papers and notes on methodology

Purification of cardiolipin for surface pressure studies

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Summary Thin-layer chromatography and surface pressure-area isotherms of commercial bovine cardiolipins showed that the samples contained contaminants. They were purified by TLC and their purity was checked by chromatography and by their monolayer properties. The molecular area of cardiolipin and its purification yield depend upon the fatty acid composition, particularly the degree of unsaturation.—**Houle, A., F. Téchy, J. Aghion, and R. M. Leblanc.** Purification of cardiolipin for surface pressure studies. *J. Lipid Res.* 1982. **23:** 496–502.

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Cardiolipin is a 1,3-diphosphatidylglycerol found in mitochondrial inner membranes and other biological membranes, all with a high metabolic activity (1, 2). The four esterified fatty acids are highly unsaturated (3), therefore they are readily autoxidizable. The two phosphoryl residues make it a negatively charged phospholipid, as compared with phosphatidylcholine which has a net charge close to zero. It is presumably because of its negative charge that cardiolipin is necessary in double-layer phospholipid membranes to which polar proteins are to be attached (4).

The data on purification of cardiolipin by silica gel chromatography are at least ambiguous (5). Acidic phospholipids are not fractionated satisfactorily and their elution seems variable beyond control. The present work aims at applying to the purification of cardiolipin a method close to that of Tancrède, Chauvette, and Leblanc (6). The purity was determined by thin-layer chromatography, monolayer properties at water-air interface, as well as by estimations of the phosphorus content and analysis of the fatty acids.

MATERIALS AND METHODS

Bovine cardiolipin was purchased in the highest available purity from four suppliers: Applied Science Laboratories (State College, PA), P. L. Biochemicals (Milwaukee, WI), Serdary Research Laboratories (Ontario, Canada), and Sigma Chemical Company (St. Louis, MO). The cardiolipin from Sigma was supplied as a sodium salt. Bovine phosphatidylcholine (99% pure) was purchased from Applied Science Laboratories. The thin-layer chromatography plates were silica gel GF, prescored, 20 × 20 cm, 0.25 mm thick, from Mandel Scientific (Québec, Canada). Chloroform, methanol (Baker Chemical, Philipsburg, NJ), and benzene (Fisher Scientific Company, Fairlawn, NJ) were reagent grade and were distilled before use. Their purity was checked as described by Tancrède et al. (6). Fifty ml of the distilled solvents were evaporated to dryness; the dry residues, if any, were dissolved in 1 ml of benzene. One hundred μl of the latter solution was spread on a Langmuir trough and, upon compression, was found to contain negligible amounts of film-forming impurities. The water was first demineralized, then twice distilled in a quartz still (surface tension, 70 mN m^{-1}; resistivity, 18 MΩ cm). All other chemicals were reagent grade and were used as such. The lipid solutions used for surface pressure measurements were prepared by weighing 0.3 to 1.0 mg of lipids on a Cahn electrobalance RG 2000 (precision better than 1%).

The surface pressure-area measurements were done in a Langmuir-type film balance (6). The trough (35.0 × 14.7 × 5.0 cm) was set in a stainless steel box. All the experiments were done in a nitrogen atmosphere at 20.0 ± 0.5°C and 50–55% relative humidity. The lipids were spread on a Tris–HCl buffer (10^{-3} M, pH 8.0). Usually 1.5×10^{16} molecules were deposited from 100 μl of benzene with a Hamilton microsyringe. The reproducibility of the results was ±2 $Å^2$ per molecule and ±0.2 mN m^{-1}. The molecular weights of cardiolipin and phosphatidylcholine were approximated to 1460 and 787, respectively. The solutions were kept in the dark, between experiments, at −20°C.

Surface potential measurements were made with an air-ionizing electrode made of ^{241}Am (20 μC) in a gold casing, purchased from the Amersham Company. It was included in a Teflon casing, itself placed in a grounded

Abbreviation: TLC, thin-layer chromatography.

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aluminum enclosure, to eliminate electrical interferences. The gold tip was set 3 to 4 mm above the interface. A platinum electrode dipped into the film-free subphase. The two electrodes were connected to a high-impedance Keithley electrometer (model 615). The results were reproductible within ±20 mV. Between experiments, the electrodes were kept in the same conditions, using water instead of subphase, in order to minimize changes of the volta potential (7).

