Novel preparation of cardiolipin from beef heart

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SUMMARY A new method is described for the isolation of beef heart cardiolipin. A lipid-protein complex, rich in cardiolipin, is obtained by a one-step solvent fractionation of the tissue total lipid extract. Cardiolipin in the complex is largely freed of protein by salt denaturation and is further purified by gel filtration on Sephadex LH-20 followed by column chromatography on bicarbonate-treated silicic acid. The highly purified product is obtained as the sodium salt in a yield of 85–100 mg/100 g of fresh tissue.

SUPPLEMENTARY KEY WORDS Sephadex LH-20 · gel filtration · proteolipid

THE ISOLATION of cardiolipin from beef heart was originally reported by Pangborn (1). Subsequently, many procedures for the preparation of this phospholipid from mammalian tissues have been described. These methods generally involve either extensive solvent fractionation (2, 3) or, alternatively, silicic acid (4–6) or DEAE column chromatography (7). More recently, cardiolipin has been purified from beef heart (8) and brain (9) by procedures which employ chromatography on silicic acid treated with sodium bicarbonate.

The present method for the preparation of cardiolipin developed out of an investigation of beef heart proteolipid (10). In the course of these studies, it was found that approximately 50% of tissue cardiolipin was recovered in the isolated lipid-protein complex and that this phospholipid accounted for 60% of the lipid moiety of the proteolipid. This observation was the basis for a new reproducible procedure for obtaining highly purified beef heart cardiolipin in good yield by a combination of solvent fractionation and column chromatography.

Analytical Methods. Determinations of phosphorus and nitrogen were performed as previously described (10). Fatty acyl ester content was assayed according to Snyder and Stephens (11) with tripalmitolein (Applied Science Laboratories Inc., State College, Pa.) as standard. Mild methanolic alkaline hydrolysis of phospholipids was performed using the conditions given by Wells and Dittmer (12). The water-soluble hydrolysis products were recovered and separated in two dimensions on Whatman filter paper No. 43, essentially according to Dawson, Hemington, and Davenport (13). GLC of fatty acid methyl esters was carried out on a column of diethylene glycol succinate on Chromosorb W (Applied Science Laboratories Inc.) with a Model 609 F & M gas chromatograph.

To determine Na and K, cardiolipin preparations in chloroform–methanol 2:1 were washed with 0.2 volume of N HCl, and the upper aqueous phase was recovered. The lower phase was washed twice with two-thirds its volume of chloroform–methanol–N HCl 3:48:47. The combined upper phases were dried in vacuo, and the residue was redissolved in 1% La_2O_3 containing 0.64 N HCl. The ions were measured on a Perkin–Elmer 303 Atomic Absorption Spectrophotometer.

Preparation of Total Lipid Extract. 200 g of fresh or frozen beef heart ventricle was minced and extracted with 19 volumes of chloroform–methanol 2:1 in a Waring Blendor for 2 min. The mixture was filtered by gravity through Whatman filter paper No. 1, and the residue on the filter was reextracted with 2 volumes of solvent. 0.2 volume of 0.9% aqueous NaCl was added to the combined filtrates, and the phases were mixed 10 times by gentle inversion. After centrifugation at 900 *g* for 15 min, the upper phase was discarded, and the lower phase was washed once with two-thirds its volume of chloroform methanol–0.9% NaCl 3:48:47 (14). The washed lower phase (2800 ml) was concentrated to one-fifth its original volume in a rotary evaporator at less than 40°C.

Solvent Fractionation. The concentrated lipid extract was cooled to −10°C, and 4 volumes of chilled diethyl ether–ethanol 1:1 were added. After 2 hr the copious precipitate of proteolipid which formed, was collected by centrifugation at −10°C for 30 min at 250 *g*, and was washed with 2 volumes of diethyl ether–ethanol 1:1. The damp residue was immediately dissolved in chloroform–methanol 2:1 at room temperature, and the volume was made to 500 ml.

Salt Denaturation of Protein. 100 ml of 0.1 M tripotassium citrate was added to the clear, yellow-red solution of proteolipid (15). The two phases were mixed 10 times by inversion and separated by centrifugation at 250 *g* for 20 min. The aqueous upper phase was removed by aspiration, and the lower phase was carefully decanted from the interface. The lower phase was shaken once with two-thirds its volume of chloroform–methanol–0.9% NaCl 3:48:47; the upper phase was discarded, and the interface was rinsed once with the same mixture. The lower phase was dried in vacuo on a rotary evaporator. The residue was taken up in 50 ml chloroform–methanol 2:1 and centrifuged to remove additional denatured protein. The supernatant was again dried in vacuo, and the residue was redissolved in 10–20 ml of the same solvent. A small amount of insoluble material was removed by centrifugation. The clear, nearly colorless solution, designated crude cardiolipin, contained 10–13 mg of P,

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

the bulk of which was accounted for by cardiolipin. Small amounts of protein and neutral lipid, presumably triglyceride, were also present.

