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PROTON BINDING BY PHOSPHATIDYL INOSITOL IN AQUEOUS DISPERSION

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SUMMARY

Electron microscopy of bakers' yeast phosphatidyl inositol, ultrasonically dispersed (22 kcycles for 1 h) in NaCl solution (0.01–0.06 M, pH 6.2) and negatively stained with phosphotungstate of the same pH and ionic strength, showed that the lipid exists as hollow spherules, most of which are 250–350 Å in diameter. The walls of the spherules are one or two lipid bilayers thick. The proton association curves (pH 10–2.5) of these spherules are unimodal below pH 6.0 and not reversible, and display a discontinuity after approx. 25 % of the binding sites are protonated (pH 3.5). Parallel electron microscopy studies have shown a conformation change at this pH. At lower pH the microspherules collapse into tubules and discs that aggregate to form onion-like structures. Comparison of proton uptake and release curves shows the irreversible nature of this change. The pK's were obtained from linear plots of the data in the Henderson–Hasselbalch and Tanford–Kirkwood forms. In the former case the pK values ranged from 1.84 to 2.89 and in the latter, pK_{int} values ranged from −1.80 to 1.42.

INTRODUCTION

It was first noted by BANGHAM *et al.*[1] that under favourable conditions of temperature and ionic strength, phospholipids in aqueous dispersions form spherical aggregates (liposomes) composed of concentric lipid bilayers. Under vigorous dispersal conditions, spherules of only one or two concentric bilayers may be formed[2]. PAPAHADJOPOULOS AND WATKINS[3] have presented evidence from ion entrapment studies that indicate the the aqueous compartments within the spherules are completely enclosed.

There have been several studies of the ion and proton binding properties of aqueous dispersions of acidic phospholipid aggregates[4–6,7], but little has been mentioned about the possible effects of aggregate morphology upon the ionization parameters. ABRAMSON and co-workers[4,6] have concluded from titration studies of phosphatidyl serine that "essentially all ionogenic groups are readily available for titration and therefore probably present on the micellar surface"[4], and have assumed in the interpretation of titration curves of phosphatidyl inositol dispersions that "all ionizable groups are exposed"[6]. In similar studies on phosphatidyl serine and phosphatidyl inositol diphosphate, HENDRICKSON AND FULLINGTON[7] have found that all

acidic groups in the lipid dispersion are fully titrable. Their studies appear to contradict the morphological and ion entrapment evidence that suggests that some of the binding sites are exposed to the inner aqueous compartment and are not accessible to the external bulk medium.

We have repeated the titration and morphological studies of aqueous dispersions of phosphatidyl inositol. Electron microscopy of ultrasonicated dispersions at neutral pH confirms the existence of micro-vesicles of bilayer construction. However, both electron microscopy and titration studies indicate a pH-dependent conformational change.

MATERIALS AND METHODS

All reagents were of analytical quality. The water used in all preparations was double glass-distilled, (the second distillation over alkaline $KMnO_4$ in a N_2 atmosphere), and stored in a reservoir with a moisture and CO_2 trap. The specific conductivity of this water was 1.5 $\mu\Omega^{-1}\cdot$cm. Buffers used for pH standardization, accurate to $\pm$0.01 pH unit, were obtained from the Fisher Chemical Co. and stored frozen under N_2 when not in use. The sodium salt of phosphatidyl inositol was prepared after the method of TREVELYAN[8,9]. The purity of the lipid was established by thin-layer chromatography in the following solvent systems: chloroform–methanol–water (95:35:4, by vol.); ethanol–chloroform–water, (5:2:2, by vol.); light petroleum (b.p. 30–60°)–diethyl ether–acetic acid (90:10:1, by vol.) and also by infrared spectroscopy and phosphorus[10], nitrogen[11], and inositol assays[12]. These analyses showed a N/P molar ration of 0.02–0.03 and an inositol/P molar ratio of 1.0. Free fatty acids could not be detected by infrared spectroscopy nor by thin-layer chromatography.

