Biochimica et Biophysica Acta, 774 (1984) 19–25
Elsevier

BBA 72139

CHARACTERISATION OF PHOSPHATIDYLCHOLINE/PHOSPHATIDYLINOSITOL SONICATED VESICLES

EFFECTS OF PHOSPHOLIPID COMPOSITION ON VESICLE SIZE

K. HAMMOND [a], M.D. REBOIRAS [a,*], I.G. LYLE [b] and M.N. JONES [a,**]

[a] *Department of Biochemistry, University of Manchester, Manchester, M13 9PL, and* [b] *Unilever Research Laboratories, Bebington, Wirral, Merseyside, L63 3JW (U.K.)*

(Received December 6th, 1983)

Key words: Phospholipid vesicle; Liposome; Lipid composition; Vesicle size; Electron microscopy; Photon correlation spectroscopy

Phosphatidylinositol (PI), dipalmitoylphosphatidylcholine (DPPC) and mixed lipid (DPPC plus PI) sonicated vesicles have been prepared covering a range of composition. The vesicles were characterised by gel filtration, electron microscopy and photon correlation spectroscopy. The dimensions of the vesicles as measured by electron microscopy were in good accord with those obtained from photon correlation spectroscopy measurements. The number average diameters of the vesicles increase on increasing the PI content and range from approx. 30–80 nm as the weight % of PI is increased from 0 to 100. Gel filtration on Sepharose 4B columns gave anomalous results indicating that PI-containing vesicles were retarded on the gel possibly due to an interaction between the inositol headgroup and the gel matrix. Electrophoretic measurements on multilamellar vesicles show that the surface charge density increases with the PI content of the vesicles upto 50 weight % PI and remains constant thereafter. The radii of sonicated vesicles also increase with PI content which reflects a decreasing liposome curvature with increasing surface charge density.

Introduction

Phosphatidylinositol (PI) is a significant component of cell membranes and its metabolism and role in calcium transport are of current interest [1–3]. There have been several studies on the affects of PI on the physical properties of bilayer systems [4–14]. In the absence of divalent cations PI is miscible with dipalmitoylphosphatidylcholine (DPPC) over the entire composition range [9] and it has been suggested that the two phospholipids mix ideally [4]. Calorimetric studies however, suggest that a 1 : 1 complex is formed between DPPC and PI which mixes ideally with excess DPPC [15].

The distribution of PI in PI/DPPC sonicated vesicles has been investigated [5,7,8]. Using an exchange protein and a PI-specific phospholipase Low and Zilversmit [8] deduced the PI was not preferentially located in either inner or outer surface of the phospholipid bilayer. Other studies have however, suggested that anionic phospholipids may be preferentially located on the outer bilayer surface [7,13] or the inner bilayer surface [5,14].

PI has been shown to have an inhibitory effect on divalent metal ion induced fusion of mixed lipid vesicles and pure PI vesicles are not fused in the presence of Ca^{2+} or Mg^{2+} [10]. It has been proposed that the size and hydration of the in-

* Present address: Departmento de Electroquimica, Facultad de Ciencias, Universidad Autonoma, Canto Blanco, Madrid 34, Spain.

** To whom correspondence should be addressed.

ositol headgroup prevents the formation of dehydrated complexes with divalent metal ions which initiate membrane fusion.

We report here a study of the characterisation of PI-DPPC mixed lipid sonicated vesicles by several techniques which preceeds an investigation into the aggregation of these vesicles by a range of metal ions. The objective of the study was to determine the effect of increasing PI content on the physical characteristics of DPPC sonicated vesicles.

Materials and Methods

Materials. L-α-Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma London Chemical Company approx. 99% pure (product number (P-6267)). Two sources of phosphatidylinositols were used, Grade 1 from soybean (as the sodium salt, approx. 98%) was obtained from Sigma and Grade 1 from wheat germ (as the sodium salt, molecular weight 846 [12]) was obtained from Lipid Products, South Nutfield, U.K. The purity of the phospholipids was checked by thin-layer chromatography. Their purity was in excess of 98% and they were used as supplied. The Lipid Products PI was found to contain fewer slow-moving contaminants than the Sigma PI and was routinely used in the experiments unless otherwise stated.