Cleaning of the TLC plates

Cleaning the silica gel of the TLC plates was found to be necessary. If actually 50 cm^2 of it were scraped off, suspended in 50 ml of chloroform–methanol 65:25 (v/v) and filtered through fine-frittered glass, and the filtrate was evaporated to dryness, and then the residue was dissolved in 1 ml of benzene, 100 μl of this latter solution contained a noticeable amount of film-forming impurities. The 100 μl was deposited on the subphase in the Langmuir trough and, when the mobile barrier reached 1 cm from the float, the surface pressure was as high as 43 mN m^{-1}, which is a pressure at which many phospholipid monomolecular films collapse.

The plates were therefore cleaned by a 12-hr continuous migration of the cleaning solvent (either chloroform–methanol–acetic acid–water 50:15:4:1 (v/v) or methanol–water 50:50 (v/v)). The plates were then dried 1 hr at 110°C. An additional cleaning was done with the solvent used for the purification of cardiolipin (see below). The plates were then activated 1 hr at 110°C, cooled in a desiccator, and used immediately. The cleanliness of the plates was checked as described above and the plates were used when the surface pressure was less than 0.5 mN m^{-1} and the barrier was 1 cm away from the float. As a control, it was checked that pure phosphatidylcholine had the same surface pressure characteristics before and after its migration on the cleaned TLC plates. The impurities remaining on the silica gel, if any, did not interact with the phospholipids (see below). Finally it was noted that weighing the residues of the filtrate, before spreading them on the Langmuir trough, gave no significant result.

The methanol–water cleaning solvent was used in order to eliminate the possibility that acetic acid could remain trapped in the silica gel. The results of the purification of cardiolipin with the TLC plates treated both ways are given below.

Purification of commercial cardiolipin

A benzene solution of cardiolipin (20 mg/ml) was spotted on two clean TLC plates (maximum of 100 μg per spot). The chromatograms were developed in one of the following solvents. (*i*) Chloroform–methanol 85:15 (v/v) was used to obtain a good separation of cardiolipin (R_f = 0.5) from contaminants migrating close to or at the solvent front; (*ii*) chloroform–methanol–water 65:25:4 (v/v), the solvent used most frequently; (*iii*) chloroform–methanol–water 75:22:3 (v/v) because it has a polarity intermediate between the two others.

When the migration was completed, a 5 × 20 cm section was broken off each plate and exposed to iodine vapour. By reference to this section, the silica gel of the remaining section (15 × 20 cm) was scraped off on a 1-cm width from the top of the cardiolipin spot and the lipid was desorbed by stirring the silica gel for 10 min in 50 ml of chloroform–methanol 65:25 (v/v). The solution was filtered through a frittered glass funnel and evaporated to dryness under a nitrogen atmosphere. Care was taken to avoid as much as possible exposure of the lipid to air.

All the analytical TLC were performed with chloroform–methanol–water 65:25:4 (v/v) as a developing solvent.

Characterization of the cardiolipin

The preparations were analyzed for phosphorus (8), and their fatty acid composition was determined by gas–liquid chromatography (Hewlett-Packard model 5830 A) using a 2-m column, 6 mm O.D., with 5% DEGS Gas Chrom Q 80/100 mesh, at 185°C, after methylation (9) of the acids.

RESULTS

Analytical TLC of the commercial samples of cardiolipin showed, in each and every case, a large spot at R_f = 0.9, characteristic of cardiolipin (10) but also long tailing and some other spots indicating the presence of oxidation products and other impurities.

Fig. 1 shows that the surface pressure-area and the surface potential-area isotherms of such samples are very different from one another. The surface pressure-area curves are widely expanded towards large molecular areas at low pressures, because of the presence of contaminants (11). The limiting molecular areas extrapolated to zero pressure are all above 160 $Å^2$ per molecule (such an extrapolated value is only an indicative approximation because cardiolipin monomolecular films are in a liquid state).

For all those reasons, it was decided that the commercial samples of cardiolipin must be purified before use. After purification, as described in the Materials and Methods section, analytical TLC showed only one spot at R_f = 0.9 free of any tailing or other contaminant spots.