Titrations

5 ml of 1 mg/ml dispersions of phosphatidyl inositol, well-stirred and under a N_2 atomsphere were titrated at constant temperature with standardized 0.05 M HCl and NaOH using a calibrated Agla micrometer syringe assembly and a Radiometer pH Meter 26 with scale expander, equipped with Radiometer electrodes K4112 and G2222B. The pH meter was calibrated at two pH values bracketing the titration range and then checked at a mid-range pH. After each titrant addition and system equilibration, the stirrer and N_2 bubbler were turned off, and the meter reading recorded. Under similar conditions titration curves of water, NaCl solutions, and phosphate buffer were reproducible to 0.05 % maximum variation. After a titration of lipid dispersion, samples were removed for phosphorus assay and for thin-layer chromatography to check the integrity of the lipid.

Ultrasonication

Samples were ultrasonically dispersed by an M.S.E. 100 W ultrasonic disintegrator equipped with an exponential titanium probe, at a frequency of 20 kcycles/sec and an amplitude of 6–8 μm in sessions of 10–15 min. Samples were kept cool by an ice-brine bath and blanketed by a N_2 atmosphere. After sonication, samples were spun at 750 rev./min for 15 min to remove titanium dust and checked by thin-layer chromatography for chemical degradation.

Electron microscopy

Electron microscopy studies were carried out using a Philips Model EM-200. Samples were negatively stained with either phosphotungstic acid or ammonium molybdate at the same pH and ionic strength as the sample using techniques described by TINKER AND PINTERIC[13].

RESULTS AND DISCUSSION

Morphology of lipid dispersions

Electron microscopy was used to investigate the dispersal conditions of ionic strength and pH of the medium and duration of sonication that favour the formation of phosphatidyl inositol micro-vesicles.

Duration of sonication

Electron microscopy was used to investigate the liposome size distribution in a sol containing 1 mg lipid per ml after sonication in 0.02 M NaCl (pH 7.5) for lengths of time up to 60 min. After an hour of sonication a fairly uniform array of microspherules is obtained (Fig. 1b). Examination of a number of electron micrographs (sample size approx. 1000 microvesicles) shows that about 75 % of the structures have diameters between 250 and 350 Å, with a wall thickness of 75–100 Å. After an hour of sonication, pH drops to about 6.2.

Ionic strength effects

Electron microscopy studies were done on samples dispersed at pH 7.5 in distilled water and NaCl solutions (0.02, 0.08, 0.15, and 0.20 M). The results indicate that dispersal in solutions of ionic strength up to 0.08 M produce structures not remarkably different from those shown in Fig. 1, while dispersal in solutions of 0.15 and 0.02 molar ionic strength produced large aggregates of lipid which could not be examined by electron microscopy because of poor stain penetration and the inability of the grid to support the aggregate.