Gel filtration was carried out using Sepharose 4B agarose gel (Pharmacia Fine Chemicals). Fine particles were removed prior to column packing by decantation from settling suspensions. *Micrococcus lysodeikticus* (dried cells) were obtained from Sigma and used to determine the void volumes of the columns.

Vesicle preparation. Mixed phospholipid vesicles were prepared by addition of the desired amounts of stock solutions (5 $mg \cdot cm^{-3}$ in chloroform) of DPPC and PI along with 50 cm^3 of chloroform/methanol solution (4:1, v/v) to a 1 litre round-bottom flask. The solvent was removed by rotary evaporation at a temperature of approx. 50 °C. The resulting lipid film was flushed with nitrogen to eliminate trace solvent for at least 30 min and the required quantity of nitrogen saturated aqueous medium added at approx. 50 °C. For the gel filtration experiments and electron microscopy the vesicle dispersions were prepared at a total lipid concentration of 0.02% (w/v). The dispersions were saturated with nitrogen, sealed and sonicated above the chain melting temperature of DPPC (41 °C) for 2.25 h in a bath sonicator.

Electron microscopy. Vesicles were examined and photographed at magnificants between 10000 and 100000 diameters at 80 kV with a Jeol Jem 100 CX electron microscope. Samples were negatively stained by mixing equal volumes of vesicle dispersion and either ammonium molybdate or uranyl acetate (both 2% (w/v) in distilled water) at 4 °C. Carbon coated copper grids were briefly immersed in the dispersions, air dried and examined within one hour of preparation. The photographic negatives (8.8 × 6.3 cm) were print-enlarged approximately three times. Vesicle images were traced onto transparent acetate sheets, numbered and their diameters measured. For ellipsoidal images the mean length of the major and minor axes were recorded. A minimum number of 200 vesicle diameters were measured for each sample.

Photon correlation spectroscopy. The *z*-average diffusion coefficients ($\langle D_z \rangle$) were measured by photon correlation spectroscopy using a 1 W argon ion laser source (Spectra Physics 164, $\lambda_0 = 514.4$ nm). The light scattered at 90° to the incident beam was collected and analysed with a 48-channel autocorrelator (Malvern K 7023) operating in scaling mode. Vesicle suspensions were filtered through 0.22 μm (or 0.45 μm) MilliPore filters to remove dust particles and diluted within the concentration range covered by dilute solution theory. The data were analysed by the method of cumulants [16] and the reciprocal of the reciprocal *z*-average diameters $(\langle d_z^{-1} \rangle)^{-1}$ [17] were calculated from the Stokes-Einstein equation

$$\left(\langle d_z^{-1} \rangle\right)^{-1} = \frac{kT}{3\pi\eta\langle D_z \rangle} \tag{1}$$

where $T = 298$ K, k is the Boltzmann constant and the viscosity of the aqueous media (η) was taken to be $8.904 \cdot 10^{-4}$ $N \cdot s \cdot m^{-2}$.

Gel filtration. Two Sepharose 4B columns were used one 69 cm × 1 cm (diametre) and one 40 cm × 1 cm (diametre). The gels were pretreated with 0.1% (w/v) DPPC vesicles in distilled water pH 6.6 to prevent phospholipid adsorption on the

gel matrix. Despite pre-treatment of the columns with lipid, recovery was found to increase with column use. Aliquots of lipid vesicles (5 cm^3) were layered on the columns and eluted with distilled water pH 6.6 (containing 0.02% (w/v) sodium azide) or 10 mM Tris in 0.1 M NaCl, pH 7.4 (containing 0.02% (w/v) sodium azide). The flow rate was approx. 5.4 $cm^3 \cdot h^{-1}$ and fractions of 1.8 cm^3 or 1.5 cm^3 were collected and their phospholipid content was assayed by a modification of the procedure of Bartlett [18]. The void and inner volumes of the columns were measured using *Micrococcus lysodeikticus* suspensions and potassium chromate, respectively.