Table 1 shows the monolayer properties of the cardiolipin from Sigma before and after purification by the different procedures described above. It was clearly ap-

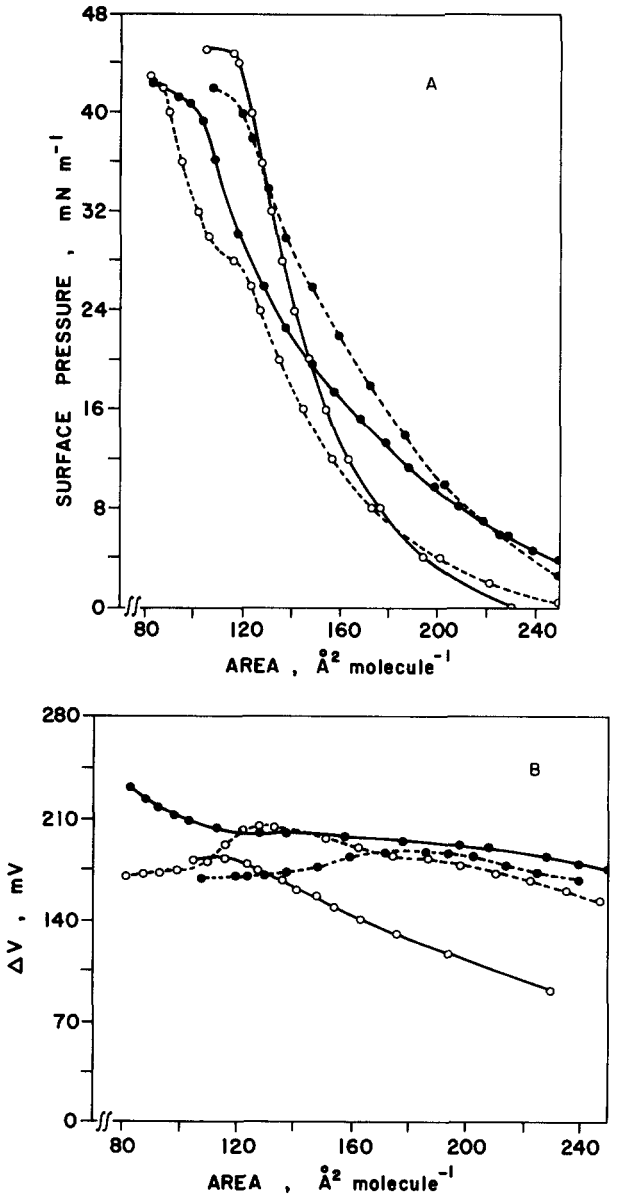

Fig. 1. Surface pressure-area (curves A) and surface potential (curves B) isotherms of commercial cardiolipin. Subphase = Tris-HCl buffer, 1×10^{-3} M; pH 8.0, 20°C. Relative humidity, 50–55%. ● - - - ●, Applied Science; ● —— ●, PL; O - - - O, Serdary; O —— O, Sigma.

parent that all the purified samples had smaller molecular areas than the crude commercial sample. Moreover, among the purified samples it was clear that one of the purification and analysis procedures yielded very little cardiolipin and one with a much smaller molecular area than the others. The three other procedures gave results which were very similar. The results were the same with chloroform–methanol–water 65:25:4 or 75:22:3 (v/v) (see Fig. 3, curve d).

The commercial cardiolipin from the other companies was purified by TLC on plates cleaned with the acetic acid-containing solvent and developed with the chloroform–methanol mixture (**Fig. 2A** and **2B**). We are aware of the low average yield of purification for this particular protocol. In this case, we may have concentrated a very small subset of molecules enriched in saturated fatty acids (vide infra) which lowered the area per molecule (see for comparison Fig. 2A and Fig. 3 curve d).