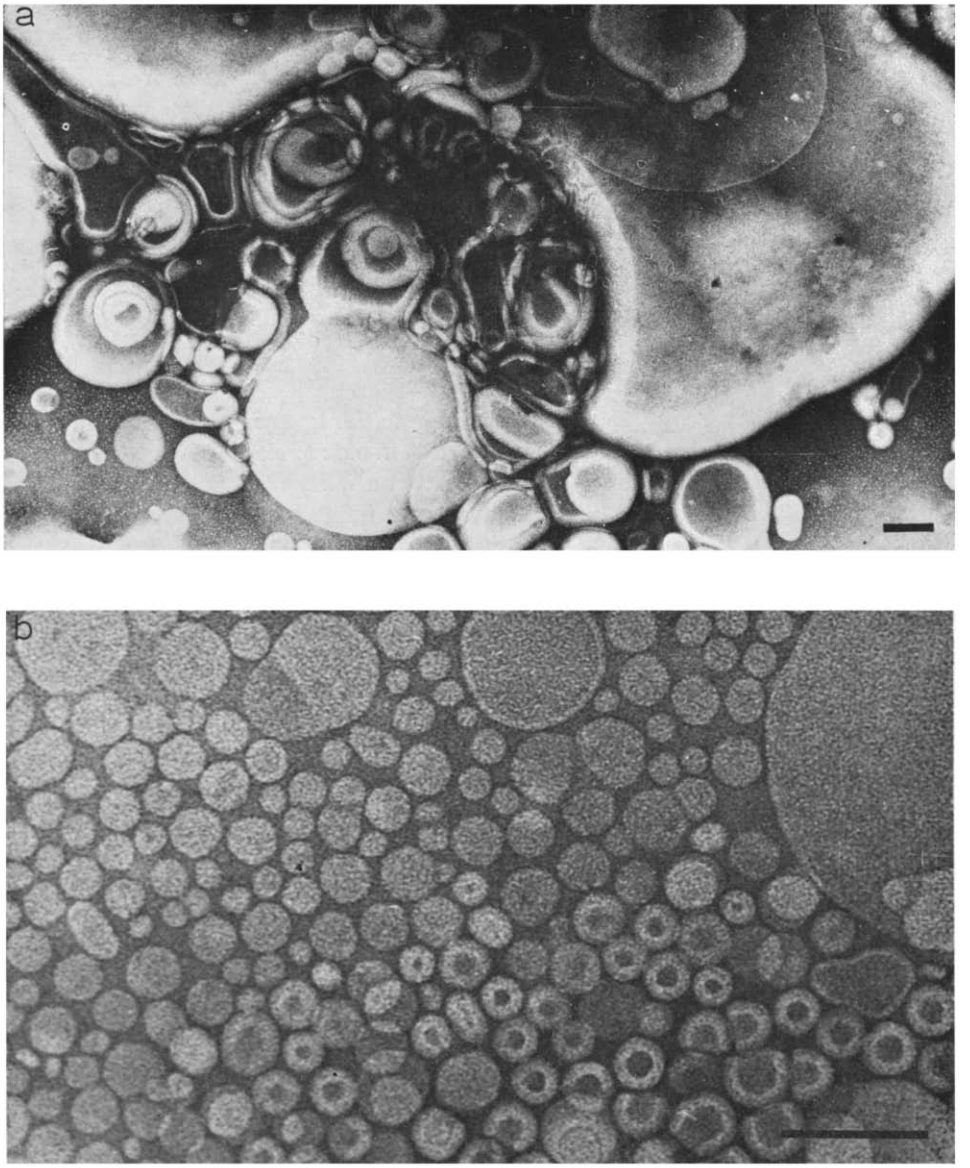
Morphological and chemical stability after sonication

It was noted that after a lapse of up to 5 h between sonication and staining for electron microscopy pictures, no structural changes in the liposomes could be detected. Thin-layer chromatography of a sample of lipid dispersed in 0.02 M NaCl in the usual way for 1 h at pH 9.1 showed no chemical degradation. A time-course hydrolysis study of this dispersion was carried out at pH 9.1, using the Radiometer titrigraph in pH-stat mode to monitor appearance of acidity. No appreciable hydrolysis was detected during an 8-h period.

Titration data

Titration curves

Blank-corrected titration curves of lipid microvesicles in 0.01, 0.02, 0.04, and 0.06 M NaCl were obtained. Illustrated in Fig. 2 are proton-association curves for sols in 0.02 M NaCl, while Fig. 3 shows similar curves for sols in 0.04 M NaCl. In one of the sols in 0.02 M NaCl and in all of the sols dispersed at higher ionic strengths, a discontinuity in the proton association curves is noted at low pH. In this region the titration curve of the lipid sols approaches that of the blank, resulting in an apparent drop in the milliequivalents of protons bound per mole of phosphatidyl inositol. This phenomenon could be caused by some process which results in the release of



Figs. 1a and 1b. Legend, see p. 487.

protons from the liposomes or by some process which traps significant amounts of water in a compartment inaccessible to titrant.

