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CONTROL OF THE STRUCTURE AND FLUIDITY OF PHOSPHATIDYLGLYCEROL BILAYERS BY pH TITRATION

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Summary

Titration of the single dissociable proton in phosphatidylglycerol bilayers not only shifts the ordered-fluid phase transition but also changes the bilayer fluidity in the region above the phase transition, and gives rise to a quite different bilayer structure in the region below the phase transition:

1. The ordered-fluid phase transition temperatures of dimyristoyl and dipalmitoyl phosphatidylglycerol bilayers have been measured as a function of bulk pH in 0.1 M salt using water-lipid partitioning spin labels. From the dissociation curve obtained, it is found that the one titratable proton has an apparent pK_a of 2.9 for bilayers of both lipids.

2. In the fully ionized state, these phosphatidylglycerols not only give very similar transition temperatures (23°C for dimyristoyl and 40°C for dipalmitoyl chains) and pre-transition temperatures to the similar chain length phosphatidylcholines, but also show identical bilayer structures at corresponding temperatures when examined by freeze-fracture electron microscopy: i.e. defects, ripples and jumbled patterns, in the ordered, pre-transitional and fluid phases, respectively.

3. When the phosphatidylglycerols are fully protonated, the bilayer pre-transition, as monitored by the spin labels, is absent and the main transition is somewhat broader and increased in temperature by approx. 17°C. Exclusively smooth bilayers are observed by electron microscopy at all temperatures. This suggests that, contrary to the situation in the charged bilayers, the phosphatidylglycerol molecules are not tilted relative to the bilayer normal in the ordered phase.

4. In fluid bilayers, above the main transition temperature, the fluidity is found to be greater when the phosphatidylglycerol molecules are charged than when uncharged, due to the increased intermolecular separation caused by electrostatic repulsion.

Abbreviations: TEMPO, 2,2,6,6,-tetramethyl-piperidine-*N*-oxyl; C_{11} spin label, 2,2-di[1-pentyl]-4,4'-[dimethyl]-3-oxazolidinyloxyl.

The results demonstrate that the structure and fluidity of charged lipid bilayer membranes can be changed isothermally without the mediation of the ordered-fluid phase transition.

Introduction

Possible mechanisms by which charged phospholipids can interact with membrane proteins and the manner in which they are involved in membrane fusion [1,2] and permeability processes [3,4] are topics of considerable interest. Aqueous lipid bilayers provide a simple model system with which the lipid chain motion and net charge on the head-group can be studied by changing the bulk pH and salt concentration [6—9]. Furthermore, by selecting a lipid class of known hydrocarbon chain composition, direct comparisons with other lipids of similar chains but differing head-groups can be drawn, thereby selectively investigating the effects of just one part of the molecule on the behaviour of the whole bilayer.

For this study, we have selected dimyristoyl and dipalmitoyl phosphatidylglycerols to investigate the ordered-fluid phase transition characteristics, bilayer fluidity and bilayer structure as a function of the bulk pH in an approximately physiological salt concentration of 0.1 M. The results show that the one dissociable proton on the phospholipid head-group can be removed between pH 1.5 and pH 10.0 with an apparent pK_a of 2.9. Using water-lipid partitioning spin labels, the ordered-fluid phase transition temperatures are seen to decrease by about 17°C upon removing this one proton from the lipid head-group, which is in good agreement with previous work on other negatively-charged lipid bilayers [5,9].

In addition to the triggering of the main transition of phosphatidylglycerols by pH, the same spin labels show that the fluidity of the bilayer can be increased isothermally by increasing the pH, whilst the bilayers remain solely within the fluid phase. The electrostatic interactions of the head-group therefore appear to be important in controlling the molecular separation [10,11] and, consequently, the hydrocarbon chain motion in the lipid bilayer.

The charged phosphatidylglycerols give very similar main phase transition characteristics and structures in the electron microscope to the corresponding chain length phosphatidylcholines [12]. From the electron microscopic evidence and the absence of a pre-transition in the uncharged bilayers, it is suggested that at low pH the lipid molecules are not tilted below the main transition, but at higher pH they are tilted below the pre-transition. If a pre-transition does occur in the uncharged bilayers, it does not involve a large free volume change nor the formation of distinct structural features in the bilayer, as seen in the charged bilayer or in phosphatidylcholines.