A comparison of Figs. 1 and 2 leads to the following observations. (*i*) At low values of the surface pressure, the curves are steeper after purification; (*ii*) at all surface pressures, the molecular area is smaller (for instance, at 20 mN m^{-1}, the molecular areas are 108 (Applied Sci-

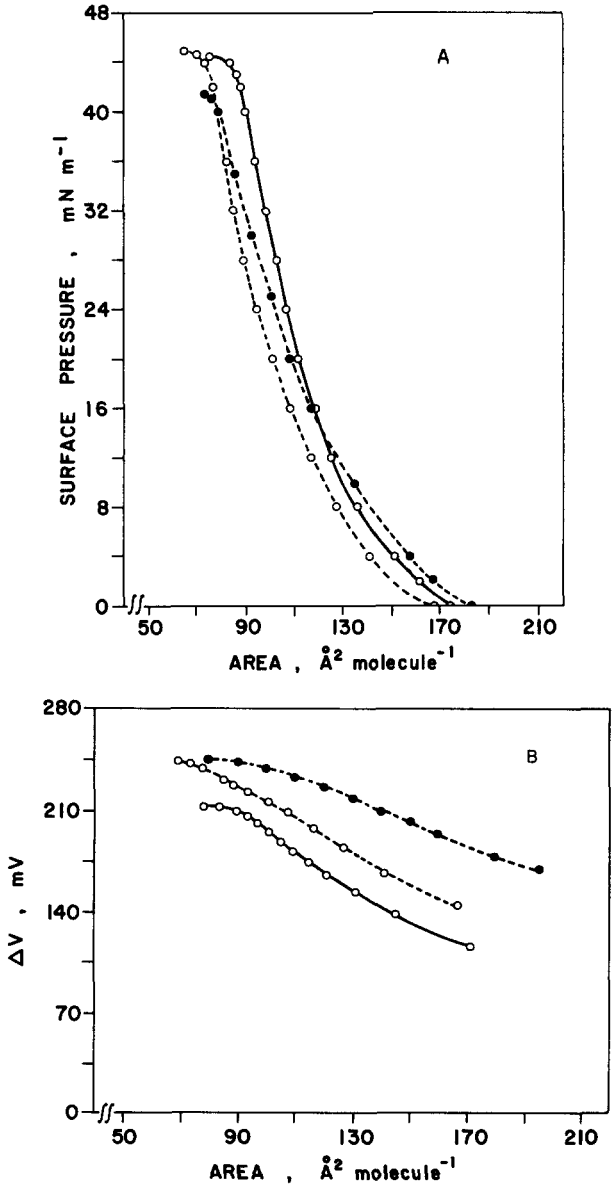

Fig. 2. Average surface pressure-area (curves A) and surface potential (curves B) isotherms of cardiolipin after TLC. Plates cleaned with chloroform–methanol–acetic acid–water 50:15:4:1(v/v) and developed in chloroform–methanol 85:15(v/v). Experimental conditions and symbols are the same as in Fig. 1.

TABLE 1. Surface pressure-area characteristics of cardiolipin from Sigma, before and after purification by TLC[a]

		Chloroform–Methanol–Acetic Acid–Water[b] 50:15:4:1 (v/v)		Methanol–Water[b] 50:50 (v/v)	
	Control	Chloroform–Methanol[c] 85:15 (v/v)	Chloroform–Methanol–Water[c] 65:25:4 (v/v)	Chloroform–Methanol[c] 85:15 (v/v)	Chloroform–Methanol–Water[c] 65:25:4 (v/v)
		molecular area ($Å^2$ molecule^{-1})			
Surface pressure, mN m^{-1}					
0	231[d]	174	233	218	224
10	171	130	168	160	166
20	147	112	144	138	143
30	133	100	131	124	130
40	123	90	121	115	120
Surface pressure of collapse, mN m^{-1}	45.0	44.0	44.8	44.6	44.0
Limiting extrapolated area per molecule, $Å^2$ molecule^{-1}	165	132	159	152	156
Average yield of purification[e]		0.06	0.37	0.11	0.47

[a] Same experimental conditions as in Fig. 1.
[b] Cleaning solvent.
[c] Developing solvent.
[d] See Fig. 1A, curve O —— O.
[e] Quantity of cardiolipin after purification/quantity of commercial cardiolipin deposited on the TLC plates.

ence), 101 (Serdary), 112 (Sigma) $Å^2$ molecule^{-1} instead of, respectively, 165, 135, and 147 $Å^2$ molecule^{-1}); (*iii*) the collapse pressure of purified cardiolipin is, in all cases, slightly higher than that of the commercial samples. All those observations lead to the conclusion that the purification has freed the cardiolipin of its contaminants.