In order to study the effect of the change in electrostatic interactions (both intermolecular, and intra- and interliposomal) during a titration, upon the morphology of the liposome, the titration procedure was paralleled and samples taken for microscopy at several values of pH. Figs. 1b–1d illustrate the change in liposome morphology as the formal unit negative charge on the lipid primary phosphate is reduced.

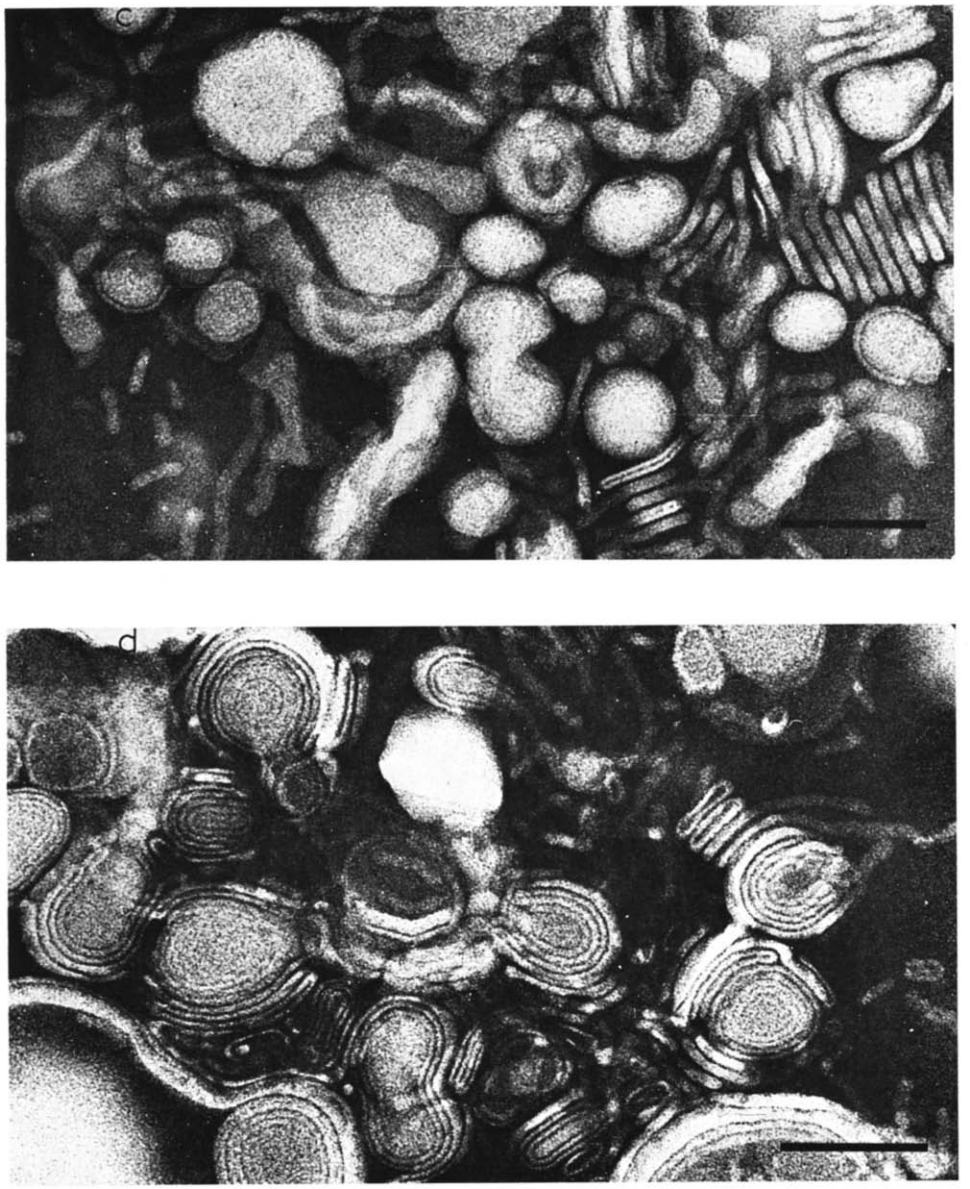


Fig. 1. Electron micrographs of 0.1 % phosphatidyl inositol dispersions in 0.02 M NaCl, negatively stained with ammonium molybdate (a and b) and phosphotungstic acid (c and d). Markers are 1000 Å units. a and b. Effect of sonication upon liposome structure. Sols were hand dispersed in a and ultrasonically irradiated for 60 min in b, at neutral pH. c and d. Effect on morphology of Sol b of lowering the pH to 3.25 (c) and 2.75 (d).