Microelectrophoresis. Measurements were made on multilamellar vesicles prepared as described above with the omission of sonication using a Rank Bros. (Bottisham, Cambridge, U.K.) microelectrophoresis apparatus with a horizontal flat cell and grey platinum electrodes. The rate of migration of the vesicles was determined by timing their transit over a known distance (d). The velocity of at least 25 vesicles was measured in both directions and the mobilities (u, $m^2 \cdot s^{-1} \cdot V^{-1}$) were calculated from the equation

$$u = \frac{dL_e}{Vt} \qquad (2)$$

where L_e is the effective length of the cell between the electrodes, V the applied voltage and t the time. The alignment of the apparatus and the positions of the stationary levels were checked with reference to the known mobilities of human erythrocytes [19].

Zeta potentials (ζ) were calculated from the Henry equation

$$\zeta = \frac{3\eta u}{2\epsilon_0 \epsilon_r f(\kappa a)} \qquad (3)$$

where η is the viscosity of the medium (taken to be $8.904 \cdot 10^{-4}$ $N \cdot s \cdot m^{-2}$ at 25 °C), ϵ_0 is the permittivity of free space ($8.854 \cdot 10^{-12}$ $J^{-1} \cdot C^2 \cdot m^{-1}$) and ϵ_r is the relative permittivity of the medium (78.5). The Henry correction factor $f(\kappa a)$ was calculated from the expansion given by Hunter [20]. Assuming that the multilamellar vesicles had a radius (a) of approx. 250 nm the value of $f(\kappa a)$ was 1.47 for the medium used (phosphate buffer pH 7.4, ionic strength 0.0344, $\kappa = 6.097 \cdot 10^8$ m^{-1}), i.e. close to the Smoluchowski condition where the particle radius (a) is large compared with the double-layer thickness (κ^{-1}).

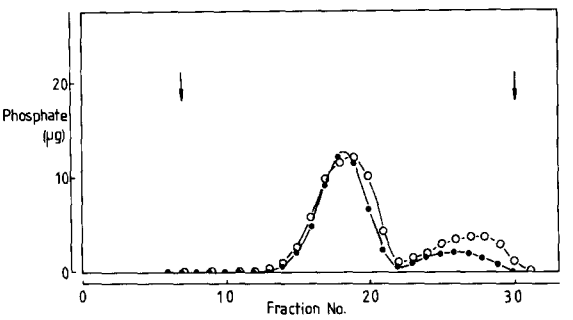

Fig. 1. Gel filtration of phosphatidylinositol (PI) sonicated vesicles on Sepharose 4B gel. ●, wheat germ PI; ○, soybean PI. The vesicles were eluted in distilled water (pH 6.6).

Results

Gel chromatography on Sepharose 4B

Initial studies on sonicated PI vesicles showed that PI was readily susceptible to oxidative degradation during sonication. Fig. 1 shows the elution profiles of PI vesicles made from soybean and wheat germ PI. Nitrogen gas was passed through the dispersions prior to sonication for 10 min. Under these conditions the samples gave two peaks. The smaller of the two was found to vary in area in successive preparations but could be eliminated entirely by increasing the length of time

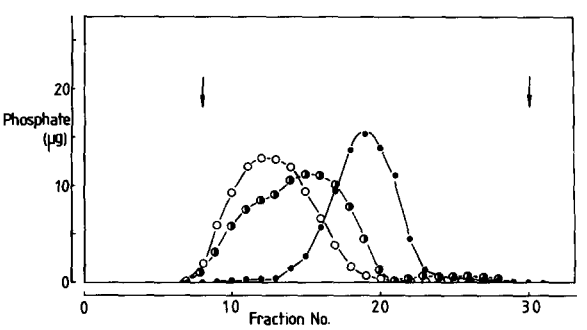

Fig. 2. Gel filtration of phosphatidylinositol (PI) dipalmitoylphosphatidylcholine (DPPC) and DPPC/PI mixed lipid sonicated vesicles on Sepharose 4B gel. The vesicles were eluted in distilled water. The arrows denote the void volume and elution volume of chromate (void plus inner volume). ○, DPPC vesicles: ● PI vesicles; ◑ DPPC/PI (50:50, by weight) vesicles.

