pH-Dissociation Characteristics of Cardiolipin and Its 2′-Deoxy Analogue

Morris Kates[a,*], **Jing-Yi Syz**[b], **David Gosser**[b] and **Thomas H. Haines**[b]

[a]Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5 and [b]Department of Chemistry, City College of the City University of New York, New York, New York 10031

Cardiolipin (CL) is found in inner mitochondrial membranes and the plasma membrane of aerobic prokaryotes. CL is tightly bound to those transmembrane enzymes associated with oxidative phosphorylation. CL has earlier been reported to have a single pK at low pH. We have titrated CL in aqueous suspension (bilayers) and in solution in methanol/water (1:1, vol/vol) and found it to display two different pK values, pK_1 at 2.8 and pK_2 initially at 7.5 but shifting upwards to 9.5 as the titration proceeds. The unusually high pK_2 might be explained by the formation of a unique hydrogen bond in which the free hydroxyl on the central glycerol forms a cyclic intramolecular hydrogen-bonded structure with one protonated phosphate (P-OH group). We have therefore chemically synthesized the 2′-deoxycardiolipin analogue, which lacks the central free hydroxyl group, and measured its pH-dissociation behavior by potentiometric titration, under the same conditions as those for CL. The absence of the hydroxyl group changes the titration dramatically so that the deoxy analogue displays two closely spaced low pK values (pK_1 = 1.8; pK_2 = 4.0). The anomalous titration behavior of the second dissociation constant of CL may be attributed to the participation of the central glycerol OH group in stabilizing the formation of a cyclic hydrogen-bonded monoprotonated form of CL, which may function as a reservoir of protons at relatively high pH. This function may have an important bearing on proton pumping in biological membranes.
Lipids 28, 877–882 (1993).

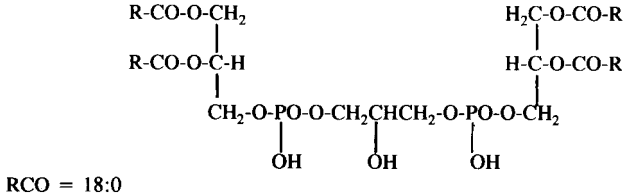
Almost a century ago, Wasserman *et al.* (1) reported the finding of "specific" antibodies in the sera of syphilitic patients by using the antigen in alcohol extracts from the spleen of a syphilitic fetus. Within a few years, Browning *et al.* (2) established that the antigen could be extracted from a variety of tissues, and Noguchi (3) discovered that the ether-soluble antigen was precipitable by acetone, a characteristic of phospholipids. The latter observation provoked Pangborn (4) to isolate and purify the antigen from beef heart by means of the Wasserman antibody assay. She named the isolated antigen "cardiolipin" (CL) and subsequently chemically characterized it as an acylated polyglycerolphosphate complex (5). CL was soon found to be present not only in mammals but in bacteria and plants (6–8).

Although CL was one of the first membrane lipids to be isolated in pure form, its structure proof and synthesis were not achieved until the 1960s. Le Cocq and Ballou (9) established its structure and configuration as di-*O*-(*sn*-3-phosphatidyl)-*sn*-1′,3′-glycerol (Fig. 1). The total synthesis of CL was soon achieved by de Haas *et al.* (10).

Few *et al.* (11), in a classic paper in which they measured pK values of surface charges of suspensions of bacterial cells and cell lipid extracts by microelectrophoresis, determined the pK_a of a sample of CL obtained from Pangborn. The pK_a of CL they reported was 1.05. They also found that the lipids of *Micrococcus lysodeikticus* displayed a pK_a of 0.4. A curious feature of the titration curves obtained by Few *et al.* (11) was that unlike those of the *M. lysodeikticus* cells and the lipids, the CL titration continued to rise after the observed endpoint.

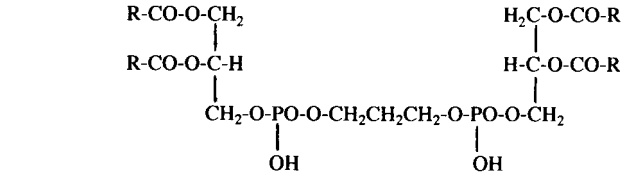
Shortly thereafter, Coulon-Morelec *et al.* (12) also reported a single pK below pH 4.0 for CL based on titration in ethanol solution using indicator dyes. In a more recent study, Seddon *et al.* (13) reported a pK of 2.8 based on the pH dependence of phase changes. In all three measurements, it was assumed that there is only one pK for the two phosphate protons, presumably because the molecule is symmetrical. This view is widely held in the literature (14–16).

CL has a variety of unique physical and chemical properties. Unlike most phospholipids, it readily forms a hexagonal II(H_{II}) phase (17), which is easily characterized by ^{31}P nuclear magnetic resonance (NMR) (18). This



RCO = 18:0

Hydrogenated cardiolipin (CL)



RCO = 16:0

2′-Deoxycardiolipin (dCL)

FIG. 1. Chemical structures of CL and dCL.

*To whom correspondence should be addressed.