In order to check the purification procedure, a sample of phosphatidylcholine was chromatographed by TLC and the developing solvent was chloroform–methanol–water (65:25:4 (v/v). Whether the plates were cleaned with one or the other of the cleaning solvents, the phosphatidylcholine had exactly the same surface pressure-area isotherm as the commercial non-treated sample; the limiting extrapolated molecular area was 75 $Å^2$ molecule^{-1} and, at 30 mN m^{-1} it was 65 $Å^2$ molecule^{-1}, which is in agreement with the values published for egg lecithin and for beef liver lecithin (12, 13). It thus appears that the purification procedure does not break down the phospholipids that are submitted to it.

Phosphorus content and fatty acid analysis

Table 2 gives the phosphorus content of different commercial and purified cardiolipin samples. The results are expressed as weight percentage of P in the samples. Except in one specified case, all the values are averages of at least three measures made on three different samples. The figures are close enough to one another to

TABLE 2. Phosphorus and fatty acid content of different commercial and purified cardiolipin samples

Sample or Reference	Cleaning Solvent with (+) or without (−) Acetic Acid	Developing Solvent with (+) or without (−) Water	Molecular Area of Cardiolipin at 20 mN m^{-1}, $Å^2$ molecule^{-1}	Phosphorus Content, wt% Cardiolipin	Fatty Acid Residues, % Total Fatty Acids							
					16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4
Commercial (Sigma)			147	3.9 ± 0.5	0.4	0.3	3.2	6.0	86.3	0.8	1.3	1.5
Purified (Sigma)	+	−	112	3.4 ± 0.2	1.5	1.4	0	7.0	89.0	0.6	0.5	0
	−	+	143	3.8 ± 0.2	0.4	0.6	0	6.1	92.0	0.7	trace	0
Commercial (Serdary)			135	3.3 ± 0.3	0.6	1.6	0.7	6.5	86.5	2.9	0.6	0.6
Purified (Serdary)	+	−	101	2.7 ± 0.2[a]	3.2	1.7	1.7	6.7	78.7	5.8	2.2	0
Shah and Schulman, 1965			149		6.7	1.9	10.3	10.9	70.2			
Shimojo and Ohnishi, 1967			104	4.0 (±0.5)	trace	11.2	0.3	10.8	76.5			1.2

[a] Only one purification.

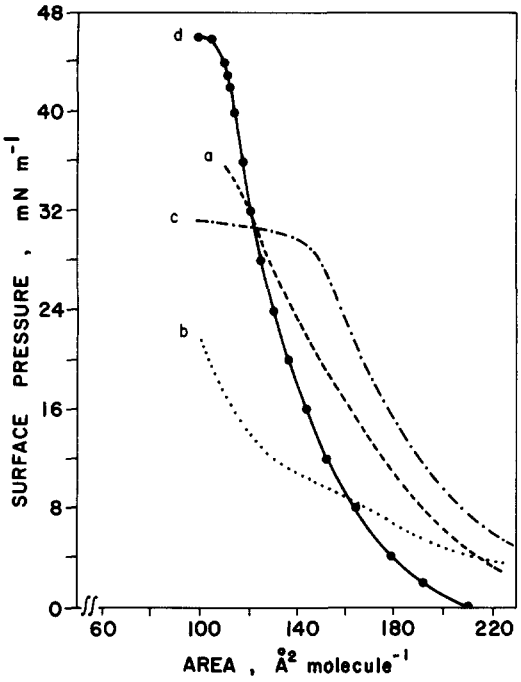

Fig. 3. Surface pressure-area isotherms of cardiolipin published by: a) Shah and Schulman (1965), subphase, NaCl, 0.02 M; pH 5.6, 25°C; b) Shimojo and Ohnishi (1967), subphase, triethanolamine–HCl 0.001 M; pH 7.0, 20°C; c) Ries (1979); and d) present work, plates cleaned with methanol–water 50:50(v/v) and developed in chloroform–methanol–water 75:22:3(v/v), subphase, Tris-HCl, 1×10^{-3} M; pH 8.0, 20°C.

confirm that in all cases it is indeed cardiolipin that was dealt with, i.e., the purification procedures did not destroy the cardiolipin molecules. Table 2 also gives the fatty acid composition of the same samples, expressed in molar ratios of each fatty acid to the sum of fatty acids. For both types of analyses, it was checked that the results with clean TLC plates were zero or close to zero.

