Biochimica et Biophysica Acta, 513 (1978) 11—20

BBA 78159

POLYMORPHIC PHASE BEHAVIOUR OF CARDIOLIPIN AS DETECTED BY ^{31}P NMR AND FREEZE-FRACTURE TECHNIQUES

EFFECTS OF CALCIUM, DIBUCAINE AND CHLORPROMAZINE

P.R. CULLIS [a,*] A.J. VERKLEIJ [b] and P.H.J.Th. VERVERGAERT [c]

[a] *Biochemistry Department,* [b] *Institute of Molecular Biology and* [c] *Department of Molecular Biology, Section Electron Microscopy, State University of Utrecht, Transitorium 3, Padualaan 8, Utrecht (The Netherlands)*

(Received March 1st, 1978)

Summary

1. The influence of Ca^{2+} on the polymorphic phase behaviour of cardiolipin has been investigated employing ^{31}P NMR and freeze-fracture techniques. The close correlation between the results obtained here and previous X-ray studies (Rand, R.P. and Sengupta, S. (1972) Biochim. Biophys. Acta 255, 484—492) confirms ^{31}P NMR as a useful analytical procedure for investigating the polymorphic phase behaviour of hydrated phospholipids.

2. Ca^{2+} induces formation of the hexagonal (H_{11}) phase via an intermediary phase which is observed at Ca^{2+}/cardiolipin ratios of less than 1 (mol/mol). This intermediary appears to consist of 'inverted' structure which lies adjacent to regions of bilayer structure.

3. The local anaesthetics dibucaine and chlorpromazine produce similar phase changes for cardiolipin as does Ca^{2+}. It is suggested that the anaesthetics interact with the membrane in their charged form and induce their effects by charge neutralization.

Introduction

In previous publications it has been shown that ^{31}P NMR is sensitive to the local motion and orientation in the phosphate group region of membrane phospholipids [1] as well as to the macroscopic polymorphic phase (bilayer, hexagonal (H_{11}) or cubic, rhombic and inverted micellar) assumed [2—5]. In an X-ray study [6] it has been shown that cardiolipin, in the presence of excess

* Present address: Biochemistry Department, University of British Columbia, Vancouver V6T 1W5, Canada.

water, assumes the bilayer phase in the absence of divalent cations, whereas in the presence of equimolar Ca^{2+} the hexagonal (H_{II}) phase is observed. In this study we have re-investigated the phase behaviour of cardiolipin employing ^{31}P NMR techniques with three objectives in mind: (1) To demonstrate the correspondence between ^{31}P NMR, freeze fracture and X-ray determinations of phase behaviour in a lipid system which has been well characterized by X-ray techniques; (2) To investigate details of the local motion and conformation in the phosphate group regions of cardiolipin, which has a unique polar headgroup structure which limits the conformation and local motion available; (3) To investigate the effects of local anaesthetics, which may be expected to decrease the charge density at the lipid-water interface, on the polymorphic phase behaviour of cardiolipin.

Materials and Methods

Cardiolipin (ex bovine heart) was obtained from Sigma, St. Louis, U.S.A. This lipid was more than 99% pure as indicated by thin-layer chromatography and was used without further purification. Dibucaine hydrochloride (Cinchocaine) was obtained from Ciba whereas chlorpromazine (Largactyl) was obtained from Specia, Paris. Liposomes were prepared from 50 mg cardiolipin dissolved in chloroform, and the chloroform was evaporated under nitrogen and subsequently stored overnight under vacuum. Subsequently, the lipid was dispersed in 0.7 ml ^2H_2O (50 mM Tris-acetic acid (p^2H 7.2)/100 mM NaCl). 2 mM EDTA was present when local anaesthetics were added. Titrations of Ca^{2+}, dibucaine and chlorpromazine were carried out by adding aliquots from 100 mM stock solutions of these agents. In the case of dibucaine and chlorpromazine the pH of the stock solutions was raised to 6.0 prior to experimentation by addition of 0.1 M NaOH. (At higher pH values the drugs tended to precipitate). In no case did the p^2H of the lipid-anaesthetic dispersion fall below 6.0. In order to check whether the effects observed could arise from divalent cation contaminants in the anaesthetic, or from the small changes in pH, a control experiment employing a 100 mM dibucaine solution containing 100 mM EDTA and 100 mM Tris (pH 7.0) was employed in one experimental sequence. The (precipitated) drug dispersion was vortexed immediately before taking aliquots for addition to the sample. Equivalent changes in the polymorphic phase behaviour of cardiolipin were observed as for the situation when the stock solution at pH 6 was employed.

