrO_4^{2-}-efflux rates are simultaneously increased, in the absence of further complicating effects, the increase for $^{22}Na^+$ should be greater than the increase for CrO_4^{2-}. In order to test this hypothesis, liposomes composed of 80 mole % phosphatidylserine with 20 mole % lecithin were prepared and the effect of haemoglobin on the efflux rates of CrO_4^{2-} and $^{22}Na^+$ was measured. Haemoglobin was selected as it has recently been demonstrated to increase the efflux rate of Rb^+ from phosphatidylserine liposomes[10]. The experiments were carried out in the same way as those using lysozyme described above, with haemoglobin at a final concentration of 10 mg/ml in the liposome dispersion. There were increases in the rates of efflux of both $^{22}Na^+$ and CrO_4^{2-}; the values obtained were $k_p/k_c = 5.6$ for CrO_4^{2-} and $k_p/k_c = 26.8$ for $^{22}Na^+$. The increase in the $^{22}Na^+$-efflux rate was greater than the increase in CrO_4^{2-} efflux rate, as predicted.

The presentation of these data as efflux rate constant ratios may tend to obscure the actual levels of $^{22}Na^+$ and CrO_4^{2-}. In all of the control samples, the total leakage of $^{22}Na^+$ after 4 h was close to 2.5% and of CrO_4^{2-} was 13–17%. The $^{22}Na^+$ level in the presence of lysozyme was raised to 10.5% with 100 mole % phosphatidylserine liposomes and to 7.0% with 40 mole % phosphatidylserine liposomes. In both cases the CrO_4^{2-} level in the presence of protein was the same as in the control samples. In the experiments carried out using haemoglobin, the $^{22}Na^+$ leak after 4 h totalled 75% of the original $^{22}Na^+$ present in the liposomes whilst the CrO_4^{2-} levels rose from 11–13% in the controls to 52–59% in the presence of protein.

The explanation of the discrimination effect described here is intended as a simple working hypothesis and it is hoped that studies now under way will elucidate further the details of these interactions.

ACKNOWLEDGEMENTS

I would like to thank Dr D. Rosen and Dr M. Perry for many useful discussions during the course of this work. I am grateful to the Medical Research Council for their financial support.

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