It is apparent that with decreasing electrostatic interaction comes a marked change in the morphology of the dispersion. The well-dispersed vesicular structure that can be formed under appropriate condition (Fig. 1b), is not maintained throughout the titration. The sequence illustrated in Figs. 1b–1d might be interpreted as follows:

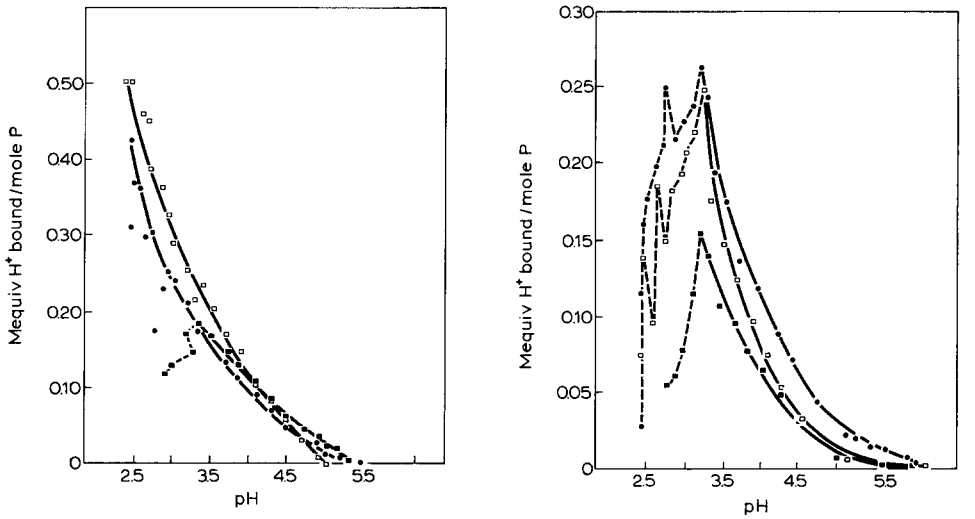


Fig. 2. Proton-association curves of phosphatidyl inositol dispersions in 0.02 M UaCl. Results of three experiments indicated by ■, □ and ●.

Fig. 3. Proton-association curves of phosphatidyl inositol dispersions in 0.04 M NaCl. Results of hree experiments indicated by ■, □ and ●.

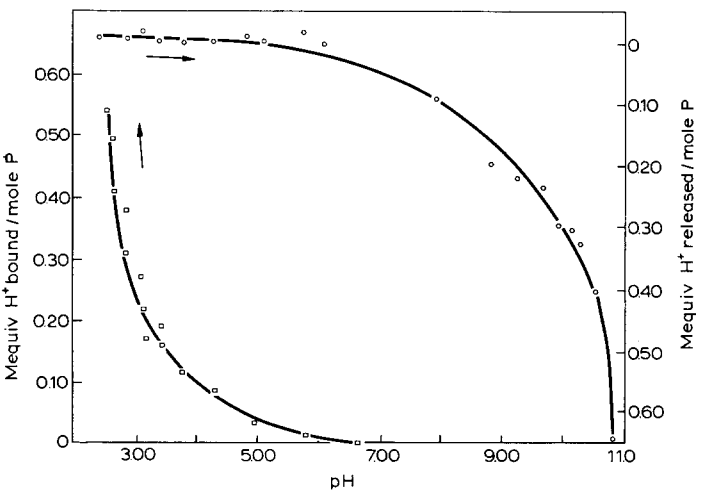


Fig. 4. Downscale and back titrati ons of phosphatidyl inositol dispersion in 0.02 M NaCl. Titration of sol from pH 6.6 to 2.5 by addit ion of 0.05 M HCl: back titration of same sol from pH 2.5 to 11 by addition of 0.05 M NaOH.

