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Cardiolipin: A stereospecifically spin-labeled analogue and its specific enzymic hydrolysis

(diphosphatidylglycerol/phospholipid/phospholipase/electron spin resonance/biological membranes)

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ABSTRACT **The spin-labeled cardiolipin 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(16-doxylstearoyl)glycero(3)phospho]-*sn*-glycerol has been prepared. The stereoselective synthesis makes use of the monolysocardiolipin 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-lyso-*sn*-glycero(3)phospho]-*sn*-glycerol, available from the stereospecific hydrolysis of cardiolipin by phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) of *Trimeresurus flavoviridis*. The results of treatment of the spin-labeled cardiolipin with the cardiolipin-specific phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) (*Hemophilus parainfluenzae*) of known specificity and with phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) of *Bacillus cereus* are consistent with the assigned structure. The spin-labeled cardiolipin is further characterized and the unique features of this diastereomer are discussed in the context of the unusual stereochemistry of the natural phospholipid.**

Cardiolipin was isolated from beef heart and identified as the Wasserman antigen by Pangborn (1) in 1942. The structure (Fig. 1), that of a diphosphatidylglycerol, was subsequently verified by synthesis (2). Shortly after its isolation, its nearly unique association with mitochondria was recognized (3). Cardiolipin has been found associated with purified intrinsic mitochondrial membrane enzymes such as cytochrome oxidase (4) and cytochrome *c* reductase (5).

Spin-labeled fatty acids (6) and spin-labeled phospholipid analogues (7) have been useful in characterizing the structure of the lipid portion of biological membranes. The recent demonstration of a boundary layer (8, 9) is a good illustration of the power of the technique. Availability of a spin-labeled cardiolipin would allow application of this technique to study associations of cardiolipin with biological membranes.

The stereochemistry of cardiolipin has been of continuing interest; LeCocq and Ballou (10) deduced from classical stereochemical arguments that cardiolipin must have the *lyxo* form, *i.e.*, both terminal glycerol moieties must possess the same absolute configuration, because the compound is optically active. The absolute configuration of cardiolipin was demonstrated by synthesis (2) and by degradation (11). Some of the consequences of the symmetry of cardiolipin have been discussed (12): "The consequences of the diastereotopicity of corresponding groups in cardiolipin are two-fold: (1) the nuclear magnetic resonances of any corresponding atoms or groups of atoms in this molecule must be anisochronous, and (2) the rate of reaction of any corresponding atom or groups of atoms in this molecule under achiral or chiral conditions must be different." The former premise has been demonstrated (12, 13). The latter premise was demonstrated (11) by the stereospecificity of the cardiolipin-specific phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4).

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MATERIALS AND METHODS

Cardiolipin (bovine), crude venom containing phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) from *Trimeresurus flavoviridis* (habu), and phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* were purchased from Sigma Chemical Co. Bacterial cardiolipin was a product of Supelco, Inc. 16-Doxylstearic acid was purchased from Syva Research Chemicals. The methyl ester of this spin-labeled fatty acid was found to be greater than 98% homogenous by isothermal gas–liquid chromatography (Hewlett–Packard model 5830A) [250°, 25 ml/min on a 6-foot (1.8-m) column of 10% Silar-5-CP 80/100 Chromsorb WHP (Applied Science)]. The Faraday technique (14), using standard diamagnetic corrections (Pascal's Constants) (15) based on the known composition of 16-doxylstearic acid, gave the theoretical number of unpaired electrons per mole for this compound. Thin-layer chromatographic plates were purchased from Supelco (Redicoats, silica gel H, no binder). All other solvents and reagents were of analytical reagent grade or better and were purchased from usual commercial sources. The solvents used in the chemical acylation were all chemically dried and redistilled before use. Monolysocardiolipin was prepared by treatment of authentic cardiolipin with phospholipase A_2 from habu crude venom according to the method of Okuyama and Nojima (16). The incubation mixture contained 100 mg cardiolipin in a volume of 100 ml of 95% ethanol. To this was added 5.0 ml of 0.15 M sodium borate buffer (pH 7.0) containing 2 mM calcium acetate and 2.0 mg of crude venom. The incubation mixture was shaken gently for 30 min at room temperature, and 50 ml of 2% acetic acid in methanol was added to quench the reaction. The mixture was evaporated to dryness under reduced pressure at 40° with the occasional addition of chloroform to completely remove all traces of acetic acid. The residue was redissolved in chloroform and the lipids were extracted (17), using 10 mM Na_2EDTA (pH 7.0) as the aqueous phase. The monolysocardiolipin was isolated from the reaction mixture by chromatography on a bicarbonate-treated silicic acid column (16). It was purified to homogeneity by preparative-thin layer chromatography using an acidic solvent system: acetone/chloroform/methanol/glacial acetic acid/water (40:30:10:10:5 vol/vol). A basic solvent system: chloroform/methanol/concentrated ammonium hydroxide (65:25:5 vol/vol) was also used. The purified monolysocardiolipin migrated as a single spot on