^{31}P NMR measurements were carried out at 36.4 MHz employing a Bruker WH-90 spectrometer equipped with temperature control and proton decoupling facilities. All spectra were obtained in the presence of high power (20 W) broad band proton decoupling. Accumulated free induction decays were obtained from up to 10 000 transients, employing a 0.2 s interpulse time and a 45° radio frequency pulse.

Freeze-fracture micrographs were obtained as described elsewhere [7]. Samples were quenched from 0°C. It should be noted that the freeze-fracture results were very reproducible for cardiolipin, and did not appear to be sensitive to the quench temperature or rate. This is in marked contrast to effects observed for unsaturated phosphatidylethanolamines (Verkleij, A.J. and Cullis,

P.R., unpublished) where preparations in the hexagonal (H_{II}) phase at the quench temperature often show bilayer structure in the micrographs. These effects may be attributed to the tendency of such lipids to assume the bilayer phase at temperatures below their hydrocarbon phase transition temperature which are often close to the quench temperature (see ref. 4). Beef heart cardiolipin, on the other hand, is very unsaturated [8] and would therefore be expected to have a much lower transition temperature, which would mitigate against such artifacts.

Results

A representative 36.4 MHz ^{31}P NMR spectrum of cardiolipin liposomes at 30°C is illustrated in Fig. 1a. The broad, asymmetric lineshape observed with a low field shoulder is similar to the ^{31}P NMR spectra observed for liquid crystalline phosphatidylcholines [2,9—12] and phosphatidylethanolamines [2,3,13] in a bilayer configuration. This is with the exception that a small narrow component is observed which is characteristic of a small population (less than 10%) of lipids in an environment where they may experience effectively isotropic

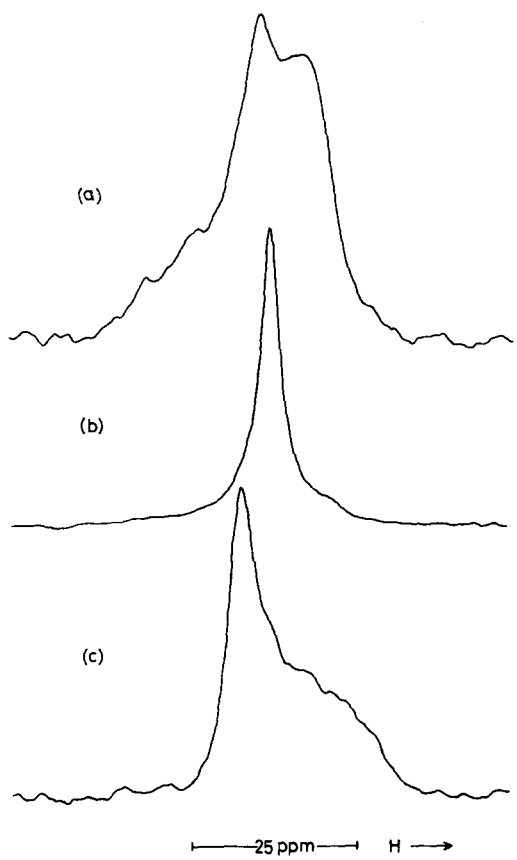

Fig. 1. 36.4 MHz ^{31}P NMR spectra at 30°C of aqueous dispersions of cardiolipin in the presence of varying amounts of Ca^{2+}. The Ca^{2+}/cardiolipin ratios (mol/mol) are: a, 0; b, 0.6; and c, 1.0.

motional averaging, and that the chemical shift anisotropy $\Delta\sigma_{CSA}^{EFF}$ (the chemical shift between the high field peak and the low field shoulder) is approx. −30 ppm, which is slightly smaller than that observed for other liquid crystalline phospholipids where $\Delta\sigma_{CSA}^{EFF} = -30$ to −40 ppm [2,9—15]. As shown in Fig. 2a freeze-fracture electron micrographs of the sample of Fig. 1a also reveal structure characteristic of multibilayer liposomal structures. Thus the ^{31}P NMR and freeze fracture results are fully consistent with X-ray studies [6] of cardiolipin in the absence of Ca^{2+} which also show bilayer structure.