Such similarities between charged phosphatidylglycerols and phosphatidylcholines may account, at least in part, for the fact that the major phospholipid of certain organisms, *Staphlococus aureas* [13] and *Acholeplasma laidlawii* [14] (which grow at pH greater than 2.9) is phosphatidylglycerol, whereas the most abundant phospholipid in mammalian systems is phosphatidylcholine.

Materials and Methods

Spin labels

The spin-label TEMPO was prepared according to the method of Rozantzev and Neiman [15]. The C_{11} spin label was prepared by Dr. W. Kühnle of this Institute.

Phosphatidylglycerols

The sodium salts of dimyristoyl and dipalmitoyl phosphatidylglycerols were synthesised from their corresponding phosphatidylcholines (Fluka, Buchs.) by the action of phospholipase D (Boehringer-Mannheim, G.F.R.) in the presence of excess glycerol [3,16]. The phosphatidylglycerols were then chromatographed on a pre-equilibrated basic silicic acid column [3]. The pure product co-chromatographed on silicic acid thin layer chromatography plates with phosphatidylglycerol (produced from egg phosphatidylcholine and purchased from Lipid Products, Surrey, U.K.) using a solvent system of $CHCl_3$/CH_3OH/25% NH_4OH (65 : 15 : 1, v/v) and developed with Dittmer spray [17] followed by charring. Typical yields were of the order of 60—80% of the original phosphatidylcholine. The sodium salt of the lipid was crystallized from acetone (20× excess) from a $CHCl_3/CH_3OH$ (2 : 1, v/v) solution and dried under vacuum (approx. 10^{-1} Torr) at room temperature (for approx. 12 h). The dry material was stored at —20°C until required.

The optical rotation was measured to be $[\alpha]_D^{24} = 6.3°$ in $CHCl_3/CH_3OH$ (2 : 1, v/v) and the structure of the phosphatidylglycerols was confirmed by 270 MHz ^1H NMR in $CDCl_3/CD_3OD$ (2 : 1, v/v) [34].

Lipid dispersions

Dry lipid was dispersed at the required concentration by gentle agitation with a spatula in buffer above the ordered-fluid phase transition temperature (T_t). Constant ionic strength buffers ($J = 0.1$ M) with low temperature coefficients [18,19] were used for all the experiments employing the C_{11} spin label. For these experiments, the lipid concentration was 1.4 mM and the pH was measured to within ±0.02 units after the dispersion had been made. For the TEMPO experiments, the lipid concentration was 66 mM and the pH was measured to within ±0.25 units. These dispersions were prepared in 0.15 M KCl/HCl at pH 1.5 and 0.15 M KCl/0.05 M Tris at pH 8.0, both buffers having an ionic strength of 0.1 M [20].

The spin labels were added to the lipid dispersions from stock solutions of 10^{-2} M and 10^{-3} M in water to final concentrations of 10^{-4} M and 10^{-5} M for TEMPO and the C_{11} spin label, respectively.

Spin label measurements

ESR measurements were made with a Varian E-12 9 GHz spectrometer equipped with a nitrogen gas flow, temperature regulation system. Samples were contained in sealed-off 100-μl capillary tubes accomodated within standard 4-mm quartz ESR tubes containing silicon oil for temperature stability. Temperatures were measured to within ±0.15°C with a thermocouple placed just above the cavity within the quartz ESR tube. Bilayer transition

curves were recorded continuously by monitoring the height of the minimum of the high-field aqueous line as the temperature was swept. The magnetic field was locked to the second derivative of the high-field aqueous peak using a Varian Field-Frequency Lock Unit. The microwave detector bias current was maintained constant by an automatic coupling-iris adjustment.

The ESR cavity was mounted horizontally and all lipid dispersions were homogenous throughout the capillary tube, which contained enough sample to protrude from the cavity. These precautions minimize any effects of settling or any other concentration variability in the section of the sample being measured.