DISCUSSION

At a phase boundary, amphiphilic lipid molecules are always oriented in such a way that the polar groups tend to dissolve in water and the hydrophobic groups tend to stand away from it (11). Thus, the polar head of cardiolipin can be assumed to be below the water–nitrogen interface (14, 15) and the four fatty acid residues then account for the surface area of cardiolipin at the interface. It is already known (16) that the surface areas of palmitoyl cardiolipin and palmitoyl phosphatidyldiacylglycerol are both equal to four times that of palmitic acid. Another confirmation is that the variations of surface potential during compression are very small, which would not be the case if the headgroup dipoles underwent interactions and re-orientations (see Fig. 2B).

Fig. 3 shows the surface pressure-area isotherms of bovine cardiolipin published by several authors (14, 15, 17). They are widely different from one another and from those obtained in this work (curve d). It must first be noticed that those isotherms were made under experimental conditions different from ours (pH and ionic strength of the subphase). Secondly, the shape of the isotherms and the discrepancies between them show that the cardiolipins used by those authors are not devoid of oxidation products or other contaminants.

Chromatography

The chromatographic behavior of cardiolipin is certainly influenced by the cations bound to this anionic lipid and/or to the adsorbent. Silica gel G is known to be rich in calcium ions (18) and the cardiolipins bought from different firms are salified with sodium or other ions. Cation exchanges could occur between adsorbent and phospholipid during the chromatographic development and modify the mobility of cardiolipin. However, it has been shown that neutral solvents convert the monovalent cardiolipin salt into a divalent one (18), so that all our purified cardiolipins are divalent salts.

Acetic acid is often used in TLC of cardiolipin (19). Nonetheless, Tancrède et al. (6) pointed out the danger of using it; it has a strong affinity for silica gel and part of it could remain trapped in the adsorbent yielding a degraded lipid. This happens with monogalactosyldiglyceride (6). In the present work, the mole fraction of acetic acid is lower than that used by Tancrède et al. (6). Moreover, an additional washing with the development solvent has been performed. Finally, two cycles of drying were always done before any cardiolipin was deposited on the TLC plates. Since it has been shown here that the phosphatidylcholine does not seem to be affected by such a cleaning procedure, it is probable that another phospholipid, such as cardiolipin, is not either.

What was called here a preparation of cardiolipin is a mixture of molecules with fatty acid residues differing by their number of carbon atoms as well as their unsaturation. Therefore it can be proposed that polar developing solvents mix, on a TLC plate, both oxidized and non-oxidized cardiolipins in the same spots. A less polar solvent would not concentrate all the cardiolipins in the same place and would give molecules free of oxidized products (3). In any case, the choice of the developing solvent must necessarily be a compromise.

Structure and surface properties

A space-filling model of cardiolipin (**Fig. 4**) shows that the polar head has a maximum surface area of 160 $Å^2$, and that when the four hydrocarbon chains are tightly packed, they cover an area of 80 to 90 $Å^2$. It has been said, above, that those fatty acid residues are responsible for the surface properties of cardiolipin. They are, there-