The addition of Ca^{2+} to produce a Ca^{2+}/cardiolipin ratio of 0.6 (mol/mol) is observed (Fig. 1b) to convert the bulk (more than 60%) of the phospholipid to a phase which is characterized by the possibility of isotropic motional averaging. It should be noted that the presence of Ca^{2+} in these concentrations did not produce appreciable precipitation, and the dispersion maintained the milky appearance of normal liposomes. As shown in Fig. 2b freeze fracture results obtained from samples with comparable Ca^{2+} content indicate the presence of regions of a lipid phase which have an irregular texture and lie immediately adjacent to regions of bilayer phase.

The addition of equimolar or excess quantities of Ca^{2+} to the cardiolipin dispersions caused complete precipitation of the phospholipid, inducing formation of macroscopic structures which had the visual appearance of small polystyrene beads. As shown in Fig. 1c the asymmetric ^{31}P NMR spectra obtained from such systems have a high field shoulder and reversed asymmetry as compared to the spectra obtained from bilayer phospholipids. Such ^{31}P NMR spectra have been previously identified with the hexagonal (H_{II}) phase [2—5]. Freeze fracture results (Fig. 2c) obtained from these systems have the characteristic striated appearance previously noted [16—18] for phospholipids in the hexagonal (H_{II}) phase. It should be noted that the freeze-fracture identifications of hexagonal (H_{II}) phase regions rely on observation of the striated pattern in all planes of the fracture face. Observation of such a pattern in only one plane could arise from a cross-section of (condensed) stacked bilayers. In summary, the ^{31}P NMR and freeze-fracture results obtained from cardiolipin in the presence of equimolar or higher Ca^{2+} concentrations are in full agreement with the X-ray studies [6] of similar systems, which also show formation of the hexagonal (H_{II}) phase.

Addition of EDTA to cardiolipin in the presence of equimolar Ca^{2+} caused the sample to become almost translucent, similar to preparations of sonicated vesicles. Freeze fracture studies indicated the presence of small (500—5000 Å diameter) unilamellar structures, which was entirely consistent with the ^{31}P NMR spectra obtained, which showed predominantly bilayer structure with a narrow component superimposed. This narrow component arises from the smaller structures where isotropic motional averaging occurs due to tumbling and/or lateral diffusion processes [19].

The value of $\Delta\sigma_{CSA}^{EFF}$ may be estimated from the ^{31}P NMR spectra obtained from cardiolipin in the hexagonal (H_{II}) phase, as the separation between the high field shoulder and the main peak ($\Delta\sigma_{CSA}^{EFF'}$) is related to $\Delta\sigma_{CSA}^{EFF}$ according to $\Delta\sigma_{CSA}^{EFF} = -2\Delta\sigma_{CSA}^{EFF'}$ [3]. From the spectrum of Fig. 1c we obtain $\Delta\sigma_{CSA}^{EEF'} \approx 20$ ppm, and thus $\Delta\sigma_{CSA}^{EFF} \approx -40$ ppm. This of course assumes that binding of Ca^{2+} does not affect the local motion and conformation in the phosphate region, or