Freeze-fracture electron microscopy

Lipid dispersions to be used for freeze-fracture electron microscopy were prepared as for the TEMPO experiments, except for the addition of the spin label. Droplets [1—2 μl] of the lipid dispersion were pipetted onto copper planchets and equilibrated at the desired temperature for approx. 2 min. The samples were quenched by plunging the planchets into liquid Freon 22 (du Pont). Fracturing was carried out on a Balzers freeze-etch device (type BA 360) at —110°C with no etching. The replicas, which were floated off in methanol/H_2O and cleaned with chloroform/methanol, were examined on a Siemens 101 electron microscope operating at 80 kV with instrumental magnification of 20 000.

Results

Transition temperature measurements

TEMPO and the C_{11} spin label are soluble in water and in fluid-phase phospholipid bilayers but much less so in solid, ordered-phase bilayers [21]. When in the aqueous phase, the spin-label gives rise to a narrow, three-line spectrum characteristic of a nitroxide undergoing rapid isotropic motion [22]. A similar, but broader spectrum arises from these spin labels when dissolved in fluid lipid. In a situation where the spin label can exist in both environments in equilibrium, for example in an aqueous, fluid lipid dispersion, the two separate spectra can be resolved from each other in the high-field line region [21].

Ordered-fluid phase transition temperatures of the phosphatidylglycerol dispersions were measured by continuously recording the decrease in the aqueous spin label as it partitioned into the fluid bilayers. This method gives a measure of the concentration of spin-label partitioning into the fluid bilayer, since the line-width of the spectrum from the aqueous spin label changes relatively little with temperature. (Measurements of the concentration of partitioned spin label are a function of line-height and line-width, and the latter is seen to vary with temperature.)

The transition temperatures measured for dimyristoyl and dipalmitoyl phosphatidylglycerol dispersions as a function of pH in 0.1 M salt solution are given in Fig. 1. These results were obtained using the C_{11} spin label which was found to give slightly broader (approx. 1—2°C) transition widths than TEMPO. Also, this spin label showed more hysteresis in the main transitions than in comparable TEMPO experiments. The results in Fig. 1 clearly show that it is

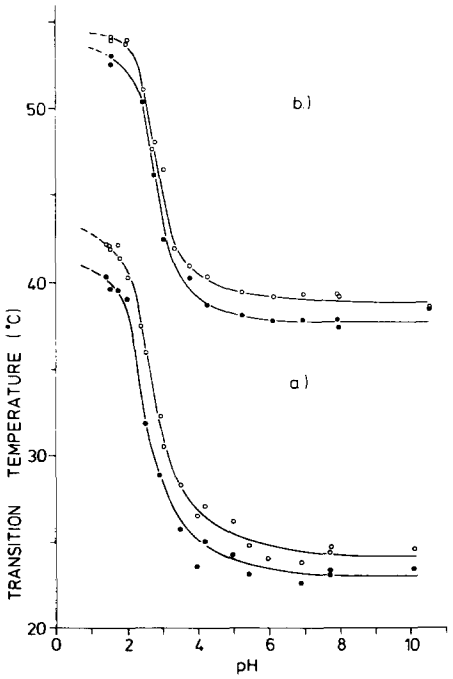

Fig. 1. The main transition temperatures of (a) dimyristoyl and (b) dipalmitoyl phosphatidylglycerol bilayers (1.4 mM) in 0.1 M KCl, as a function of pH, measured using the C_{11} spin label (10^{-5} M). Open circles, T_t on heating; full circles, T_t on cooling.

possible to titrate the proton of the phosphate group of the phosphatidylglycerols to give an effective pK_a of 2.9. It was found that in measuring the transition temperatures of the lipid bilayers at pH <1.7, some spin label reduction occurred. Also, after experiments at low pH (pH <1.7) and high temperature ($T > T_t$) with dipalmitoyl phosphatidylglycerol the lipid was found to have been decomposed to give approx. 2—4% lysophosphatidylglycerol and fatty acid chains as estimated by thin layer chromatography.