Abbreviations: BH, beef heart; CL, cardiolipin (diphosphatidylglycerol); CL-BH, cardiolipin from beef heart; 18:0-CL (H_2-CL-BH), hydrogenated beef heart cardiolipin; H_2-CL-EC, hydrogenated cardiolipin from *E. coli; cy*-17:0 and *cy*-19:0, 9,10-methylenehexadecanoic and octadecanoic acid, respectively; DAG, diacylglycerols; dCL, 2′-deoxycardiolipin (diphosphatidyl-1,3-propanediol); 16:0-dCL, synthetic deoxycardiolipin with palmitoyl (16:0) as acyl groups; DPG, dipalmitoylglycerol; DPPA, dipalmitoyl phosphatidic acid; FAB-MS, fast-atom bombardment mass spectrometry; FTIR, Fourier transform infrared spectroscopy; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PPD, phosphatidylpropanediol; PPOP, phosphatidylpropanediol-phosphate; TLC, thin-layer chromatography.

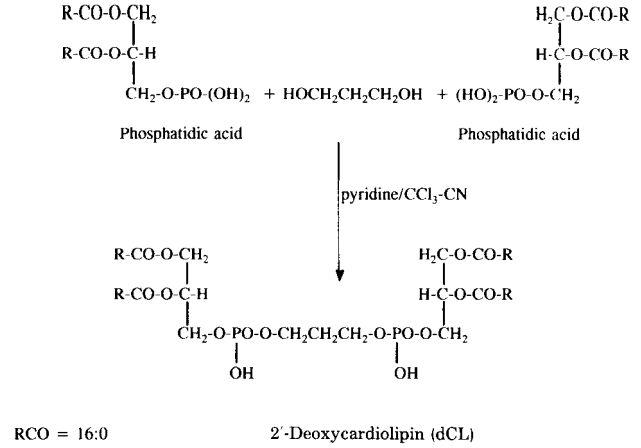


FIG. 2. Scheme for the synthesis of 2′-deoxycardiolipin.

characteristic is promoted by a fatty acid chain composition rich in polyunsaturated fatty acids, as is found in mitochondrial CL. A lesser known and more poorly understood chemical quality is the low degree of accessibility of the free hydroxyl group to acetylation in pyridine using vigorous conditions (19,20). In this connection, recent Fourier transform infrared (FTIR) studies of CL have shown (21) that the central free hydroxyl group can form intramolecular hydrogen bonds with the phosphate groups, which might explain the difficulty in acetylating the C-OH group.

In mitochondria, CL is located largely on the matrix side of the inner membrane (for a review, see Ref. 22). CL is known to bind tightly to inner mitochondrial membrane-bound enzymes, such as cytochrome oxidase (23), the F_0F_1ATPase (24), and the ADP/ATP exchange protein (25), and to play an essential role in the optimal functioning of cytochrome c oxidase (26) and F_0F_1-ATPase (27,28). Nonetheless, the specific role of this lipid still requires clarification.

Knowledge of the pH-dissociation characteristics of CL may be of interest in connection with its biological structure-function relationships, particularly that of a proton reservoir in energy transducing membranes. We therefore examined the pH potentiometric titration of CL and found that, instead of titrating as a simple dibasic acid, it displayed two widely separated pK values, one at 2.8 and a second, anomalous pK above physiological pH. In order to explain the existence of the second pK, we synthesized 2′-deoxycardiolipin (dCL) (Figs. 1 and 2). This permitted us to examine the role of the central free hydroxyl group of the connecting glycerol in the anomalous CL titration. The dCL titrated as a typical dibasic acid with two closely spaced pK values below pH 4. Comparison of the titration behavior of dCL and CL suggests that the free hydroxyl of the connecting glycerol in CL participates in stabilizing the monoprotonated form of the CL molecule, thus enabling it to function as a proton reservoir at high pH (see Discussion section).