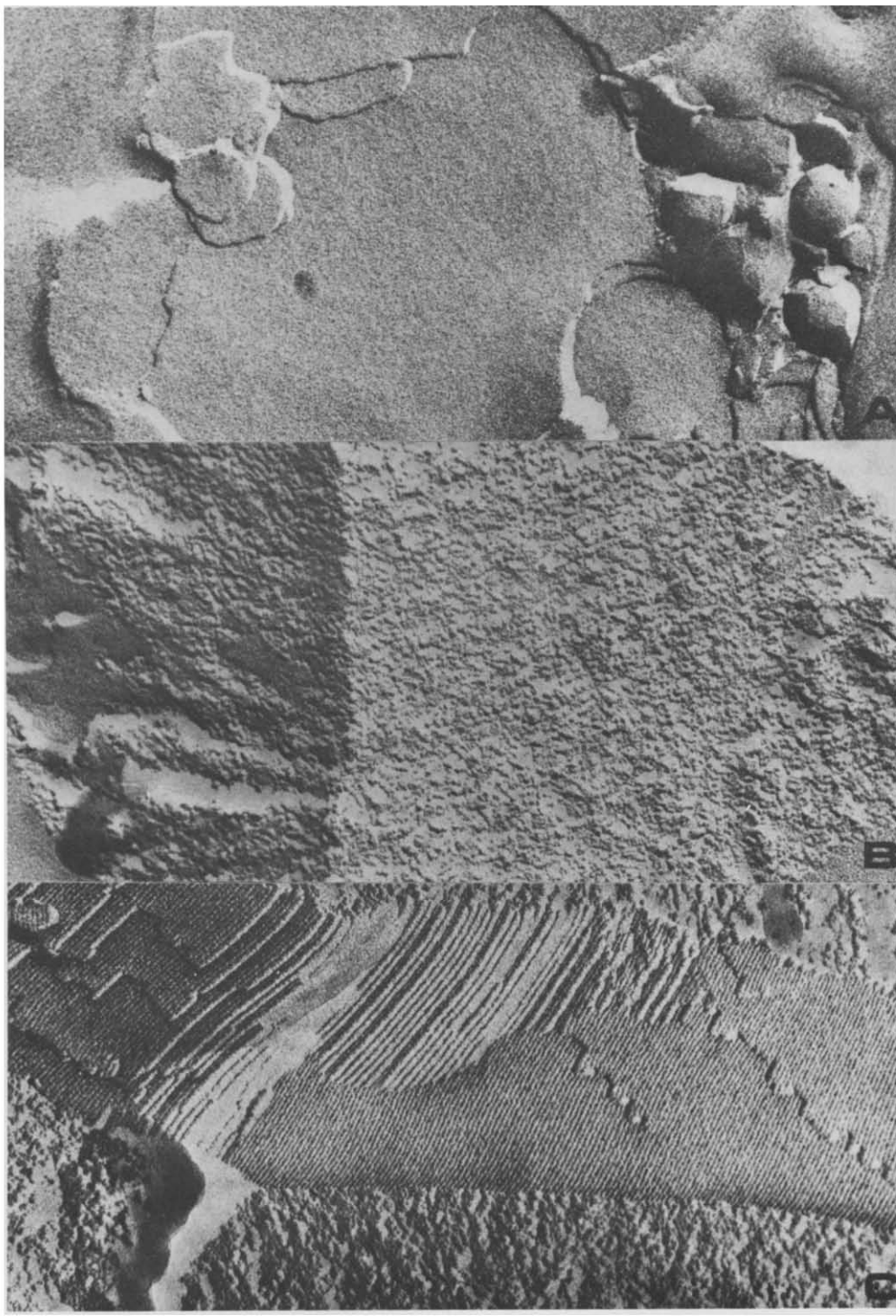

Fig. 2. Freeze-fracture electron micrographs of cardiolipin in the presence of varying amounts of Ca^{2+}. The Ca^{2+}/cardiolipin ratios are (mol/mol): a, 0; b, 0.6; and c, 1.0. Magnification 100 000×. Samples were quenched from 0°C.

the rigid lattice value of the chemical shift anisotropy tensor. Such an assumption would appear justified in view of the observation of ^{31}P NMR spectra for 12 : 0/12 : 0 phosphatidylglycerol in the presence of equimolar Ca^{2+} (at 70°C) which have the normal 'bilayer' shape and values of $\Delta\sigma_{CSA}^{EFF}$ in the range −30 to −35 ppm [2]. The value of $\Delta\sigma_{CSA}^{EFF}$ thus obtained for cardiolipin (−40 ppm) is somewhat larger than that obtained from the liposome spectra (−30 ppm, Fig. 1a) and is possibly more reliable, as the liposome spectra indicate the presence of a small amount of non-bilayer phase with which bilayer phospholipid may experience rapid exchange [4]. This would reduce the value of $\Delta\sigma_{CSA}^{EFF}$ observed.