Abbreviations: Cardiolipin is diphosphatidylglycerol or 1-(3-*sn*-phosphatidyl)-3-(3-*sn*-phosphatidyl)-*sn*-glycerol. Monolysocardiolipin designates either 1-[1-acyl-2-lyso-*sn*-glycero(3)phospho]-3-(3-*sn*-phosphatidyl)-*sn*-glycerol or 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-lyso-*sn*-glycero(3)phospho]-*sn*-glycerol. Dilysocardiolipin designates 1-[1-acyl-2-lyso-*sn*-glycero(3)phospho]-3-[1-acyl-2-lyso-*sn*-glycero(3)-phospho]-*sn*-glycerol. 16-Doxylstearic acid is the spin-labeled stearic acid analogue, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyl-*N*-oxyl.

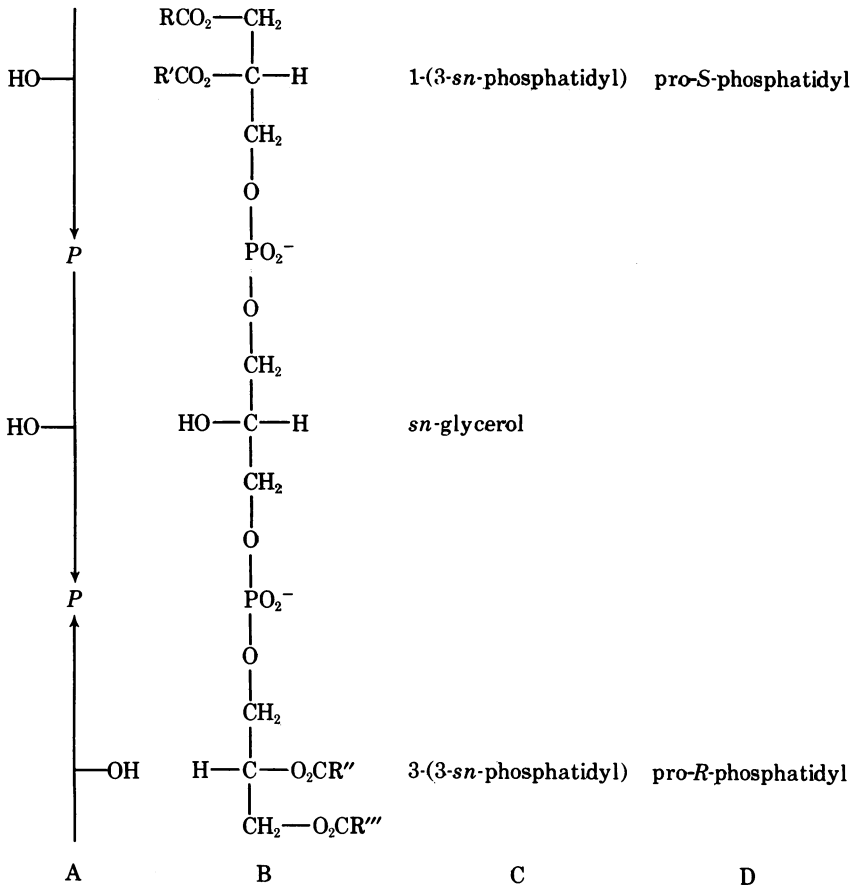

FIG. 1. Stereochemistry of cardiolipin. Column A shows the absolute configuration of the glycerols comprising the cardiolipin, whose Fischer projection is shown in B. The arrowhead designates the 3 carbon of *sn*-glycerol; *P* = phospho. Note the absence of a plane of symmetry in this *lyxo* configuration. Column C names the groups according to the IUPAC–IUB Commission on Biochemical Nomenclature (24). Column D gives the names according to the Cahn–Ingold–Prelog conventions (25).

thin-layer chromatograms in the two solvent systems. A starting amount of 136 μmol of cardiolipin phosphorus (100 mg lipid, dry weight) typically provided 10–14 μmol pure monolysocardiolipin (7–10% yield).

The monolysocardiolipin was acylated by first preparing the symmetric anhydride (18) of 16-doxylstearate. The acylation was carried out using Na_2O catalyst; however, we added the Na_2O in a solution of bis(2-methoxyethyl) ether at a concentration such that 0.25 ml of suspension contained 0.43 mol Na_2O per mol of phospholipid. This produced better mixing of the catalyst and reactants. The bis(2-methoxyethyl) ether was removed under reduced pressure and the reaction mixture was returned to the sonicating oil bath and allowed to react for 36 hr. The yields (~20% based on phosphorus) are comparable to those obtained by the acylation of lysophosphatidylcholine. More recently we have employed the method of Boss *et al.* (19) for this acylation, with improved yields (50–60%). The spin-labeled cardiolipin was purified from the reaction mixture by extraction (17) followed by preparative thin-layer chromatography in the acidic solvent system.