MATERIALS AND METHODS

Chemicals. All solvents were glass distilled prior to use, except for pyridine and triethylamine, which were dried over fresh potassium hydroxide pellets and used without distillation. Dipalmitoyl phosphatidic acid (DPPA, disodium salt) and *Escherichia coli* CL [Na salt; fatty acid analysis by gas-liquid chromatography (GLC): 16:0, 43.0%; *cy*-17:0, 24.2%; *cy*-19:0, 20.6%; 14:0, 1.4%; 16:1, 1.6%; 18:0, 1.6%; 18:1, 5.2%] were purchased from Sigma Chemical Co. (St. Louis, MO); beef heart (BH) CL (Na salt) was purchased from Sigma (fatty acid analysis by GLC: 18:1, 6.7%; 18:2, 92.2%; 16:0, 0.5%; 16:1, 0.4%; 18:0, 0.2%) or from Avanti (Pelham, AL). Adams' catalyst ($PtO_2 \cdot xH_2O$, Gold label), 1,3-dibromopropane and trichloroacetonitrile were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Hydrogenation of CL. CL-BH (*ca.* 25 mg) or *E. coli* CL (*ca.* 13 mg) were hydrogenated in methanol (10 or 5 mL, respectively) in the presence of 170 or 100 mg, respectively, of Adams' catalyst in an all-glass hydrogenator by stirring in an atmosphere of hydrogen gas at room temperature (22–24°C) until uptake of hydrogen ceased (*ca.* 2–3 h). The mixture was centrifuged, and the platinum catalyst washed twice with 1 mL of chloroform/methanol (1:1, vol/vol). The combined supernatants were diluted with the appropriate amounts of chloroform and 0.2N HCl (final ratio, chloroform/methanol/water, 1:2:0.8, by vol) to form a biphasic system (29); the chloroform layer was removed, neutralized by addition of 1 N ammonium hydroxide in methanol, diluted with benzene and concentrated to a small volume under a stream of nitrogen. The ammonium salt of hydrogenated CL was then precipitated by the addition of 10 vol of acetone and cooling at −10°C; it was collected by centrifugation, reprecipitated from chloroform by addition of acetone, washed with cold acetone and finally dried *in vacuo;* the yield of hydrogenated CL was 80–85%; thin-layer chromatography (TLC) R_f, 0.48 in solvent A (R_f of unhydrogenated CL, 0.48). Fatty acid analysis by GLC: BH hydrogenated CL (H_2-CL-BH): 14:0, 1.0%; 16:0, 9.3%; 16:1, 2.5%; 18:0, 76.6%; 18:1(*t*), 9.4%. *E. coli* hydrogenated CL (H_2-CL-EC): 14:0, 2.8%; 16:0, 50.5%; *cy*-17:0, 19.1%; 18:0, 8.9%; *cy*-19:0, 12.9%. Positive fast-atom bombardment mass spectrometry (FAB-MS) of H_2-CL-EC showed major ion peaks at 523, 551, 563, 579, 591 and 607 *m/z,* corresponding, respectively, to 14:0/14:0, 16:0/16:0, 16:0/*cy*-17:0, 16:0/18:0, 16:0/*cy*-19:0 and 18:0/18:0 molecular species of diacylglycerols (DAG), consistent with the fatty acid analytical data. Negative FAB-MS did not yield any diagnostic ion peaks.

Chromatography. TLC of CLs and the deoxy analogue was carried out on 20 × 20 cm plates coated with silica gel 60A K6 (0.25 mm thick; Whatman International Co., Maidstone, England) using chloroform/methanol/conc. (14M) ammonium hydroxide (65:35:5, by vol) as solvent (Solvent A). GLC of fatty acid methyl esters, prepared by methanolysis of CL or dCL samples in methanolic HCl (30), was carried out on a column of SP-2330 (Supelco, Inc., Bellefonte, PA) at 185°C on a PYE Unicam (Cambridge, England) gas chromatograph.

Physical measurements. ^1H and ^{13}C NMR spectra were measured in $CDCl_3$ on a Bruker (Karlsruhe, Germany) CXP-300 spectrometer. FAB-MS was carried out on a VG-7070E (Vacuum Generators, Inc., Manchester, England) mass spectrometer with digital RL 02 data system and printonic printer.

Potentiometric titration. Samples (6–15 mg, 4–11 μmoles) of H_2-CL-BH, H_2-CL-EC, or 16:0-dCL, were converted to their respective protonated form using an

acidified Bligh and Dyer (29) procedure (see Ref. 30) and suspended in 2 mL of 0.05 M KCl by vortexing and sonication with a needle tip sonicator (Sonic Dismembranator, Quigley-Rochester, Inc., Rochester, NY). The samples of free acids formed milky-to-translucent dispersions in 0.05 M KCl, which were titrated with 0.05N KOH in 0.05 M KCl using a glass electrode, accurate over the pH range 2–12, and a pH meter (Radiometer, Copenhagen, Denmark). The KOH solution was delivered in increments with a syringe microburet (1 mL; Micro-Metric Instrument Co., Cleveland, OH). In some experiments, samples were sonicated in a cup horn type sonicator (Ultrasonics, Inc., Model W-385, Plainview, NY), with a Lauda K-2/R water circulator (Brinkmann Instruments, Westbury, NY), and titrated with an Orion digital pH meter (Model 601A) with a microelectrode (Microelectrodes Inc., Model No. MI-410, Londondery, NH), using a Hamilton syringe (2 mL) and a syringe pump (Razel Scientific Instruments, Stamford, CT) for constant delivery of the aqueous 0.05M KOH.

Titrations were also done on solutions of the protonated forms of CL or dCL (4–9 μmoles) in 2 mL of methanol/aqueous 0.05 M KCl (1:1, vol/vol). It was difficult to suspend the free acid form of the natural, unsaturated BH-CL in water by sonication and vortexing. This lipid was therefore titrated only in methanol/0.05 M KCl (1:1, vol/vol).

All titrations were done at room temperature (22–24°C) and care was taken to minimize exposure of samples to pH conditions above 8. Total consumption of alkali was in the range of 95–105 and 85–100% of the theoretical values calculated for CL and dCL, respectively. Immediately after titration, samples were recovered (85–100%, depending on the fatty acid composition) by acetone precipitation of the ammonium salt form (30); little or no degradation was observed by TLC using Solvent A.