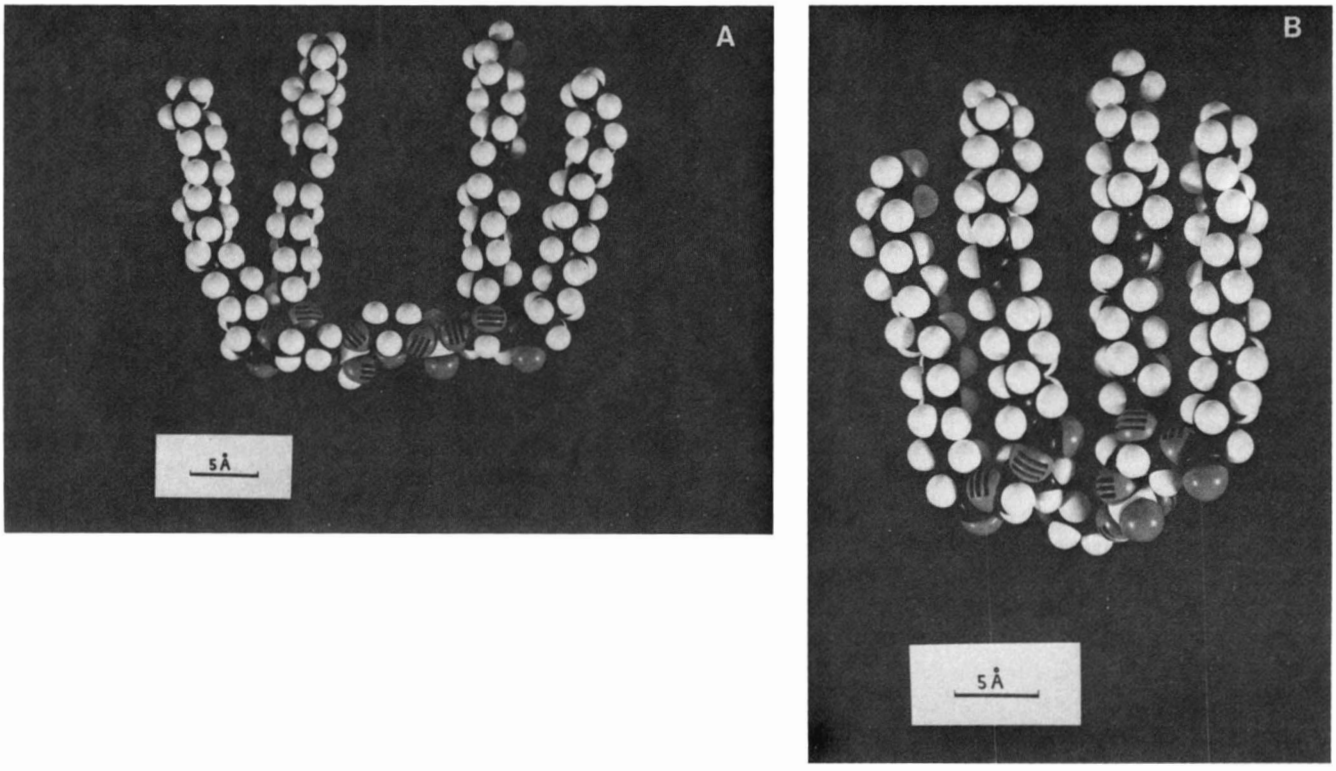

Fig. 4. Molecular model of cardiolipin. Its orientation at a nitrogen–water interface (A) in an expanded film and (B) in a condensed film.

fore, responsible for the differences of isotherms for cardiolipin from different companies; the cardiolipins from different sources have different fatty acids (Table 2). Otherwise, the cardiolipin from one company always gives similar isotherms, within a ± 4 Å^2 molecule^{-1} span.

Actually, phosphatidylcholine and other phospholipids can be separated by TLC into fractions differing by their fatty acyl residues (20). Monolayers of phosphatidylcholine show different isotherms, depending on the saturation of their fatty acids; for the same number of double bonds, the surface area is much larger when they are distributed between the two hydrocarbon chains than when they are all on one chain only (13, 21, 22). Feher, Collins, and Healy (23) have described similar results with monolayers of fatty acids. Table 2, in the present work, shows a correlation between the molecular areas at 20 mN m^{-1} and the percentage of unsaturated fatty acids; for molecular areas of 101, 112, and 143 Å^2 molecule^{-1}, those percentages are 95, 98.5, and 99.5, respectively.

Therefore, it is clear by now that the procedures described here yield cardiolipin, that it is as pure as technically possible, and that the differences between the surface pressure-area isotherms of cardiolipins from different sources stem from differences in their fatty acid composition, particularly the degree of unsaturation.

The fatty acid composition of the cardiolipin samples was determined by Dr. P. E. Dussault from the Department of Biochemistry, Université Laval, Québec. We wish to thank Dr. Dussault for his skillful help. The authors thank Dr. P. Tancrède for helpful discussions. We appreciate the financial support of the Natural Science and Engineering Research Council Canada and of the Ministère de l'Education du Québec. This work was made possible by the cultural exchange program between Belgium and Québec.

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