Spin-labeled cardiolipin was characterized by determination of the ratio of fatty acyl ester groups (20) to lipid phosphorus (21), using AMP as the primary standard, and by treatment with phospholipase C and the cardiolipin-specific phospholipase D of *Hemophilus parainfluenzae* (11, 22). The labeled cardiolipin was treated with phospholipase C from *B. cereus* (23). The labeled cardiolipin (0.69 μmol phosphorus) was mixed with bovine cardiolipin (1.6 μmol phosphorus) as carrier. The lipid was added as a suspension in 0.4 ml of Tris·HCl (pH 7.3) to the incubation mixture containing 2.0 units of enzyme and 1.0 ml of diethyl ether in a total volume of 1.6 ml. The mixture was incubated at 37° for 120 minutes, then evaporated to dryness under vacuum with the addition of $CHCl_3$ to aid in the removal of water. The lipids were extracted from the dried residue with the solvent system of Bligh and Dyer (17).

The reaction products were separated by thin-layer chromatography on silica gel G in petroleum ether/diethyl ether/acetic acid (80:20:1 vol/vol) to isolate the diacylglycerol product or on silica gel H in the previously described acidic solvent system to isolate the phosphatidylglycerol phosphate product. The zones corresponding to each compound were scraped and extracted (17). The extracts were evaporated under N_2 to dryness, taken up in 0.5 ml of $CHCl_3$, and degassed with argon, and the spectra were recorded. The concentration of spin-labeled cardiolipin and its derivatives was estimated from the centerfield peak height (generally full scale) divided by the receiver gain. The peak height was an accurate measure of concentration under these conditions from the minimum detectable signal up to gain settings of 4×10^3 (spectral broadening from spin–spin interactions were observed at higher concentrations of spin label). In the experiments reported, as little as 0.5% of the total nitroxide radical could have been detected. Electron spin resonance spectra were collected using a Varian E-3 ESR spectrometer at a modulation amplitude of 1.0.