The influence of increasing amounts of the local anaesthetics dibucaine and chlorpromazine on the polymorphic phase assumed by cardiolipin is shown in Figs. 3 and 4, respectively. Behaviour which is very similar to that observed when Ca^{2+} is added is obtained, with the exception that approximately twice the molar ratios of anaesthetic to cardiolipin are required to produce equivalent effects. The precipitate formed for anaesthetic/cardiolipin ratios of 2 (mol/mol) was also visually very similar to the precipitate formed in the presence of equimolar Ca^{2+}. Freeze fracture electron micrographs obtained from cardiolipin

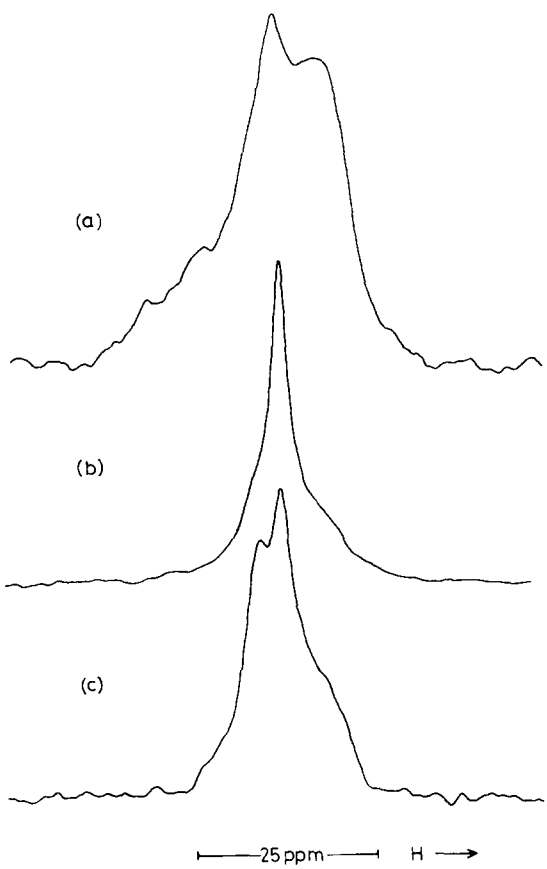

Fig. 3. 36.4 MHz ^{31}P NMR spectra at 30°C of aqueous dispersions of cardiolipin in the presence of varying amounts of dibucaine. The dibucaine/cardiolipin ratios (mol/mol) are: a, 0; b, 0.9; and c, 2.