Titration curves were computer-simulated using the general equation (31) for either monoprotic or diprotic systems. For the CL titrations, the curve after the first endpoint was calculated according to the approximation:

$$pH = pK_a(eff) + \log\{[A^-]/[HA]\} \quad [1]$$

where $pK_a(eff)$, the effective pK_a, varies as the titration of CL proceeds. In this case, it is not a constant as it is in the general equation (31). The theoretical curve for dCL was calculated on the basis of the equation:

$$V_b = \frac{V_a\,\alpha\gamma - V_b\,\beta F_a + F_a V_a \alpha}{\alpha(F_b\gamma)} \quad [2]$$

where V_b = volume of base added; V_a = initial volume of acid; F_a = initial formality of the acid; F_b = normality of base; $\alpha = (H^2)/(K_1K_2) + (H)/(K_2) + 1$; $\beta = (H^2)/(K_1K_2) - 1$; and $\gamma = (K_W)/(H) - H$; $H = [H^+]$ and K_1 and K_2 = the first and second dissociation constants of a diprotic acid.

The water-soluble phosphate esters, glycerol-1,3-diphosphate and 1,3-propanediol-1,3-diphosphate, were titrated in water as described for CL and dCL.

Syntheses of glycerol-1,3-diphosphate. Glycerol-1,3-diphosphate was synthesized as described previously (32) by condensation of 1,3-dibromo-2-palmitoyloxypropane with silver dibenzylphosphate (33) followed by removal of the benzyl groups by catalytic hydrogenolysis (Pd/C) and the palmitoyl group by mild alkaline hydrolysis; the product was isolated as the dibarium salt and converted to the free acid by treatment with Dowex-50 (H^+) ion-exchange resin and to various salt forms by neutralization with the appropriate base (32).

Synthesis of 1,3-propandiol-1,3-diphosphate. 1,3-Propandiol-1,3-diphosphate was synthesized by a procedure analogous to that for glycerol-1,3-diphosphate, in which 1,3-dibromopropane (0.377 g, 1.87 mmol) was condensed with silver dibenzylphosphate (1.4 g, 3.6 mmol) in anhydrous benzene (13.5 mL) under reflux with stirring for 24 h, followed by hydrogenolysis of the benzyl groups of the blocked product in ethanol (4 mL) with Pd/C catalyst (200 mg). The final product was isolated as the dibarium salt (28.4 mg, 0.055 mmol; 3% yield) and converted to the free acid form and to other salt forms as described for glycerol-1,3-diphosphate (32).

Synthesis of 2′-deoxy-cardiolipin (16:0-dCL). The synthesis of 16:0-dCL was carried out as summarized in Figure 2. DPPA disodium salt (67 mg, 0.1 mmol) was converted to the free acid form (30), dried in a desiccator over KOH pellets, mixed with an excess of 1,3-propanediol (140 mg, 1.84 mmol) and dissolved in dry pyridine or triethylamine (10 mL) at 50°C. The condensation was then brought about by adding trichloroacetonitrile (4 mL) to the clear tan solution and stirring in a nitrogen atmosphere at 50°C for 20 h (34). The dark brown mixture was diluted with an equal volume of benzene, concentrated in a rotary evaporator at 40–50°C and dried in a desiccator over concentrated sulfuric acid and then KOH pellets. The black residue was dissolved in 12 mL of chloroform/methanol (1:1, vol/vol), diluted with 5.4 mL of 1 N HCl and the mixture was centrifuged. The chloroform layer was removed, washed with an equal volume of methanol/water (10:9, vol/vol), made alkaline with 0.2 N ammonium hydroxide in methanol and concentrated to dryness *in vacuo*. The residue was dissolved in 0.5 mL of chloroform, diluted with 5 mL of acetone, and cooled on ice. The black precipitate of crude product, 16:0-dCL, was centrifuged, washed with cold acetone and reprecipitated from a small volume of chloroform (0.5 mL) by the addition of 10 vol of cold acetone. The combined acetone supernatants were concentrated to a small volume and diluted with acetone to yield a second crop (yield of combined crops, 36.5 mg).

This crude 16:0-dCL, containing unreactied DPPA, was purified by preparative TLC using Solvent A, eluted from the silica with chloroform/methanol/water (1:2:0.8, by vol) and isolated as the ammonium salt as described above. The yield of TLC-pure 16:0-dCL ammonium salt was 14 mg (0.01 mmol, 10% from DPPA); R_f 0.60 in Solvent A. Fatty acid analysis by GLC: 14:0, 1%; 16:0, 95.5%; 16:1, 1%; 18:0, 1.6%; 18:1, 0.9%. ^1H NMR ($CDCl_3$): 0.86 (*t*, terminal CH_3, 12H), 1.23 [acyl CH_2, C-4 to C-(ω − 1), 96H], 1.55 (acyl C-3, 8H), 1.77 (*s*, unidentified, 4H), 2.08 (broad single peak, HOH), 2.28 (*t*, acyl C-2, 8H), 3.67 (*s*, unidentified, 3H), 3.9 (*m*, CH_2-O-P, 8H), 4.13 (*t*, P-O-C-CH_2-C-O-P, 2H), 4.33 (*d*, CH_2-O-acyl, *sn*-1-glycerol, 4H), 5.18 (*t*, CH-O-acyl, *sn*-2-glycerol, 2H). Negative FAB-MS: 811 (unidentified, possibly [M − dipalmitoylglycerol (DPG) + OH + Na − H]$^-$ or [phosphatidylpropanediol phosphate (PPDP) + Na − H]$^-$); (base peak) 705 [M − H −