Actual transition temperature curves recorded by the continuous scanning method for dimyristoyl and dipalmitoyl phosphatidylglycerol bilayers in the fully protonated and fully ionized forms are given in Fig. 2. It can be seen that a very pronounced pre-transition is observed in the negatively-charged state with both lipids. These pre-transitions are similar to those observed calorimetrically for phosphatidylcholines [23,24], and for dipalmitoyl phosphatidylglycerol [7,25]. On vigorously vortex mixing a phosphatidylglycerol dispersion above its main transition temperature, it was seen to become much less cloudy. Such dispersions were found to give broad, assymetric main transitions with no distinct pre-transition. This is presumably due to the formation of small vesicles whose size limits the degree of cooperativity of the transition [26]. Broadening of the main transition is observed as the pH of the dispersion is lowered into the titration region (pH 4.0—2.0) with both spin labels, until at pH 2.0 the uncharged lipid shows no pre-transition, as detected in these experiments. Such an effect is clearly not due to the formation of small vesicles as the electron micrographs show (Fig. 4). Also given in Fig. 2 are the temperatures of the main and pre-transitions for dimyristoyl and dipalmitoyl phosphatidylglycerols.

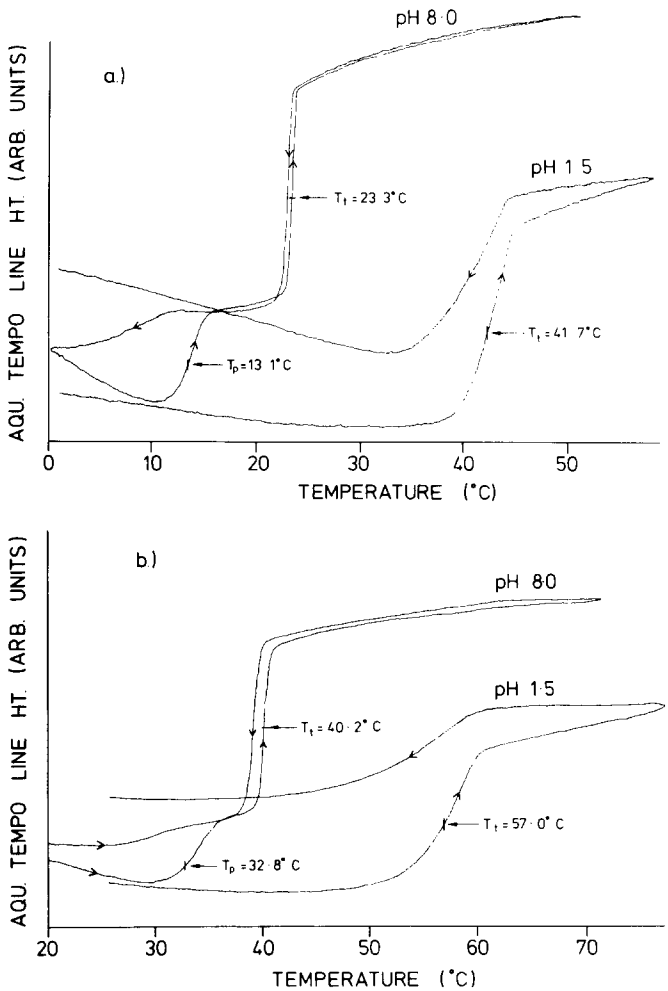

Fig. 2. Transition temperature curves for charged and uncharged (a) dimyristoyl and (b) dipalmitoyl phosphatidylglycerol bilayers (66 mM), in 0.1 M KCl, measured as described in the text using the TEMPO spin label (10^{-4} M).

As in Fig. 1, these are the temperatures at which the degree of transition is 0.5, as previously defined [26].

Measurements of bilayer fluidity

Fig. 2 gives only an arbitrary measure of the decrease in the amount of TEMPO in the aqueous phase, although the lipid concentration and instrumental settings were the same for each experiment. However, it is clear that more TEMPO partitions into the fluid, charged phosphatidylglycerol bilayers than into the fluid bilayers at pH 1.5 at any one temperature. In Table I are listed the values of the water-lipid partition coefficient, f, for TEMPO [21] measured at specific temperatures. Clearly at any one temperature above both T_t values, at which the bilayers at both pH values are fluid, the spectral parameter, f, measured for the uncharged bilayers is always lower

TABLE I

VALUES OF THE TEMPO PARTITION COEFFICIENT f AT GIVEN TEMPERATURES FOR DIMYRISTOYL AND DIPALMITOYL PHOSPHATIDYLGLYCEROLS AT pH 1.5 AND 8.0 IN 0.1 M KCl

See text for details.