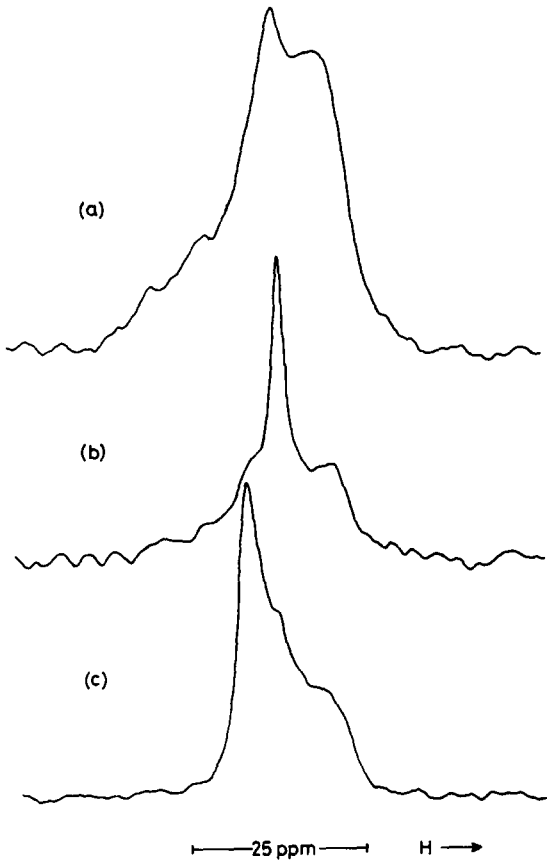

Fig. 4. 36.4 MHz ^{31}P NMR spectra at 30°C of aqueous dispersions of cardiolipin in the presence of varying amounts of chlorpromazine. The chlorpromazine/cardiolipin ratios employed are: a, 0; b, 0.9; and c, 2.0.

dispersions containing sufficient dibucaine to induce the phase that gives rise to the narrow ^{31}P NMR signal showed the presence of short regions of hexagonal (H_{11}) phase, as shown in Fig. 5, which would be expected to allow isotropic motional averaging.

As noted in Fig. 6, the appearance of non-bilayer phases in cardiolipin induced by Ca^{2+} bears an approximately stoichiometric relation to the amount of Ca^{2+} added, indicating that formation of the non-bilayer configurations arises due to charge neutralization. Similary, the appearance of non-bilayer phases induced by the anaesthetics is also approximately stoichiometric assuming a single positive change for each anaesthetic molecule. This suggests that it is the positively charged form of the anaesthetic which is associated with the lipid and which produces the observed phase changes again primarily by charge neutralization.

A final point concerns the value of $\Delta\sigma_{CSA}^{EFF'}$, which may be observed to be approx. 18 ppm in the presence of anaesthetic sufficient to induce the hexago-

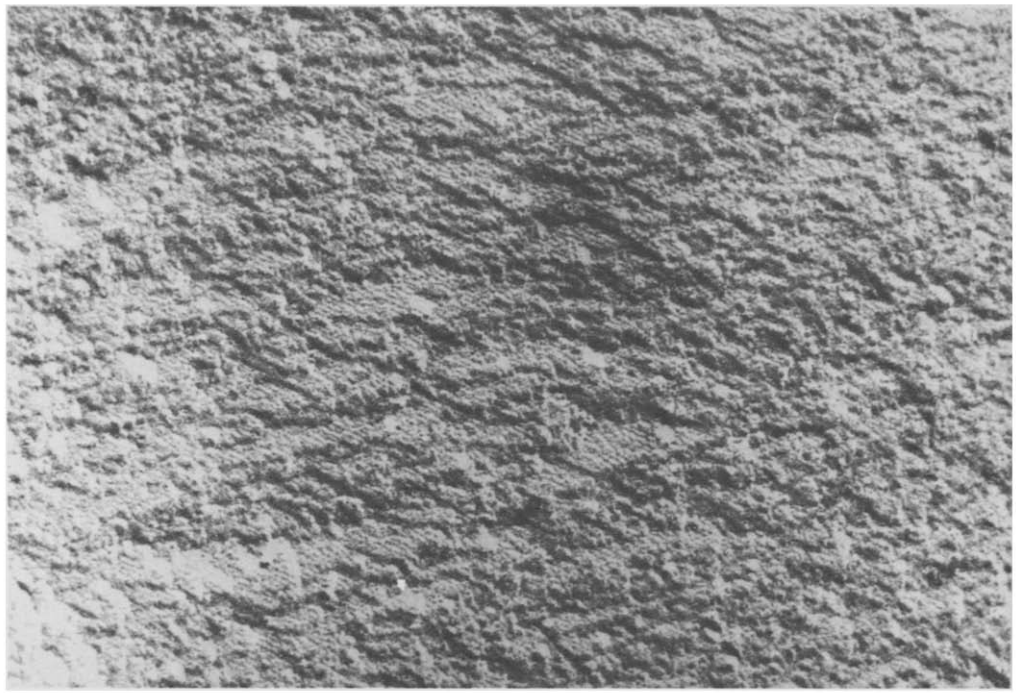

Fig. 5. Freeze-fracture electron micrograph of cardiolipin in the presence of dibucaine (dibucaine/cardiolipin ratio = 1.5 mol/mol). Magnification 100 000×. Sample was quenched from 0°C.

nal (H_{11}) phase (Figs. 3c and 4c). This indicates that $\Delta\sigma_{CSA}^{EFF} \approx -36$ ppm and thus strongly implies that the local motion and conformation in the phosphate regions of cardiolipin is not significantly affected by the presence of anaesthetic.