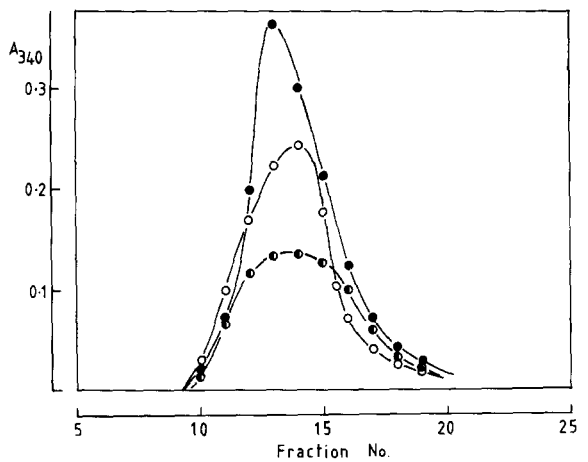

Fig. 3. Gel filtration of phosphatidylinositol (PI) dipalmitoylphosphatidylcholine (DPPC) and DPPC/PI mixed lipid sonicated vesicles on Sepharose 4B gel. The vesicles were eluted in Tris buffer (pH 7.4) containing 0.1 M NaCl. ○, DPPC vesicles: ●, PI vesicles; ◐ DPPC/PI (50:50, by weight) vesicles.

during which N_2 was passed through the dispersion to 30 min, and was hence attributed to oxidation products of PI.

Typical elution profiles of PI, DPPC and mixed lipid (50% by weight PI/DPPC) vesicles in distilled water are shown in Fig. 2. On Sepharose 4B DPPC vesicles elute with a K_d of 0.49. The elution volumes of the vesicles were independent of the initial concentration in the range 0.01 to 0.1% (w/v) as shown in Fig. 4. Running the vesicles in a buffer of ionic strength 0.1 reduced their elution volumes and changed the order of elution (Fig. 3) although the differences between the elution volumes for PI, DPPC and mixed lipid vesicles was relatively small. In salt for DPPC vesicles K_d is 0.20 and for PI vesicles K_d is 0.15, as in distilled water the mixed lipid vesicles eluted between the pure components. The elution profiles of the mixed lipid vesicles (Fig. 2) displayed a slight shoulder which suggested the possibility that the vesicle population might be heterogeneous with respect to phospholipid composition as well as to size. To investigate this, fractions on either side of the peak were pooled and analysed by thin-layer chromatography. The TLC plates showed that there was no detectable difference in lipid composition across the elution profile indicating an absence of phospholipid heterogeneity.

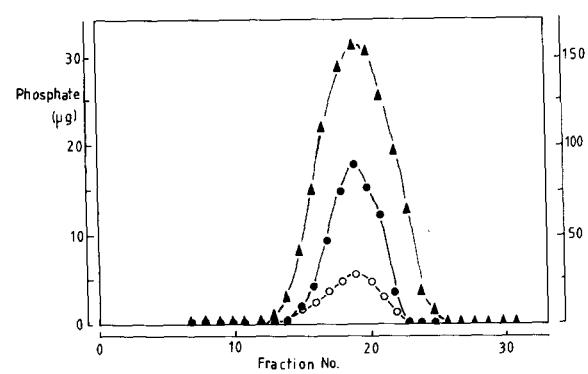

Fig. 4. Effect of initial vesicle concentration on elution profile of phosphatidylinositol, sonicated vesicles on Sepharose 4B gel. ●, lipid concentration 0.1% w/v (right hand vertical axis); ▲, lipid concentration 0.04% w/v (left hand vertical axis); ○, lipid concentration 0.01% w/v (left hand vertical axis). The vesicles were eluted in distilled water.