DPPA + H_2O]$^-$ or [phosphatidylpropanediol (PPD) − H]$^-$; 647 [M − PPD + OH]$^-$ or [DPPA − H]$^-$; Positive FAB-MS: 707 [M − DPPA + H_3O]$^+$; 551 [M − PPDP + H]$^+$ or [DPG − OH]$^+$; calcd. for 16:0-dCL (free acid), $C_{73}H_{142}O_{16}P_2$, M = 1336; PPDP, $C_{38}H_{76}O_{12}P_2$, M = 786; PPD, $C_{38}H_{75}O_9P$, M = 706; DPPA, $C_{35}H_{69}O_8P$, M = 648; DPG, $C_{35}H_{68}O_5$, M = 568. No parent ion peak for 16:0-dCL was detected by negative or positive FAB-MS.

The free acid form of 16:0-dCL was prepared by the acidified Bligh and Dyer (29) procedure (see Ref. 30). The sodium salt was prepared by titration of the free acid form of 16:0-dCL0 with 0.05N NaOH in methanol to pH 7.5, followed by precipitation from chloroform solution with acetone.

Synthesis of the unsaturated deoxy analogue of CL. A synthesis of the unsaturated deoxy analogue of CL was carried out as described above for 16:0-dCL, but starting with commercial phosphatidic acid (PA) (0.08 mmole) derived from egg lecithin (Serdary, London, Ontario, Canada). The yield and TLC R_f values of the unsaturated dCL (yellow oil) were the same as for 16:0-dCL. Fatty acid composition: 14:0, 0.4%; 16:0, 39.2%; 16.1, 1.9%; 18:0, 10.9%; 18:1, 36.2%; 18.2, 9.8%; 18:3, 0.2%. ^{1}H NMR: 0.86 (*t*, terminal CH_3, 12 H), 1.23 (acyl-CH_2, C-4 to C-(ω − 1), 92 H), 1.56 (acyl C-3, 8 H), 1.75 (*s*, unidentified, 4 H), 2.00 (*q*, allylic methylene, 8 H), 2.28 (*q*, acyl C-2, 8 H), 2.76 (*t*, diallylic methylene, 2 H), 3.70 (*s*, unidentified, 4 H), 3.96 (*m*, CH_2-O-P, 8 H), 4.17 (*q*, PO-C-CH_2-C-OP, 2 H), 4.39 (*d*, CH_2-O-acyl, *sn*-1-glycerol, 4 H), 5.21 (*t*, H-C-O-acyl, *sn*-2-glycerol, 2 H), 5.32 (*m*, olefinic C-H, 5 H).

RESULTS

Syntheses. Because of the symmetry of the 2′-deoxy analogue of CL (16:0-dCL), its synthesis could be achieved by a single-step condensation of di-16:0-PA with 1,3-propanediol in the presence of pyridine or triethylamine and trichloroacetonitrile (Fig. 2). The 16:0-dCL was obtained in about a 10% overall yield but was contaminated with a black substance that co-precipitated with it and could only be removed by preparative TLC in Solvent A. The 16:0-dCL had a much higher mobility on TLC (R_f 0.60) in Solvent A than either natural CL or the hydrogenated CL (R_f 0.48), which can be attributed to the absence of the central OH group. Both the ^{1}H NMR and the FAB-MS spectra were consistent with the expected chemical structure of the dCL (Fig. 1). Under the conditions used here for FAB-MS, 16:0-dCL did not show a parent ion peak, the only negative ion peaks observed being those for the cleavage products, DPPA, PPD and possibly PPDP.

Potentiometric titration. H_2-CL-BH (18:0-CL) formed bilayer phases in either sonicated aqueous dispersion or in methanol/water (1:1, vol/vol) (21). On titration (Fig. 3), 18:0-CL showed the presence of one strong acid group, with apparent pK = 3.2, accounting for one equivalent of acid per mole, and a weak acid group with a surprisingly high pK accounting for a second equivalent of acid. The titration curve of the unhydrogenated BH-CL in methanol/water (1:1, vol/vol) was virtually superimposable on that of the 18:0-CL in water. Titration of the former in aqueous medium was not possible because this phospholipid forms hexagonal II phased dispersions in water (21), in which the polar head groups are not available for titration. In contrast, 16:0-dCL titrated as a normal dibasic acid in either sonicated aqueous dispersion (Fig. 3) or in methanol/water (1:1, vol/vol) (data not shown). It titrates two equivalents of base with an apparent pK of about 3.

Computer-simulated titration curves. An attempt was made to obtain more precisely the pK values of 18:0-CL (Fig. 3) by computer simulation assuming reasonable values for both the lower pK and the upper pK. It was apparent that this was not possible since a single value for pK_2 could not be obtained. As is shown in Figure 4, the experimental data crossed through a family of theoretical curves corresponding to pK_2 values varying from 7.5 to 9.5. Furthermore these values fit the lower portion of the curve best when pK_1 was assumed to be 2.8. It can be seen that the experimental curve differs from the theoretical curves by a "flattening" that is expected for titrations at polyanionic surfaces, on the basis of the calculations made by Tanford and Kirkwood (35) from Gouy-Chapman considerations. The second pK of CL appears to follow the formula: pK_2 = 7.5 + (fractional equivalent/2), approximating a linear increase in its pK value between 7.5 and about 9. CL thus "buffers" the protons throughout this range, entrapping protons up to a pH of 9.0 or greater.