RESULTS AND DISCUSSION

The structure of cardiolipin is shown in Fischer projection in Fig. 1. The absolute configurations of the three glycerol moieties

Table 1. Characterization of monolysocardiolipin and spin-labeled cardiolipin as compared with authentic cardiolipin

Compound	μmol ester/ml	μmol lipid phosphorus/ml	Ester/lipid phosphorus ratios	
			Actual	Theoretical
Monolysocardiolipin	20	13	1.5 ± 0.1	1.5
Spin-labeled cardiolipin	3.9	1.7	2.3 ± 0.1	2.0
Authentic cardiolipin	11	5.1	2.2 ± 0.1	2.0

Values for ester and phosphorus represent the mean for three preparations, and the estimated significance is shown for the ratios.

comprising the cardiolipin backbone are indicated to the left. Two alternative systems of nomenclature are shown on the right. Although there are some advantages to the *R/S* convention,‡ we will adopt the most recent recommendations of the IUPAC–IUB for our discussion. Natural cardiolipin possesses no plane of symmetry§ (3-*sn*-phosphatidyl converts to 1-*sn*-phosphatidyl across a mirror plane. A 180° rotation in the plane of the paper is not a symmetry operation: the two 3-*sn*-phosphatidyl groups are interconverted, but the 2′ hydroxyl of the middle glycerol is interchanged with hydrogen). The two 3-*sn*-phosphatidyl groups are in diasteromeric environments and are in principle chemically distinguishable.¶.

From phospholipase A_2 treatment‖ of bovine cardiolipin (**1** in Fig. 2), monolysocardiolipin, an intermediate** in the complete hydrolysis to dilysocardiolipin, can be isolated (16). Monolysocardiolipin was characterized by ester/lipid phosphorus analysis (Table 1) and by thin-layer chromatography in two solvent systems. Only one of two diastereomeric monolysocardiolipins (which may not be resolved by thin-layer chromatography) should be present, **2** or **2′** (Fig. 2), depending on how effectively the phospholipase A_2 can distinguish the 1-(3-*sn*-phosphatidyl) from the 3-(3-*sn*-phosphatidyl) groups of cardiolipin.

The formation of a single diastereoisomer was demonstrated in a two-step process. The first step was the acylation of the monolysocardiolipin (**2** or **2′** or a mixture of **2** and **2′**) with 16-doxylstearic anhydride. This reaction leads to spin-labeled cardiolipin (**3** or **3′**, or a mixture of **3** and **3′**). The chromatographically homogenous, spin-labeled compound (less than 5% of the total nitroxide radical could be found outside the zone containing the spin-labeled cardiolipin) was characterized by ester/lipid phosphorus analysis (Table 1) and by thin-layer chromatography in two solvent systems (the spin-labeled cardiolipin exhibited an R_F slightly greater than bovine cardiolipin but the same as observed for *Escherichia coli* cardiolipin. The difference seems attributable to the replacement of a polyunsaturated fatty acid with the saturated, spin-labeled analogue.) The electron spin resonance spectrum (Fig. 3) is that anticipated for a lipid acylated with a doxylstearoyl group (7). A value of 1.09 nitroxide radicals per 2 mol phosphorus was obtained by comparing the centerfield peak height of the spin-labeled cardiolipin in chloroform with that given by a known weight of 16-doxylstearic acid in the same solvent.

We have considered the possibility that other spin-labeled cardiolipins could result if acylation had occurred at the 2 hydroxyl group on the central glycerol of monolysocardiolipin (Fig. 1) exclusively or together with acylation where the acyl group had been removed by hydrolysis. Acylation at both the lysophosphatidyl group and at the central glycerol would provide a biradical, acyl cardiolipin. The electron spin resonance spectrum of the product we obtained (Fig. 3) is that of a monoradical. The criteria of one nitroxide radical per two atoms of phosphorus and the chromatographic identity of the product we obtained as cardiolipin in a solvent system capable of resolving acyl cardiolipin and cardiolipin (2) allow us to rule out the possibility of acylation at both positions.