Gel Filtration on Sephadex LH-20. The crude cardiolipin solution was applied to a column of Sephadex LH-20 which had been packed and extensively washed with chloroform–methanol 2:1. The column was eluted with this solvent at a flow rate of 1 ml/min. Fractions were examined by TLC on 0.25 mm thick Silica Gel H plates (Analtech, Inc., Wilmington, Del.) developed in chloroform–methanol–acetic acid–water 80:13:8:0.3 (16). Spots were visualized with iodine vapor or by spraying the plates with 50% H_2SO_4 followed by heating at 100°C. Most residual protein emerged immediately after the void volume (Fig. 1), closely followed by neutral lipid, lecithin, and phosphatidylethanolamine. Cardiolipin was eluted in later fractions and was partially separated from phosphatidylinositol which emerged last. Those fractions which contained cardiolipin, but no detectable phosphatidylethanolamine, were pooled and dried in vacuo. The residue was redissolved in 8 ml of chloroform. The product contained 75–80% of P applied to the column and was composed of 85% cardiolipin and 15% phosphatidylinositol, with only traces of protein.

Chromatography on Bicarbonate-Treated Silicic Acid. A 2 × 20 cm column was prepared from a silicic acid–Hyflo Supercel mixture treated with $NaHCO_3$ (17) and washed with chloroform. The solution of partially purified cardiolipin (about 0.5 mg of lipid P per g of adsorbent) was applied, and 300 ml chloroform was run through the column at a flow rate of 1.0–1.5 ml/min,

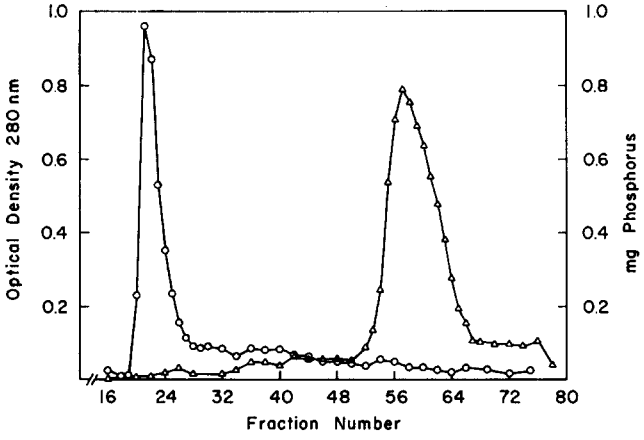

FIG. 1. Gel filtration of crude cardiolipin on Sephadex LH-20. A solution of crude cardiolipin (9.8 mg P) in chloroform–methanol 2:1 was placed on a 3 × 100 cm column which was then eluted with the same solvent mixture. Fractions (10 ml) were examined: (*a*) absorption at 280 nm, -O-, (*b*) P content, -△-, and (*c*) TLC. For further details, see text. In a typical separation, neutral lipid and lecithin were found in fractions 24–26, phosphatidylethanolamine in fractions 28–32, cardiolipin in fractions 30–64, with the peak in fraction 57, and phosphatidylinositol in fractions 57–76.

TABLE 1 FATTY ACID COMPOSITION OF CARDIOLIPIN

	Preparation 1	Preparation 2
	mole %	
16:0*	0.6	0.6
16:1	2.6	2.6
18:0	0.6	0.9
18:1	5.9	5.4
18:2	78.7	81.6
18:3	6.6	6.1
Other	5.2	3.1

* Number of carbon atoms: number of double bonds.

maintained if necessary with gentle N_2 pressure. The cardiolipin was then eluted with 300 ml of chloroform–methanol 9:1, and the effluent was dried in vacuo. The product was dissolved in a few milliliters of chloroform and stored at −10°C. No decomposition of the preparation was apparent after storage for up to 4 months as judged by TLC.

Characterization of the Product. The over-all yield of cardiolipin in three preparations ranged from 85 to 98 mg per 100 g of fresh tissue. The product was homogeneous on TLC plates developed in chloroform–methanol–acetic acid–water 80:13:8:0.3 unless the plates were heavily overloaded; in this case a trace of phosphatidylinositol could be identified.

Analyses of these preparations were as follows:

calculated (for tetralinoleyl disodium cardiolipin):
C, 65.15; H, 9. 52; P, 4.16; N, 0.00
found: Preparation 1: C, 61.39; H, 9.02; P, 3.79; N, 0.11
Preparation 2: C, 63.61; H, 9.80; P. 3.80; N, 0.09
Preparation 3: C, 63.42; H, 8.91; P, 4.04; N, 0.06
Molar ratios:
calculated: ester/P, 2.00; Na/P, 1.00; K/P = 0
found:
Preparation 1: ester/P, 1.80; Na/P, 1.05; K/P = 0
Preparation 2: ester/P, 1.94; Na/P, 0.92; K/P = 0
Preparation 3: ester/P, 2.11; Na/P, 0.73; K/P = 0

The fatty acid composition of two of the preparations is shown in Table 1. The distribution of P in alkaline hydrolysis products in three preparations was as follows: cardiolipin 98.2–98.7%, phosphatidylinositol, 0.2–1.6%; phosphatidylethanolamine (detected in one preparation only), 0.8%; unidentified, trace.