In contrast, the titration of 16:0-dCL (Fig. 3) can be computer-stimulated (Fig. 5) by a theoretical curve for a typical dibasic acid having two strongly acidic groups with a pK_1 of 1.8 and pK_2 of 4.0. The "flattening" of the experimental curve here can also be accounted for by the surface charge effects desribed by Tanford and Kirkwood (35). Unlike the CL titration which displays an actual shift in the pK_2, dCL exhibits only a slight flattening of its titration curve (Fig. 5). This shows that the Gouy-Chapman effects are insufficient to provoke the dramatic pK_2 shift observed with CL (Fig. 4). It should also be noted that the results of Few *et al.* (11) show an upward drift after the pK_1 not unlike the titration reported here. Few *et al.* (11) obtained BH mitochondrial CL from Pangborn (5). Since there is no mention of hydrogenation, we must assume the CL was polyunsaturated and hence, we cannot be certain of the state of the CL (H_{II}, bilayer, *etc.*). Nevertheless, the upward drift of the titration curve obtained by Few *et al.* (11) appears to be consistent with our results.

Two other "titrations" have been reported in the literature for cardiolipin. Seddon *et al.* (13) reported a pK below 2.8 based upon the pH dependence of phase changes. This measurement could not be expected to detect a second pK unless there was a discrete phase change associated with it. These investigators assumed at the outset that there was only one pK and that CL is dibasic (16). The other attempt to measure the pK of CL (12) was made by titration in ethanol solution using indicator dyes. In this case a single pK was reported to be below 4.0 although a precise number could not be assigned.

Finally, the glycerol-1,3-diphosphate and 1,3-propanediol-1,3-diphosphate, model compounds for the polar headgroups of 18:0-CL and 16:0-dCL, respectively, both titrated as tetrabasic acids having two strong acid groups with pK 2.25 or 2.35, respectively, and two weak acid groups with pK 6.3 or 7.1, respectively (data not shown). The free hydroxyl on the glycerol-1,3-diphosphate does not appear to alter significantly the titration characteristics of the

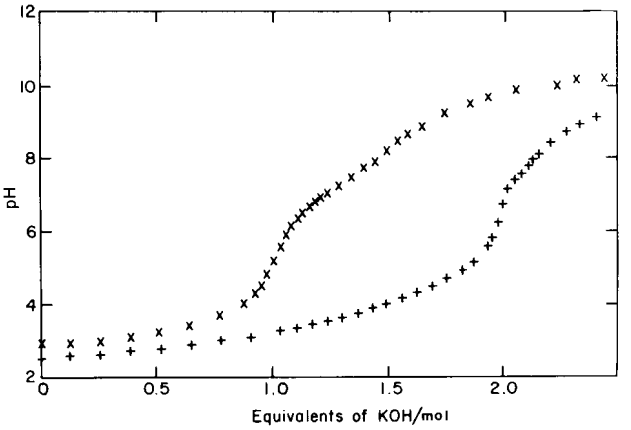


FIG. 3. pH-Titration curves of sonicated aqueous dispersions of hydrogenated beef heart cardiolipin (18:0-CL) (X) and of synthetic 2'-deoxycardiolipin (16:0-dCL) (+).

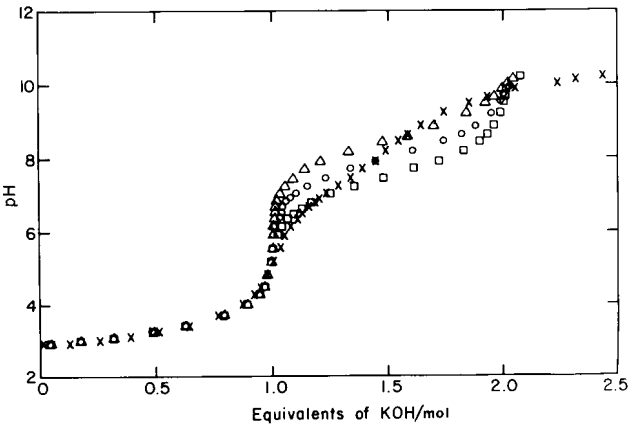


FIG. 4. Computer simulation of the titration of 18:0-CL: experimental curve (X); theoretical curves for a family of dibasic acids with pK_1 = 2.8, and pK_2 = 7.5 (□), 8.0 (△), 8.5 (○). Abbreviation as in Figure 3.