	Temperature (°C)			
	10	20	30	45
Dimyristoyl phosphatidylglycerol				
pH 1.5	0.016	0.018	0.041	0.310
pH 8.0	0.016	0.080	0.346	0.431
	25	35	45	60
Dipalmitoyl phosphatidylglycerol				
pH 1.5	0.041	0.041	0.043	0.391
pH 8.0	0.041	0.108	0.484	0.532

than that measured for the charged bilayers at pH 8.0. The fluidity is, therefore, higher in the charged fluid bilayers than in the uncharged fluid bilayers. A similar effect is also seen in the pre-transition region of the charged bilayers. At any temperature below T_t for bilayers at pH 1.5, the values of f are very much lower than for bilayers at pH 8.0 above T_p. These measurements of f at the lower pH are, in fact, almost identical to those for TEMPO in water alone.

Electron microscopy

The structures of fully ionized dimyristoyl and dipalmitoyl phosphatidylglycerol bilayers appear similar to those already reported for phosphatidylcholines of the same chain length [12]. Fig. 3a shows a freeze-fracture electron micrograph of a dimyristoyl phosphatidylglycerol dispersion at pH 8.0 quenched from above the main transition temperature, and displays the jumbled structures characteristic of fluid phosphatidylcholine bilayers.

When quenched from between T_p and T_t, these bilayers at pH 8.0 appear rippled or banded. Large areas of very regular ripples are observed, as shown in Fig. 3b, interspersed with areas of not so regularly organised ripples as shown in Fig. 3c. Since between the more pronounced irregular bands in Fig. 3c very regular bands are seen, it is possible that the features of Fig. 3c are an intermediate stage in forming regular ripples. Such ripples have been previously observed with dimyristoyl phosphatidylglycerol bilayers at pH 7.4 [2].

The regular ripples of dimyristoyl phosphatidylglycerol in Fig. 3b have a periodicity of approximately 215 ± 20 Å, and for dipalmitoyl phosphatidylglycerol the periodicity was measured to be 295 ± 20 Å. The values are somewhat larger than those reported for the corresponding phosphatidylcholines [2].

There are certain general features of the regular banded areas that are observed in Fig. 3b. The ripples occasionally change direction, often through an angle of 60°, without causing any disruptions in the bilayer. Also, the banded pattern is seen to extend perpendicularly throughout stacked bilayers, although