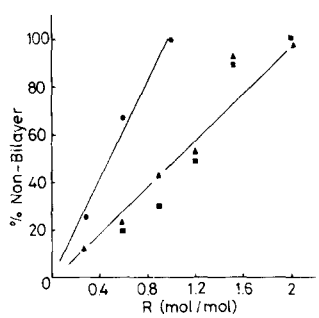

Fig. 6. Percentage of cardiolipin in non-bilayer phases at 30°C as a function of: ●, Ca^{2+} content; ▲, dibucaine content and ■, chlorpromazine content. Estimated by cutting and weighing the various components of the ^{31}P NMR spectra.

Discussion

The close correlation between the polymorphic phase behaviour of cardiolipin as detected by ^{31}P NMR with the freeze fracture results and previous X-ray studies further confirms the ^{31}P NMR technique [3] as a useful diagnostic tool to investigate the polymorphic phase behaviour of phospholipids. New information concerning the mechanism whereby Ca^{2+} induces the hexagonal (H_{II}) phase in cardiolipin is also gained. The observation of an intermediate phase characterized by isotropic averaging on the NMR time scale (10^{-5} s) corresponds to observation by freeze fracture techniques of regions of irregular topography on the fracture face and/or regions of short hexagonal (H_{II}) phase. Such structure is presumably the precursor to the long inverted cylinders of the hexagonal (H_{II}) phase formed at higher Ca^{2+} contents. Perhaps the most interesting aspect of this intermediary phase is that the structures formed are in close juxtaposition to regions of bilayer phase. This suggests that the bilayer phase and the inverted phase are in local equilibrium, depending on the Ca^{2+} concentration. Such possibilities are obviously relevant to recent proposals that local regions of 'inverted' non-bilayer lipid structures may occur in biological membranes acting as intermediaries in membrane fusion phenomena [20]. It may be argued that observations for the cardiolipin Ca^{2+} system are not representative of the behaviour of lipids in biological membranes, in that cardiolipin is usually only present in small amounts. However, bilayer to hexagonal (H_{II}) phase transitions can also be triggered by Ca^{2+} in phosphatidylethanolamine/phosphatidylserine mixtures [21] and thus the observations for the cardiolipin system may well be of more general significance, particularly to inter-membrane interactions such as occur during fusion processes.

A further interesting aspect of the results presented here concerns the unique polar headgroup structure of cardiolipin (see Fig. 7) which severely limits the possible conformations and local motions in the regions of the phosphate groups. In particular, the 'bridge' segment between the two phosphate groups must be oriented parallel to the plane of the membrane, and the local motion would be expected to be restricted to coupled torsional oscillations about the bond axes. Thus the fact that values of $\Delta\sigma_{CSA}^{EFF}$ are observed for cardiolipin which are in the same range as those reported for other phospholipids (phosphatidylcholines and phosphatidylethanolamines [9—15]) whose motion

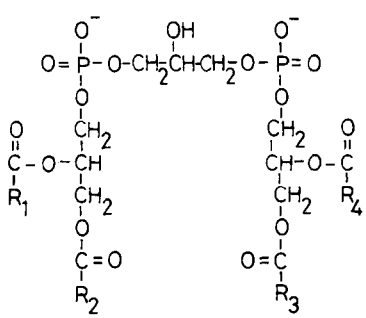

Fig. 7. Structure of cardiolipin in the headgroup region.

in the phosphate region is less obviously restricted, has two implications. First, values of $\Delta\sigma_{CSA}^{EFF}$ in the range of −30 to −40 ppm are fully consistent with an orientation of the polar headgroup parallel to the plane of the membrane (as has been shown recently for phosphatidylcholine [22]) and secondly, such values are consistent with local motion in the phosphate region consisting primarily of coupled, restricted torsional oscillations about the bond axes. This latter point is consistent with the results of recent ^2H NMR studies [23].