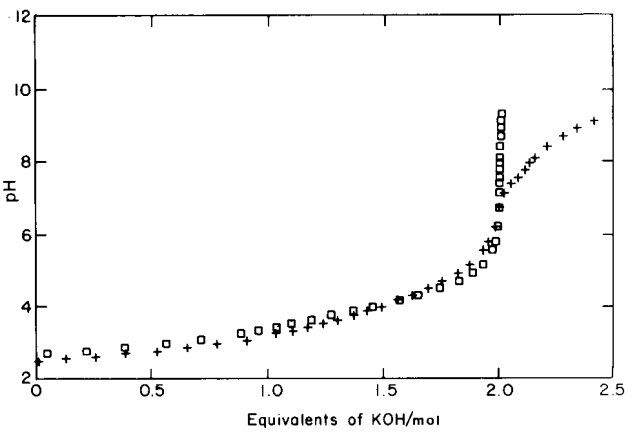


FIG. 5. Computer simulation of the titration of (16:0-dCL): experimental results (+); theoretical curve (□) for a dibasic acid with pK_1 1.8, pK_2 4.0. Abbreviation as in Figure 3.

phosphate groups since the propanediol-1,3-diphosphate displays essentially the same titration curve. However, see the discussion that follows.

DISCUSSION

The titration of the 18:0-CL presented in this paper is apparently the first electrometric titration of CL. Unlike previous attempts to titrate CL (11–13), the present measurements specifically examine the number of equivalents titrated at each pK. Our titration yielded two significant and surprising results. The first is that there are two pK values, the second pK (pK_2) being above physiological pH even at its lowest value. The second unexpected result is that the pK_2 itself changes rather dramatically as the titration proceeds. Furthermore, we have shown that in view of the comparison of the titration of CL to that of the dCL both of these unexpected results are due to the presence of the free hydroxyl on the connecting glycerol in CL.

The fact that dCL has two strong acid groups while CL has only one strong acid group may be attributed to the absence of the central OH in dCL, and to the presence of the central C-OH group in CL. The most likely way that this hydroxyl group can so significantly affect the pK of the phosphate groups is to form an intramolecular hydrogen bond with one of the P-OH groups (Fig. 6), analogous to the lipid acid anions described by Haines (36). The existence of such an intramolecular hydrogen bond has been demonstrated (37,38) by FTIR spectroscopy in phosphatidylglycerophosphate (now identified as phosphatidylglyceromethylphosphate, Ref. 39), as well as in CL (21).

The water-soluble headgroup moieties, glycerol-1,3-diphosphate and propanediol-1,3-diphosphate, do not display a significant difference in titration, as described in the Results section. This suggests that the difference between titrations of 18:0-CL and 16:0-dCL are due to the conformation of the headgroup of CL allowing the alignment of the C-OH and P-OH groups under constraint by the presence of the acyl groups (Fig. 6). It is this latter

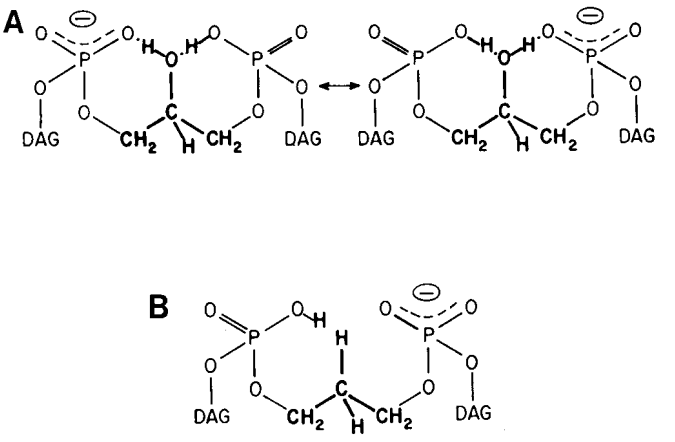


FIG. 6. A, proposed intramolecular hydrogen bonded acid-anion structure of cardiolipin at pH 7.5–9.5; and B, the structure of deoxycardiolipin at pH 2.0 (DAG, diacylglycerol). Note that structure A can trap a proton above pH 7.5 by forming an acid ion, while structure B cannot trap a proton, *i.e.*, cannot form an acid anion.

feature that also provides a mechanism to explain how the pK_2 of CL increases during the titration from 7.5 to 9.5 (Fig. 4).

The titration of dCL displays a pK_2 somewhat higher than its pK_1, presumably due to the suppression of ionization by the increased charge density on the surface as can be predicted from the work of Tanford and Kirkwood (35). In contrast, CL displays a shifting pK_2 as the titration proceeds. This suggests that the pK_2 of CL is sensitive to the charge density in two ways, first in the same way that dCL is, and second by a tightening of the H-bonded ring (Fig. 6). Presumably the second effect is significantly greater than the first; not only does a small increase in charge density provide a large increase in pK_2 but as the titration proceeds and the charge density increases, the pK shifts.

The biological significance of these observations pertains primarily to the capacity of CL to bind protons at relatively high pH. This might allow CL to buffer protons and provide a proton reservoir at high pH for participation in proton conduction in energy-transducing membranes as discussed by Haines and Kates (manuscript in preparation). These statements run counter to the suggestion that CL might provide protons at low pH (13).

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Natural Sciences and Engineering Research Council of Canada (to M.K.), and by a grant from CUNY FRAP (to T.H.H.). The assistance of S. Desilva and B. Bhullar in the synthesis of the deoxycardiolipins is gratefully acknowledged. Appreciation is also extended to Dr. N. Moldoveanu for help in preparation of the hydrogenated cardiolipins, to C. Kazakoff for the mass spectra and to R. Capoor for the NMR spectra.