at pH 5.8 the fully negatively charged phosphatidyl inositol, as a result of electrostatic repulsions, is well-dispersed as vesicles. As charge is decreased by addition of protons, and electrostatic interactions eliminated, the spherules collapse into tubules or plates which associate to form tightly packed concentric spheres structurally analogous to onions. This striking change renders a large portion of the acidic groups inaccessible and must account for the anomalous association curves.

Irreversibility of the conformational change

A lipid sample, prepared in the usual way, was titrated to low pH (2.5). The same sample was then back-titrated with base to high pH (11). Fig. 4, the blank-corrected proton uptake and release curves of the same lipid sol in 0.02 M NaCl, illustrates the irreversible nature of the conformation change. This hysteresis effect shows the acidic groups buried within the onion-like aggregate are not accessible to titrant in the bulk medium until a considerable chemical potential difference has developed across the lamellar membranes.

Treatment of data

The interpretation of the proton binding curve of a polyelectrolyte should take into account the mutual interactions of binding sites and the effects of these interactions upon the proton affinity of the polyelectrolyte. This is done implicitly in the modification of the Henderson–Hasselbalch equation by KATCHALSKY AND SPITNIK[14],

$$\mathrm{pH} = \mathrm{p}K - m \log \frac{\bar{h}}{1 - \bar{h}} \tag{1}$$

The apparent dissociation constant K and the parameter m are phenomenological constants independent of $\bar{h}$, the mean molar concentration of protons bound per mole of sites, at a constant ionic strength and a particular configuration of binding sites[14]. K increases and m decreases with increasing ionic strength, and for polymeric acids $m > 1$.

The general theory of polyelectrolyte behaviour, developed by LINDERSTRØM-LANG[16] and TANFORD AND KIRKWOOD[17], modifies the apparent dissociation constant K by the introduction of K_{int}, an intrinsic dissociation constant which is the limiting value of K when no electrostatic interactions exist:

$$K = K_{\mathrm{int}} e^{-f(\bar{z})} \tag{2}$$

where $f(\bar{z})$ is a function of $\bar{z}$, the average charge per binding site, and is related to the free energy of the particular configuration of binding sites[18–20]. A convenient linear form of the Tanford–Kirkwood equation is,

$$\mathrm{pH} + \log \frac{\bar{h}}{1 - \bar{h}} = \mathrm{p}K_{\mathrm{int}} + 0.434\, f(\bar{z}) \tag{3}$$

The proton-binding curves were analyzed in the region where liposome morphology is maintained (between pH 7 and 3.5). It was assumed that the system before conformational change was reversible.

Henderson–Hasselbalch treatment of data

Fig. 5 shows a Henderson–Hasselbalch fit of the proton association data for lipid dispersions in 0.02 M NaCl. The two apparently smooth proton-association curves shown in Fig. 2 display, in this linear transform, the same discontinuity existing in the apparently anomalous curve in Fig. 2. These discontinuities are also present in the Tanford–Kirkwood linear transforms of the proton-association curves (see below). These results are typical of all ionic strengths. Table I summarizes the least-squares estimate of the Henderson–Hasselbalch parameters pK and m in the linear region.

The goodness of fit is the estimated uncertainty of the experimental points as a percentage. The pK and m values are dependent to some degree upon ionic strength. Note also that m values decrease with ionic strength. The exceptional case of 0.01 M can be attributed to the great change in ionic strength during the titration.

TABLE I

HENDERSON–HASSELBALCH PARAMETERS

A tabulation of the parameters pK, m, and S (the estimated uncertainty of the experimental points as a percentage), arising from fits of the proton association data for phosphatidyl inositol to the Henderson–Hasselbalch equation in the linear region for four ionic strengths (I) expressed as molarity.