Electron microscopy and photon correlation spectroscopy

Fig. 5 shows typical vesicles diameter-distribution curves for PI, DPPC and mixed lipids (50% by weight PI:DPPC) vesicles as measured from negatively stained electron micrographs. Fig. 6 shows a typical electron micrograph of PI vesicles. From the distribution curves it is clear that the size of the vesicles follows the sequence DPPC < DPPC/PI < PI. This result was substantiated by photon correlation spectroscopy which also showed that the size of the vesicles increased with PI content. Table I shows the number-average ($\langle d_n \rangle$),

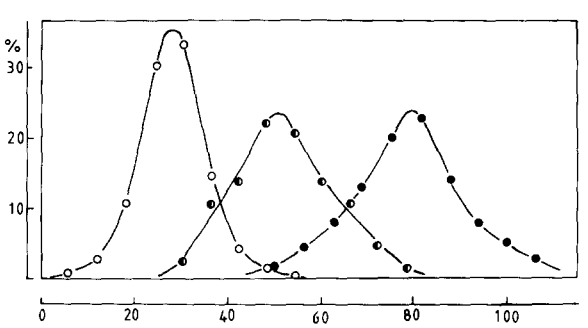

Fig. 5. Diameter distribution curves as measured by electron microscopy of phosphatidylinositol (PI) dipalmitoylphosphatidylcholine (DPPC) and DPPC/PI mixed lipid sonicated vesicles. ○, DPPC vesicles; ● PI vesicles; ◐ DPPC/PI (50:50 by weight) vesicles.

TABLE I

DIAMETERS OF DPPC, PI AND DPPC/PI SONICATED VESICLES

Vesicle composition DPPC/PI, weight ratio (%)	Electron microscopy				Photon correlation spectroscopy	
	$\langle d_n \rangle$ (nm)	$\langle d_w \rangle$ (nm)	$\langle d_z \rangle$ (nm)	$\frac{\langle d_z \rangle}{\langle d_n \rangle}$	$(\langle d_z^{-1} \rangle)^{-1}$ (nm)	$(\langle d_z^{-1} \rangle)^{-1}$ (nm) [a]
100:0	29	30	32	1.1	36	50
75:25					41	70
50:50	58	61	64	1.1	74	100
25:75					78	120
0:100	79	81	83	1.1	84	160

[a] After storage for 42 days.

weight-average ($\langle d_w \rangle$) and z-average ($\langle d_z \rangle$) diameters of the vesicles as a function of composition. The z-average diameters correlated reasonably well with $(\langle d_z^{-1} \rangle)^{-1}$ obtained by photon correlation spectroscopy.

The vesicles were stable for at least one week after preparation however, after storage at 25 °C for 42 days there was a significant size increase presumably due to aggregation and/or fusion, as shown by the data in the last column in Table I.

Microelectrophoresis

The electrical characteristics of vesicle surfaces was investigated using large multilamellar vesicles in a low ionic strength buffer (phosphate, $I = 0.03$, pH 7.4). The buffer was necessary to ensure adequate conductivity of the system for electrophoresis. Fig. 7 shows that the electrophoretic mobilities (u) of the vesicles increase with PI content upto approximately 50% and remain constant thereafter. The mobilities were used to calculate the zeta-potentials of the surfaces which reach a limiting value of −68 mV. Ohki et al. [9] observed an increase in electrophoretic mobility with increasing (yeast) PI content of DPPC/PI liposomes produced by vortexing with glass beads under similar conditions of pH and ionic strength. Their mobilities were larger than ours which may be due to their liposomes being smaller.