REFERENCES

1. Wasserman, A., Neisser, A., and Bruck, C. (1906) *Dt. Med. Wschr. 32,* 745–746.
2. Browning, C.H., Cruickshank, T., and Gilmour, W. (1911) *J. Path. Bact. 15,* 361–362.
3. Noguchi, H. (1911) *Z. Immunitäsforsch. Exp. Therap. 9,* 715–720.
4. Pangborn, M. (1942) *J. Biol. Chem. 143,* 247–256.
5. Pangborn, M. (1947) *J. Biol. Chem. 168,* 351–361.
6. Faure, M., and Maréchal, J. (1962) *Compt. Rend. Acad. Sc. Paris 256,* 4518–4520.
7. Gray, G.M., and MacFarlane, M.G. (1958) *Biochem. J. 70,* 409–425.
8. Benson, A.A., and Strickland, E.H. (1960) *Biochim. Biophys. Acta 41,* 328–333.
9. Le Cocq, J., and Ballou, C.E. (1964) *Biochemistry 3,* 976–980.
10. de Haas, G.H., Bonsen, P.P.M., and van Deenen, L.L.M. (1966) *Biochim. Biophys. Acta 116,* 114–124.
11. Few, A.V., Gilby, A.R., and Seaman, G.V.F. (1960) *Biochim. Biophys. Acta 38,* 130–136.
12. Coulon-Morelec, M.J., Faure, M., and Maréchal, J. (1962) *Bull. Soc. Chim. Biol. 44,* 171–183.
13. Seddon, J.M., Kaye, R.D., and Marsh, D. (1983) *Biochim. Biophys. Acta 734,* 347–352.
14. Cable, M.B., Jacobus, J., and Powell, G.L. (1978) *Proc. Natl. Acad. Sci. USA 75,* 1227–1231.
15. Powell, G.L., and Jacobus, J. (1974) *Biochemistry 13,* 4024–4026.
16. Marsh, D. (1990) *Handbook of Lipid Bilayers,* p. 85, CRC Press, Boca Raton.
17. Luzatti, V., and Chapman, D. (1968) *Biological Membranes,* Academic Press, New York.
18. Batenburg, A.M., Hibbeln, J.C.L., Vekleij, A.J., and de Kruijff, B. (1987) *Biochim. Biophys. Acta 903,* 155–165.
19. Rose, H.G. (1964) *Biochim. Biophys. Acta 84,* 109–113.
20. Courtade, S., Marinetti, G.V., and Stotz, E. (1967) *Biochim. Biophys. Acta 137,* 121–134.
21. Hübner, W., Mantsch, H., and Kates, M. (1991) *Biochim. Biophys. Acta 1066,* 166–174.
22. Daum, G. (1985) *Biochim. Biophys. Acta 822,* 1–42.
23. Robinson, N.C., Zborowski, J., and Talbert, L.H. (1990) *Biochemistry 29,* 8962–8969.
24. Eble, K.S., Coleman, W.B., Hantgan, R.R., and Cunningham, C.C. (1990) *J. Biol. Chem. 265,* 19434–19440.
25. Horvath, L.I., Drees, M., Klingenberg, M., and Marsh, D. (1990) *Biochemistry 29,* 10664–10669.
26. Dale, M.P., and Robinson, N.C. (1988) *Biochemistry 27,* 8270–8275.
27. Kagawa, Y., Kandrach, A., and Racker, E. (1973) *J. Biol. Chem. 248,* 676–684.
28. Serrano, R., Kanner, B.J., and Racker, E. (1976) *J. Biol. Chem. 251,* 2453–2461.
29. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911–917.
30. Kates, M. (1986) *Techniques of Lipidology,* 2nd edn, pp. 110–111, Elsevier, Amsterdam.
31. Harris, D.C. (1987) *Quantitative Chemical Analysis,* 2nd edn., W.H. Freeman and Co., New York.
32. Kates, M., Palameta, B., and Chan, T.H. (1966) *Can. J. Biochem. 44,* 707–712.
33. Cutler, Jr., F.A., Coubere, J.P., Lukes, R.M., Fisher, J.F., Mertel, H.E., Herschman, J., Chemerda, J.M., Sarett, L.M., and Pfister, K., III (1958) *J. Am. Chem. Soc. 80,* 6303–6320.
34. Ali, S., and Bittman, R. (1989) *Chem. Phys. Lipids 50,* 11–21.
35. Tanford, C., and Kirkwood, J.G. (1957) *J. Am. Chem. Soc. 79,* 5333–5339.
36. Haines, T.H. (1983) *Proc. Natl. Acad. Sci. USA 80,* 160–164.
37. Stewart, L.C., Kates, M., and Smith, I.C.P. (1988) *Chem. Phys. Lipids 48,* 177–188.
38. Stewart, L.C., Kates, M., Yang, P.W., and Mantsch, H.H. (1990) *Biochem. Cell Biol. 68,* 266–273.
39. Kates, M., Moldoveanu, N., and Stewart, L.C. (1993) *Biochim. Biophys. Acta 1169,* 46–53.

[Received December 7, 1992, and in final revised form July 31, 1993; Revision accepted July 31, 1993]