Finally the results presented here clearly show that local anaesthetics dibucaine and chlorpromazine may strongly influence the polymorphic phase assumed by negatively charged lipids, and suggest in the case of cardiolipin that the anaesthetics interact with the negatively charged membrane in their charged form, inducing their effects by neutralizing the electrostatic repulsion between the lipids. Although it is too early to speculate on the possible biological significance of such anaesthetic induced changes in lipid structure, the results obtained here offer new possibilities for mechanisms of anaesthetic action. This is in addition to previous models which largely involve changes in membrane fluidity in the presence of anaesthetics [24—26].

Acknowledgements

P.R.C. would like to thank the European Molecular Biology Organization for financial support (1977). We would also like to thank Dr. B. de Kruijff for helpful discussions.

References

1 Cullis, P.R. and McLaughlin, A.C. (1977) Trends Biochem. Sci. 2, 196—199
2 Cullis, P.R. and de Kruijff, B. (1976) Biochim. Biophys. Acta 436, 523—540
3 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207—218
4 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 513, 31—42
5 Cullis, P.R., van Dijck, P.W.M., de Kruijff, B. and de Gier, J. (1978) Biochim. Biophys. Acta 513, 21—30
6 Rand, R.P. and Sengupta, S. (1972) Biochim. Biophys. Acta 255, 484—492
7 Ververgaert, P.H.J.Th., Verkleij, A.J., Elbers, P.F. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 311, 320—329
8 Gulik-Krzywicki, T., Rivas, E. and Luzzati, V. (1967) J. Mol. Biol. 27, 303—322
9 Cullis, P.R., de Kruijff, B. and Richards, R.E. (1976) Biochim. Biophys. Acta 426, 433—446
10 McLaughlin, A.C., Cullis, P.R., Berden, J.A. and Richards, R.E. (1975) J. Magn. Res. 20, 146—166
11 Gally, H.U., Niederberger, W. and Seelig, J. (1975) Biochemistry 14, 3647—3652
12 Griffin, R.G. (1976) J. Am. Chem. Soc. 98, 851—853
13 Seelig, J. and Gally, H.U. (1976) Biochemistry 15, 5199—5204
14 McLaughlin, A.C., Cullis, P.R., Hemminga, M.A., Hoult, D.I., Radda, G.K., Ritchie, G.A., Seeley, P.J. and Richards, R.E. (1975) FEBS Lett. 57, 213—218
15 Cullis, P.R., de Kruijff, B. and Richards, R.E. (1976) Biochim. Biophys. Acta 426, 433—446
16 van Dijck, P.W.M., de Kruijff, B., van Deenen, L.L.M., de Gier, J. and Demel, R.A. (1976) Biochim. Biophys. Acta 455, 576—587
17 Papahadjopoulos, D., Vail, W.J., Pangborn, W.A. and Poste, G. (1976) Biochim. Biophys. Acta 448, 265—283
18 Deamer, D.W., Leonard, R., Tardieu, A. and Branton, D. (1970) Biochim. Biophys. Acta 219, 47—60
19 Cullis, P.R. (1976) FEBS Lett. 70, 223—228
20 Cullis, P.R. and Hope, M.J. (1978) Nature 271, 672—674
21 Cullis, P.R. and Verkleij, A.J. (1978) Biochim. Biophys. Acta, submitted
22 Büldt, G., Gally, H.U., Seelig, A., Seelig, J. and Zaccai, G. (1978) Nature, in press
23 Seelig, J., Gally, H.U. and Wohlgemuth, R. (1977) Biochim. Biophys. Acta 476, 109—119
24 Seeman, P. (1972) Pharmacol. Rev. 24, 583—655
25 Lee, A.G. (1976) Nature 262, 545—548
26 Papahadjopoulos, D., Jacobson, K., Poste, G. and Shepherd, F. (1975) Biochim. Biophys. Acta 394, 504—519