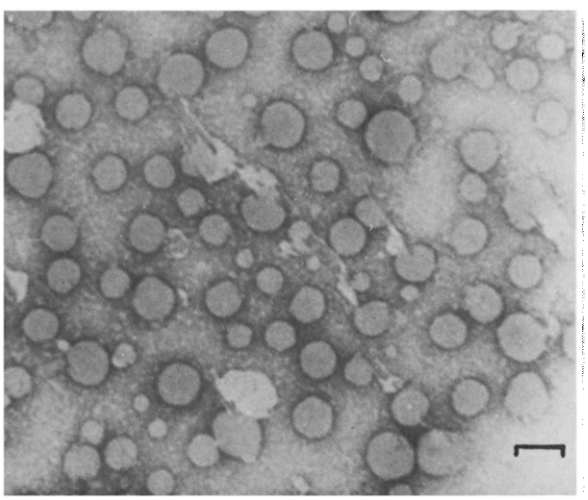

Fig. 6. Electron micrograph of sonicated PI vesicles negatively stained with uranyl acetate. The bar (left hand corner) corresponds to 200 nm.

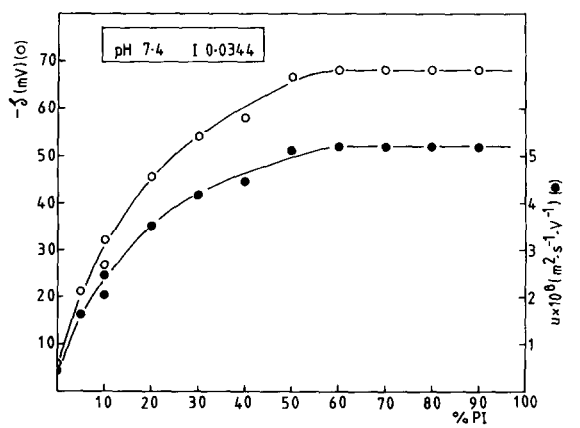

Fig. 7. Electrophoretic properties of phosphatidylinositol/dipalmitoylphosphatidylcholine multilamellar vesicles as a function of composition at 25 °C. Right hand axis (○) zeta potential (ζ), left hand axis (●) electrophoretic mobility. The vesicles were in a phosphate-saline buffer (pH 7.4), $I = 0.0344$.

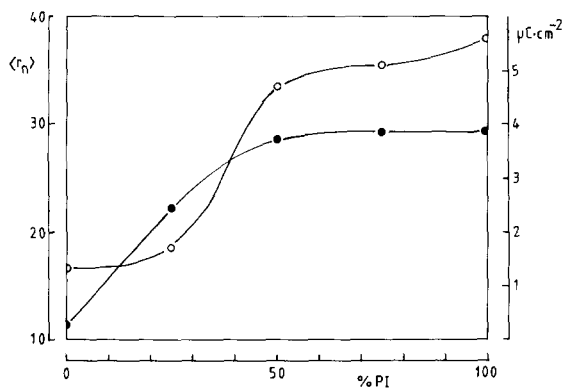

Fig. 8. Surface charge density (●, right hand axis) of multilamellar vesicles and number average radii (○, left hand axis) of sonicated vesicles as a function of composition.

Discussion

The electron microscopy and photon correlation spectroscopic data show that DPPC/PI vesicles increase in size with increasing PI content. The gel filtration data for the vesicles in water are inconsistent with this conclusion; PI vesicles elute after the DPPC vesicles. This elution order is just reversed when the vesicles are eluted in salt solution. The K_d values for DPPC vesicles are similar under both conditions 0.24 (in water) and 0.20 (in salt) whereas they are significantly different for PI vesicles 0.49 (in water) and 0.15 (in salt). This implies that it is the elution volumes of PI vesicles that are anomalous in water rather than those of the DPPC vesicles. It follows that the Sepharose 4B gel withholds PI vesicles, although addition of salt partially reduces this putative adsorption the elution volume of the PI vesicles is much closer to that of the DPPC vesicles than would be expected for an approximately 3-times larger radius. Since the vesicles are negatively charged and any residual charge on the agarose gel is likely to be negative it follows that the effect is unlikely to be predominantly electrostatic but more likely arises from an interaction between the inositol residue of PI and the gel matrix.