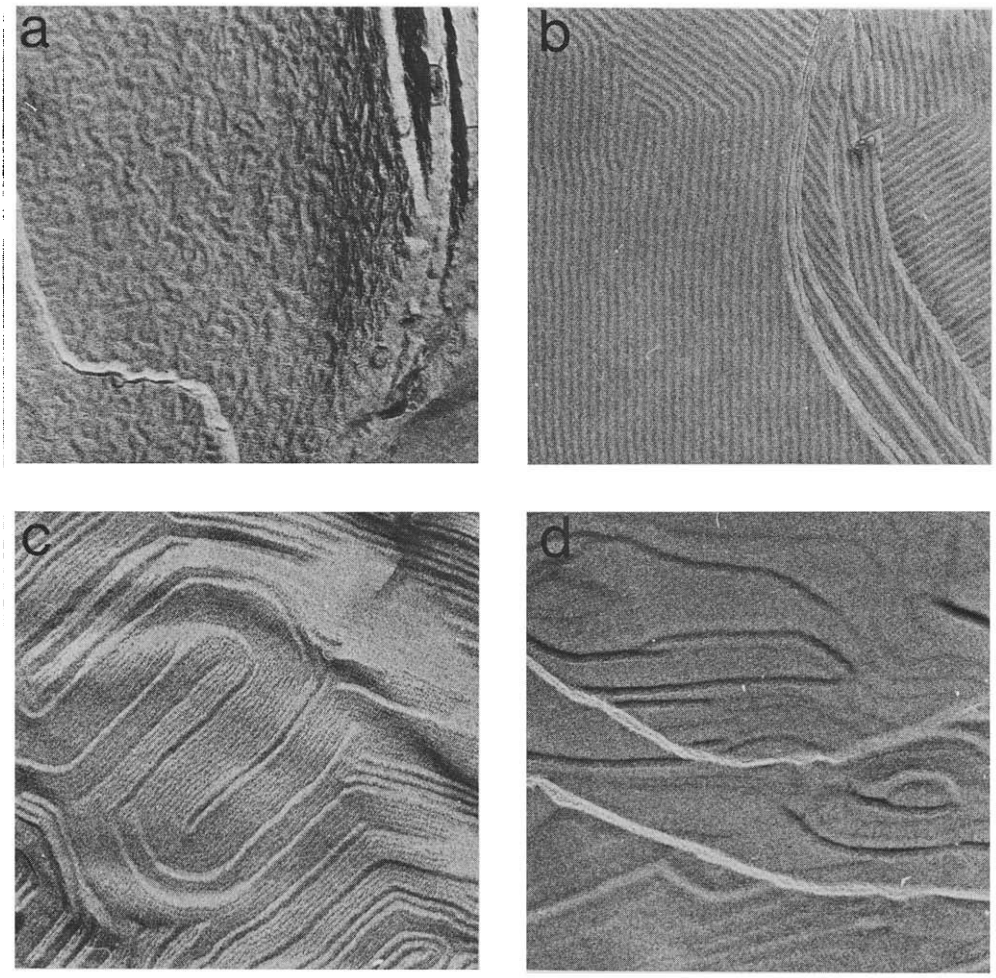

Fig. 3. Freeze-fracture electron micrographs of dimyristoyl phosphatidylglycerol bilayers at pH 8.0 in 0.1 M KCl quenched from (a) 30 ± 1°C, (b) 20 ± 1°C, (c) 20 ± 1°C, and (d) 10 ± 1°C. Magnification is approx. 66 000X.

not necessarily retaining its direction from one stack to the next.

Fig. 3d shows dimyristoyl phosphatidylglycerol bilayers at pH 8.0 quenched from below the pre-transition temperature. These gel phase bilayers show no rippled or jumbled structures but large smooth areas bounded by very irregularly spaced ridges.

Bilayers of fully protonated dimyristoyl and dipalmitoyl phosphatidylglycerols show no similar structures when quenched from above or below the main transition temperature and then examined by freeze-fracture electron microscopy. Smooth fracture faces were observed at all temperatures. Fig. 4, a and b, show dimyristoyl phosphatidylglycerol bilayers quenched from 30 and 45°C. No defects or ripples are observed in Fig. 4a and no jumbled structures

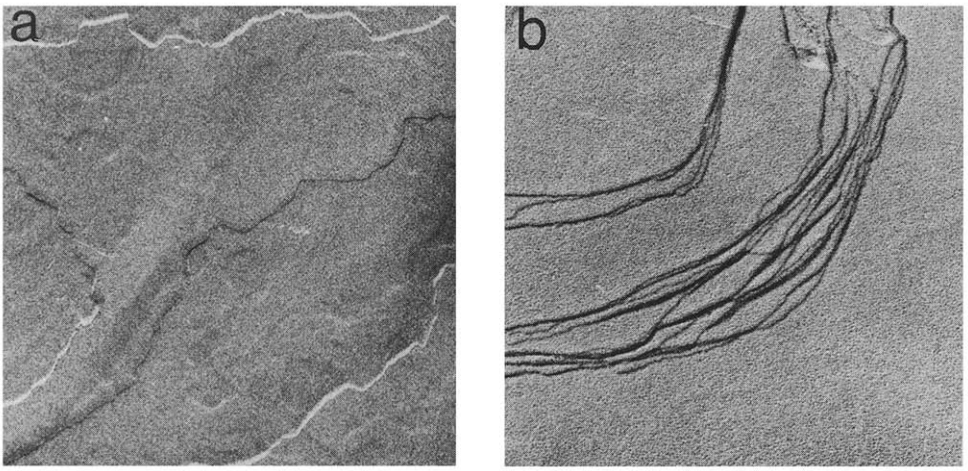

Fig. 4. Freeze-fracture electron micrographs of dimyristoyl phosphatidylglycerol bilayers at pH 1.5 in 0.1 M KCl quenched from (a) 30 ± 1°C and (b) 45 ± 1°C. Magnification is approx. 66 000×.

are seen in Fig. 4b, although the preparative procedure was the same for freeze-fracture samples at pH 1.5 and 8.0.