I (M)	pK	m	S (%)
0.01	2.89 ± 0.07	1.47 ± 0.06	6.02
	2.54 ± 0.12	1.46 ± 0.09	7.98
	2.69 ± 0.11	1.53 ± 0.10	10.97
0.02	2.46 ± 0.07	1.77 ± 0.10	7.43
	2.21 ± 0.06	1.79 ± 0.06	7.87
	2.26 ± 0.12	1.89 ± 0.10	11.20
0.04	2.48 ± 0.08	1.42 ± 0.08	6.86
	1.84 ± 0.08	1.82 ± 0.08	5.31
	2.43 ± 0.03	1.70 ± 0.02	4.50
0.06	2.27 ± 0.05	1.35 ± 0.04	5.86
	2.04 ± 0.13	1.39 ± 0.11	9.13
	2.48 ± 0.07	1.15 ± 0.06	8.18

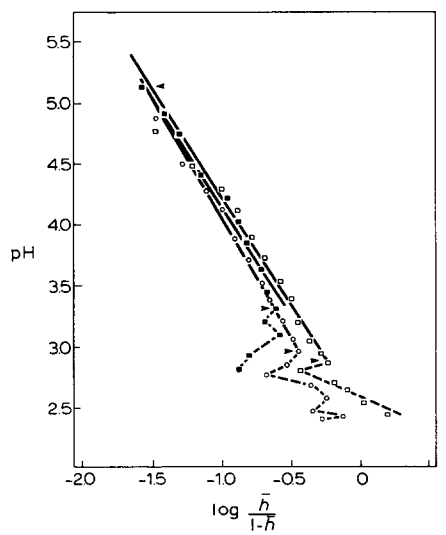


Fig. 5. Henderson–Hasselbalch plots of the data of Fig. 2. Points labelled □, ■ and ● correspond to the points labelled □, ■ and ●, respectively, in Fig. 2. Regions of linear fits are defined by the arrows. Dotted lines connect consecutive data points outside linear region.

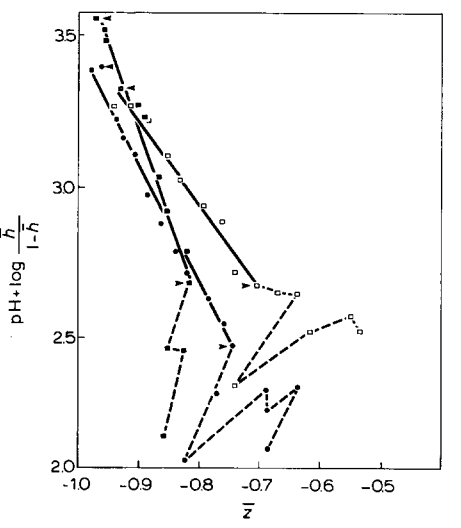


Fig. 6. Tanford–Kirkwood plots of the data of Fig. 2. Points labelled □, ■ and ● correspond to the points labelled □, ■ and ●, respectively in Fig. 2. Regions of linear fits are defined by the arrows. Dotted lines connect consecutive data points outside linear region.

Tanford–Kirkwood treatment of data

Fig. 6 is a representative Tanford–Kirkwood fit of the proton association data for lipid dispersions in 0.02 M NaCl. Charge per site in the totally unassociated state is given a pre-assumed value of —1. Thus the average charge per site at some particular degree of association can be determined by

$$\bar{z} = \bar{h} - 1 \tag{4}$$

By examinatlon of Eqn. 3 it can be seen that the numerical value of the quantity (pH + log $(\bar{h}/(1-\bar{h}))$ *minus* the value at $\bar{z} = 0$ is the numerical value of the function f, for some particular value of $\bar{z}$. Fig. 6 shows that in the region $-0.7 < \bar{z} < -1$, f is a linear function of site charge, in other words $f(\bar{z}) = w\bar{z}$. Since in the system ionic strength and temperature are constant, the changes in the form of f at the discontinuity are indicative of conformational changes.