The possibility of the major product of acylation being an acyl monolysocardiolipin, with a spin-labeled acyl group on the 2 position of the middle glycerol, can be refuted as follows: Acyl cardiolipin is not a substrate for the phospholipase C of *B. cereus*, presumably because of functionalization of the 2 hydroxyl group of the central glycerol. The spin-labeled cardiolipin we have prepared is a good substrate for this phospholipase C providing, after 25% hydrolysis, diacylglycerol as the only spin-labeled product. This product cannot be obtained from the postulated spin-acylated monolysocardiolipin. The resistance of the 2 hydroxyl of monolysocardiolipin to acylation is consistent with the previously observed resistance of cardiolipin to further acylation (2).

The second step demonstrating the formation of a single diastereomer was treatment of the spin-labeled cardiolipin with phospholipase A_2 (habu). If the spin-labeled product had been a mixture of **3** and **3′** (the result of nonselective phospholipase A_2 hydrolysis of **1**), the same nonselectivity would result in monoylsocardiolipin containing spin label; *i.e.*, both **2** and **2′** would be formed from **3** and **3′**. After 80% hydrolysis of spin-labeled cardiolipin with good recovery of products and under conditions in which as little as 0.5% of the total nitroxide radical

‡ The *R/S* convention defines a sequence of groups according to priority rules based on atomic number. The assigned nomenclatures are then independent of their representations on paper. Moreover, the problems in nomenclature raised by cardiolipin, *i.e.*, two identical and chiral groups associated with a prochiral center, have been discussed explicitly (25). The *sn* nomenclature defines the numbering of molecules containing glycerol based on one particular Fischer projection of glycerol (26). Using the *sn* conventions, cardiolipin can be named as above, although the peculiar symmetry of cardiolipin has not yet been discussed explicitly.

§ In the subsequent discussion we will assume that the phosphatidyl groups of the bovine cardiolipin are identical (R = R′ = R″ = R‴ in Fig. 1), an assumption that need not hold *in vivo*.

¶ Chiral reagents, *i.e.*, enzymes, can distinguish between prochiral groups, *i.e.*, the 1- and 3-carbon atoms of glycerol (27). In particular, the phospholipase D could, in principle, distinguish the two *enantiomeric* phosphatidyl groups of (unnatural) cardiolipins, *i.e.*, the forms possessing a plane of symmetry (12). Thus the enzymic reactions to be discussed here cannot prove the diastereomeric nature of the phosphatidyl groups of cardiolipin; they do, however, demonstrate the intramolecular diastereomeric nature of the groups of spin-labeled cardiolipin.

‖ The phospholipase in the crude snake venom from *Trimeresurus flavoviridis*, the Indian pit viper or habu, has not yet been as well characterized as other phospholipases. However, this venom attacks cardiolipin at a much faster rate than the *Crotalus adamanteus* and *Naja naja* venoms, and the conditions of attack and the products have been well characterized (16). We have designated the activity as phospholipase A_2 in analogy with other snake venom phospholipases; in fact, our conclusions regarding cardiolipin stereospecificity would not materially change were habu venom found to contain phospholipase A_1. Our work and previous work (16) rule out the possibility of other types of phospholipase activities.

** A sequence in which both **2** and **2′** were formed but **2′** was very rapidly hydrolyzed to dilysocardiolipin might lead to the accumulation of **2**. The kinetic data of Okuyama and Nojima (16) show that monolysocardiolipin is formed at the same rate as cardiolipin is hydrolyzed. Dilysocardiolipin accumulates later. Their data are consistent with the suggestion that the phospholipase A_2 preferentially attacks the 3-(3-*sn*-phosphatidyl) as indicated above.