Discussion

Various properties of aqueous dispersions of phosphatidylglycerol bilayers have already been reported by other workers [2—4,6,7,11,25,27]. The present study, however, represents the first systematic investigation of the titration behaviour and of the temperature dependence of the freeze-fracture electron microscopic features of phosphatidylglycerol bilayers. Several extremely interesting and important results emerge from this latter approach.

The data of Fig. 1 clearly show that the ordered-fluid phase transition temperatures of phosphatidylglycerol bilayers titrate with the dissociable proton of the phosphate group, as has been observed previously in other negatively charged bilayer systems [5,8,9]. The apparent pK_a of the phosphate is 2.9 and yields a value of $pK_a^{(o)} = 1.0$ for the intrinsic pK_a of the phosphatidylglycerol molecule, after correcting for surface charge effects according to the equation: $pK_a - pK_a^{(o)} = 0.86 - \log_{10} n_m$, where $n_m = 0.1$ is the ionic strength of the assumed 1 : 1 electrolyte [9]. A somewhat similar titration behaviour has been reported for monolayers of dilauryl and dipalmitoyl phosphatidylglycerol at constant surface pressure [35].

The difference in transition temperature between the uncharged and charged bilayers is seen from Fig. 1 to be: $\Delta T_t^{max} = -17.8°C$ and $-15.0°C$ for dimyristoyl and dipalmitoyl phosphatidylglycerol, respectively. These values are of the same order of magnitude as those found for dimyristoyl phosphatidic acid [5], dimyristoyl methylphosphatidic acid [9] and dipalmitoyl phosphatidylserine [8]. Träuble et al. [9] have interpreted this shift in transition temperature as the difference in the change of electrostatic surface energy which occurs as the bilayer expands laterally at the phase transition. The shift

is then given by: $\Delta T_t^{max} = -2$ kT. $(N_{av}/\Delta S^*)$. $\Delta f/f$, where ΔS^* is the entropy change at the phase transition in the uncharged state, f is the mean area per lipid molecule and Δf is the change in f at the transition [9]. Using values † of $\Delta S^* = 18$ cal · mol^{-1} K^{-1}, $T = 305$ K and $\Delta S^* = 23$ cal · mol^{-1} K^{-1}, $T = 320$ K for dimyristoyl and dipalmitoyl phosphatidylglycerol, respectively, the measured changes in transition temperature can be accounted for by essentially the same value of $\Delta f/f$: 0.266 for dimyristoyl and 0.270 for diplamitoyl phosphatidylglycerol. This value agrees well with values of $\Delta f/f$ of between 0.22 and 0.30 measured for dipalmitoyl phosphatidylcholine using X-ray diffraction [28] or spin labels [29]. However, the range of uncertainty in $\Delta f/f$ is too great, particularly with regard to the uncharged bilayers, to be able to definitively attribute the full transition temperature shift to electrostatic effects. In particular, it seems likely that changes in the pattern of intermolecular hydrogen bonding may also contribute to the titration behaviour of the transition temperature. Differences in van der Waals interaction as a result of different molecular spacings and tilt (see below) in the charged and uncharged states will probably also make a sizeable contribution. A shift in transition temperature of about 15°C between pH 3 and 7 has previously been reported for dilauryl phosphatidylglycerol bilayers [6], in qualitative agreement with the present titration results. The reported lack of shift in transition temperature of dimyristoyl phosphatidylglycerol bilayers [4] presumably arises because the low pH point of the measurement had not yet entered the titration region.

A more novel aspect of the present work is the observation that changes in membrane fluidity can be brought about by changing the ionization state of the lipid without triggering the phase transition. The data in Table I for dimyristoyl phosphatidylglycerol at 45°C and dipalmitoyl phosphatidylglycerol at 60°C demonstrate that at a given temperature, the spin-label partitioning is greater into charged than into uncharged bilayers even though both are above their respective transition temperatures. This type of fluidity regulation by changing pH may be of direct biological significance since it does not involve the potentially disruptive effects which may attend the triggering of a lipid phase transition, and indeed does not require that the lipids have a well defined phase transition.