TABLE II

TANFORD–KIRKWOOD PARAMETERS

A tabulation of the parameters of pK_{int}, w, and S (the estimated uncertainty of the experimental points as a percentage), arising from fits of the proton association data for phosphatidyl inositol to the Tanford–Kirkwood equation in the linear region for four ionic strengths (I) expressed as molarity.

I (M)	pK_{int}	w	S (%)
0.01	—0.487 ± 0.23	9.82 ± 0.59	5.18
	—1.28 ± 0.42	10.86 ± 1.04	4.09
	0.110 ± 0.15	8.08 ± 0.39	3.15
0.02	0.751 ± 0.14	6.32 ± 0.40	3.96
	—0.462 ± 0.13	9.03 ± 0.36	4.13
	—1.80 ± 0.28	12.77 ± 0.70	5.48
0.04	0.194 ± 0.22	6.89 ± 0.57	3.15
	—1.63 ± 0.53	10.99 ± 1.34	6.60
	—0.544 ± 0.22	9.44 ± 0.59	3.39
0.06	0.270 ± 0.26	6.08 ± 0.66	5.25
	—0.240 ± 0.64	6.82 ± 1.60	8.21
	1.42 ± 0.14	3.26 ± 0.36	2.39

Table II summarizes the least-squares estimates of the intercept (pK_{int}) and slope in the linear region. The great variability shown by the pK_{int} and w values for any particular ionic strength precludes comment upon the ionic strength dependence of these parameters. The pK_{int} values are remarkably low for a primary phosphate proton dissociation. Compare the mean pK_{int} of —0.422 from Table II with the first $pK = 2.12$ for *o*-phosphoric acid[21].

An explanation for this unusual phenomenon might lie in an understanding of ion binding to sites on a hydrocarbon surface and the surface contributions to the intrinsic free energy.

DISCUSSION

Since different site interactions arise from different configurations of sites, only binding data from a single reproducible configuration of sites are unambiguously interpretable. Appropriate conditions were found that led to the formation of phosphatidyl inositol dispersions that were nearly homogeneous in size and morphology. Prolonged (1 h) sonication of lipid samples in low ionic strength, nonacidic conditions produced small (250–350 Å diameter) discrete vesicles (as opposed to nested vesicles).

Under acidic conditions, single dispersed spherules aggregate to form concentric onion-like structures. Evidence for this conformation change came directly from electron microscopy and indirectly from the titration data. The titration data display a discontinuity in the pH region of conformational change. This discontinuity is obscured in some proton-association curves at lower ionic strength by the exponential nature of the curve but is apparent in linear transformations of the data.

The goodness of fit to the Henderson–Hasselbalch equation is in contrast to the Tanford–Kirkwood plots. This goodness of fit is a fortuitous one. A log–log plot of any monotonic function results in a reasonably straight line, and for this reason the Tanford–Kirkwood plot is the more valuable for the examination of titration data. The variation evident in the Tanford–Kirkwood plots of the data can be attributed to variation in liposome morphology produced by the vagaries of sonication, and to measurement problems introduced by potentiometric investigations of highly-charged small particles in suspension. KRUYT[22] and FEINSTEIN AND ROSANO[23] have outlined some of these problems.

If the sharp apparent decrease in $\bar{h}$ after conformational change can be attributed to the inaccessibility of aqueous compartments within the "onions", then it can be said that the liposome membrane is impermeable to protons after conformational change. This view is further supported by the hysteresis curve. The data give no information about the proton permeability of the vesicles at higher pH. At the onset of the conformational change, only about 25 % of the binding sites have been titrated, and we have no evidence as to the spatial location of these sites in the vesicles.

We conclude by suggesting that in the case of aqueous dispersions of phosphatidyl inositol the assumption that all ionizable groups are exposed is contrary to morphological evidence, and an assumption that all groups are titrable is contrary to evidence presented in the titration studies. It is possible that all groups are equivalent before conformational change (*i.e.* the membrane is freely permeable), but the data do not provide comment on this.

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