The increase in size of the vesicles with increasing PI content reflects the packing constraints imposed particularly on the inner bilayer by the hydrated size and charge of the PI molecules which would favour a reduced bilayer curvature. An indication of the effect of PI on the charge status of vesicle surfaces is obtained from the electrophoretic data for multilamellar vesicles (Fig. 7). The mobility and zeta potentials of the vesicles reach limiting values between 50 and 60% PI content (i.e., a PI : DPPC molar ratio in the range 0.87 to 1.3). The surface charge density over the plane of shear was calculated from the equation [21]

$$\sigma(\mu C \cdot cm^{-2}) = 11.74\, C^{1/2} \sinh(19.46\, \zeta) \qquad (4)$$

where C was taken as 0.0344 M. Fig. 8 shows σ as a function of weight % PI for the multilamellar vesicles. Aso shown is the variation of the sonicated vesicle number-average radii calculated from the z-average diameters and the known polydispersity $\langle d_z \rangle / \langle d_n \rangle$ (Table I). Increasing surface charge density would be expected to lead to an increase in vesicle radius to reduce electrostatic repulsion. The results in Fig. 7 confirm this expectation in that there is a steep rise in vesicle radius between a PI content of 30–50% and a smaller change thereafter. The limiting surface charge density (3.83 $\mu C \cdot cm^{-2}$) corresponds to an area per charge of 418 Å^2. Taking an area per phospholipid molecule in the region of 50–60 Å^2 suggests that as the PI content is increased from 50 to 100% the degree of ionisation decreases from 0.26 to 0.13. This decrease in degree of ionisation is consistent with the view that as in the case of an ionic micelle the limiting surface charge density is maintained by counterion adsorption.

Acknowledgements

We thank Dr. P.G. Cummins and Mr. E.J. Staples, Unilever Research Port Sunlight Laboratory, for assistance with the photon correlation spectroscopic measurements, Miss Rita Stubbersfield for assistance with the electron microscopy and the British Council for financial assistance to M.D.R.

References

1 Michell, R.H. (1979) Trends Biochem. Sci. 4, 128–131
2 Honeyman, T.W., Strohsnitter, W., Scheid, C.R. and Schimmel, R.J. (1983) Biochem. J. 212, 489–498
3 Merridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473–482

4 Litman, B.J. (1973) Biochemistry 12, 2545–2554
5 Berden, J.A., Barker, R.W. and Radda, G.K. (1975) Biochim. Biophys. Acta 375, 186–208
6 Sundler, R., Alberts, A.W. and Vangelis, P.R. (1978) J. Biol. Chem. 253, 5299
7 Massari, S., Pascolini, D. and Gradenigo, G. (1978) Biochemistry 17, 4465–4469
8 Low, M.G. and Zilversmit, D.B. (1980) Biochim. Biophys. Acta 596, 223–234
9 Ohki, K., Sekiya, T., Yamauchi, T. and Nozawa, Y. (1981) Biochim. Biophys. Acta 644, 165–174
10 Sundler, R. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 649, 743–750
11 Sundler, R., Düzgüneş, N. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 649, 751–758
12 Ter-Minassian-Saraga, L. and Madelmont, G. (1982) J. Coll. Int. Sci. 85, 375–388
13 Michaelson, D.M., Horowitz, A.F. and Klein, M.P. (1973) Biochemistry 12, 2637–2644
14 Voktorov, A.V., Vasilenko, I.A., Barsukou, L.I., Eustigneeva, R.P. and Bergelson, L.D. (1977) Dokl. Akad. Nauk, S.S.S.R. 234, 207–210
15 Hammond, K., Lyle, I.G. and Jones, M.N. (1984) J. Coll. Int. Sci. in the press
16 Koppel, D.E. (1972) J. Chem. Phys. 57, 4814–4820
17 Forget, J.L., Booth, C., Canham, P.H., Duggleby, M. and King, T.A. (1979) J. Polym. Sci. 17, 1403–1411
18 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
19 Bangham, A.D., Flemans, R., Heard, D.H. and Seaman, G.V.F. (1958) Nature 182, 642–644
20 Hunter, R.J. (1981) Zeta Potential in Colloid Science, p. 71, Academic Press, New York
21 Hunter, R.J. (1981) Zeta Potential in Colloid Science, p. 28, Academic Press, New York