Differences in partitioning between the two charge states below the phase transition are also indicated in Table I. At 20°C for dimyristoyl phosphatidylglycerol and at 35°C for dipalmitoyl phosphatidylglycerol the partitioning into the charged bilayers is greater than that into the uncharged bilayers at the same concentration. This effect is clearly associated with the well defined pretransition in the charged state (see Fig. 2) and the absence of such effects in the uncharged state. The greater partitioning (i.e., greater free volume) in the pretransitional region presumably also implies a greater degree of mobility in the charged state than in the uncharged state exists also in the ordered bilayer phase.

† The value of ΔS^* for dipalmitoyl phosphatidylglycerol is obtained from the transition enthalpy, ΔH, of dipalmitoyl phosphatidylglycerol in the charged state [7] assuming that ΔH does not change appreciably on titration, as is found for the analogue phosphate diester, dimyristoyl methylphosphatidic acid [30]. The value of ΔS^* for dimyristoyl phosphatidylglycerol is obtained from the same data, making the further assumption that the decrease in ΔH with chain length is the same as for the corresponding phosphatidylcholines [23].

The effects below the transition are seen more clearly from the electron microscopy. In the charged state, the freeze-fracture micrographs reveal three distinct types of bilayer surface pattern corresponding to the regions below the pre-transition, between the pre-transition and the main transition, and above the main transition, respectively (Fig. 3, a, b and d). This is exactly the same as the situation reported recently for phosphatidylcholines [12]. The pre-transitional rippled structure (cf. Fig. 3b) in phosphatidylcholines has been associated with a two-dimensional monoclinic lattice by X-ray diffraction [31] and presumably the charged phosphatidylglycerols have the same structure in the pretransition region. The same diffraction study [31] also demonstrated that the molecules were tilted relative to the bilayer normal in phosphatidylcholine bilayers below the pretransition. Gebhardt et al. [32] have pointed out that, since not all the molecules will be inclined in the same direction in the tilted structure, one would expect to observe crystalline defects in the bilayer structure. Such dislocations are seen in phosphatidylcholine bilayers [12,32] and the defect structures observed in Fig. 3d are presumably of similar origin, suggesting that charged phosphatidylglycerol bilayers below the pre-transition also have the same tilted molecular configuration. By similar topological arguments it is seen that the occurrence of smooth fracture faces in Fig. 4 implies that the molecules are not tilted relative to the bilayer normal in uncharged phosphatidylglycerol bilayers. Thus, changing pH is capable of quite radically altering the bilayer structure below the phase transition.

In summary (see Fig. 5), changes in the pH of the aqueous phase are capable of both altering the fluidity of phosphatidylglycerol bilayers above the phase transition and of altering the bilayer structure below the phase transition, without mediation of the transition itself. These effects could be of considerable importance for the regulation of structure and function in biological membranes since they can be manifest in a charged lipid system which shows no phase transition and do not give rise to the very abrupt disruptive effects characteristic of first order phase transitions.

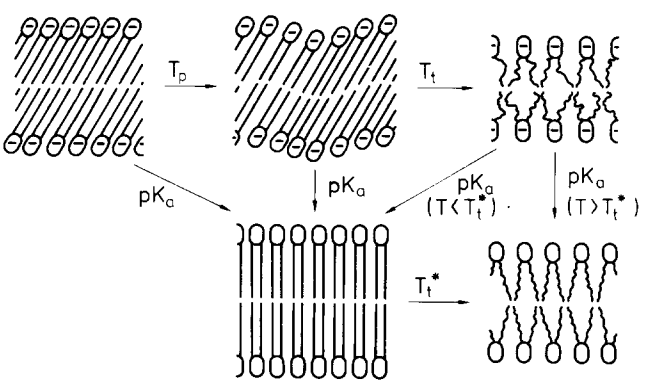

Fig. 5. Schematic summary of the major temperature- and pH-induced changes taking place in phosphatidylglycerol bilayers. The tentative structures in the ordered phases: tilted, non-tilted, rippled or smooth, are based on electron microscopy. (Gebhardt et al. [32] have suggested an alternative non-tilted, rippled phase). All phase transitions, titration behaviour and differential fluidity effects are based on spin label partitioning.

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