The nature of the nitrogen-containing contaminant is unknown. No amino acids were detected when a 4 mg sample of purified cardiolipin was subjected to acid hydrolysis followed by paper chromatography of the water-soluble hydrolysis products. The presence of a significant amount of a sphingosine-containing phospholipid is unlikely since more than 99% of P in the product was converted to a water-soluble form on alkaline hydrolysis.

Discussion. Existing methods for the isolation of cardiolipin are generally quite laborious. Moreover, column chromatography of this substance on untreated silicic acid or DEAE, according to several published procedures, involves the use of acidic conditions which could degrade the phospholipid. The present procedure is simple, reproducible, and avoids exposure of the product to acid.

A disadvantage of the method is that only 50% of cardiolipin in the tissue total lipid extract is recovered in the precipitated proteolipid. Variations in conditions of solvent fractionation failed to improve the recovery of cardiolipin in the lipid-protein complex. Nonetheless, the over-all yield of product is comparable with that of other procedures, and the precipitation step is a rapid and efficient means for concentrating the phospholipid.

The use of Sephadex LH-20 to fractionate phospholipids has not been previously described, although recently the separation of the phospholipids of *Escherichia coli* on DEAE Sephadex LH-20 has been reported (18). In the present method, gel filtration of crude cardiolipin on Sephadex LH-20 not only substantially purifies the product, but also has the advantage that the column may be reused if it is exhaustively washed with the eluting solvent after each chromatographic separation. In a series of fractionations on the same column, the volume of solvent required to elute cardiolipin varied slightly. However, the sequence of elution and the extent of separation of phospholipids were always the same.

Attempts to chromatograph crude cardiolipin on bicarbonate-treated silicic acid without prior gel filtration failed to yield a homogeneous product. In the procedure adopted, cardiolipin was eluted from the silicic acid column as a sharp peak. The traces of phosphatidylinositol usually seen in the final preparation were present only in those fractions which contained cardiolipin. The presence of this impurity in the product was not the result of overlapping peaks since the bulk of phosphatidylinositol was eluted from the column by a different solvent mixture.

This study was supported by U.S. Public Health Service Grant NS-00130 and was performed during the tenure of an Established Investigatorship to Dr. Joseph Eichberg from the American Heart Association.

Manuscript received 13 January 1970; accepted 3 April 1970.

References

1. Pangborn, M. C. 1942. *J. Biol. Chem.* **143:** 247.
2. Pangborn, M. C. 1945. *J. Biol. Chem.* **161:** 71.
3. Faure, M., M. J. Morelec-Coulon, J. Maréchal, and L. LeBorgne. 1959. *Bull. Soc. Chim. Biol.* **41:** 101.
4. McKibbin, J. M., and W. E. Taylor. 1952. *J. Biol. Chem.* **196:** 427.
5. MacFarlane, M. G. 1961. *Biochem. J.* **78:** 44.
6. Gray, G. M., and M. G. MacFarlane. 1958. *Biochem. J.* **70:** 409.
7. Rouser, G., G. Kritchevsky, D. Heller, and E. Lieber. 1963. *J. Amer. Oil Chem. Soc.* **40:** 425.
8. Takahashi, T., T. Hashimoto, and I. Hara. 1967. *Bull. Soc. Chim. Biol.* **49:** 65.
9. Yabuuchi, H., and J. S. O'Brien. 1968. *J. Neurochem.* **15:** 1383.
10. Eichberg, J. 1969. *Biochim. Biophys. Acta.* **187:** 533.
11. Snyder, F., and N. Stephens. 1959. *Biochim. Biophys. Acta.* **34:** 244.
12. Wells, M. A., and J. C. Dittmer. 1966. *Biochemistry.* **5:** 3405.
13. Dawson, R. M. C., N. Hemington, and J. B. Davenport. 1962. *Biochem. J.* **84:** 497.
14. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
15. Webster, G. R., and J. Folch. 1961. *Biochim. Biophys. Acta.* **49:** 399.
16. Skipski, V. P., M. Barclay, E. S. Reichman, and J. J. Good. 1967. *Biochim. Biophys. Acta.* **137:** 80.
17. Rathbone, L., and P. M. Maroney. 1963. *Nature (London).* **200:** 887.
18. Dittmer, J. C. 1969. *J. Chromatogr.* **43:** 512.