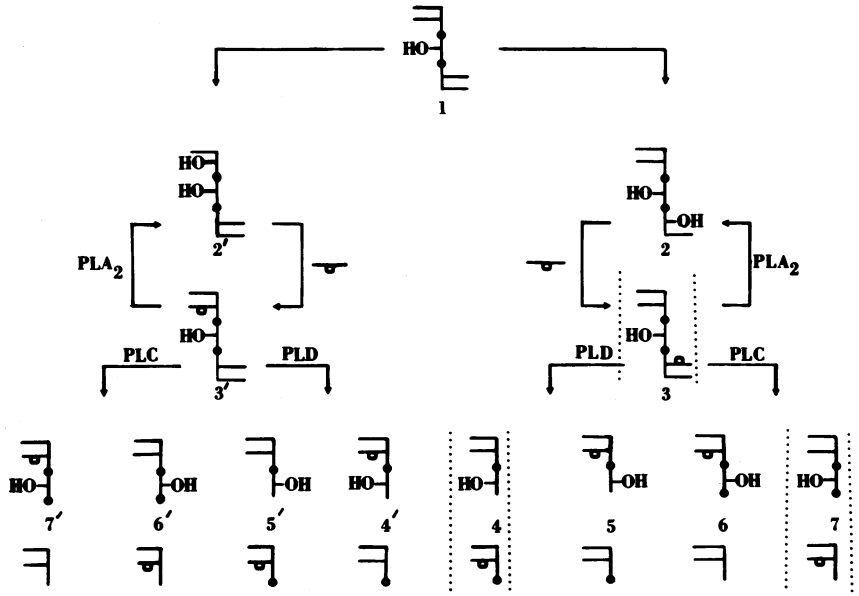

FIG. 2. Synthesis of stereospecifically spin-labeled cardiolipin and proof of structure. ●, Phospho. Structure 1 is cardiolipin in the same projection as Fig. 1. The two halves of the diagram are symmetrical, the left corresponding to phospholipase A_2 hydrolysis of the 1-(3-*sn*-phosphatidyl) group, the right, of the 3-(3-*sn*-phosphatidyl) group. The acylations of the monolysocardiolipins to form the spin-labeled cardiolipins 3 and 3′ are designated by the spin-label symbol ⌐⌐. Conversions back to 2 and 2′, respectively, by phospholipase A_2 (PLA_2) are shown. The products from treatment of 3 and 3′ with phospholipase C (PLC) of *B. cereus* and cardiolipin-specific phospholipase D (PLD) are indicated. The phosphatidylglycerol or phosphatidylglycerol phosphate are shown uppermost. Products 4 and 4′ are consistent with the known specificity of the phospholipase D. The correct structure of the stereospecifically labeled cardiolipin and the observed products are designated with dotted lines.

recovered could have been detected, we find no detectable nitroxide radical associated with monolysocardiolipin (Table 2). The data of Table 2 also rule out any acyl migration in the monolysocardiolipin (*sn*-1 to *sn*-2) between its preparation and acylation with 16-doxylstearate. We conclude that the phospholipase A_2 hydrolyses shown in Fig. 2, of cardiolipin, 1, to monolysocardiolipin, either 3 or 3′, were stereospecific for the 1-(3-*sn*-phosphatidyl) or for the 3-(3-*sn*-phosphatidyl) group.

The cardiolipin-specific phospholipase D specifically releases the 3-(3-*sn*-phosphatidyl) group from natural cardiolipin (11). The phosphatidylglycerol formed is the naturally occurring stereoisomer [1-(3-*sn*-phosphatidyl)-*sn*-glycerol]. Hydrolysis of spin-labeled cardiolipin provided spin-labeled phosphatidic acid only (Table 3). The isolated phosphatidylglycerol was not appreciably spin labeled, *i.e.*, less than 3.5%. Phospholipase D treatment of 3′ (Fig. 2) yields 4′ or 5′. Products 4′ do not contain spin-labeled phosphatidic acid; products 5′ do not contain the natural isomer of phosphatidylglycerol. Spin-labeled cardiolipin 3 is the only structure able to provide the experimentally observed products of phospholipase D hydrolysis, spin-labeled phosphatidic acid and the natural stereoisomer of phosphatidylglycerol. Phospholipase A_2 hydrolysis thus must occur predominantly, if not exclusively, in the 3-(3-*sn*-phosphatidyl) group of cardiolipin.

The stereochemical course of the phospholipase C hydrolysis under the conditions we employed on 3 (previously unknown) is now determinable. Spin-labeled diacylglycerol appears in product 7.

Should there be reason to expect differences in the chemistry of the diastereomerically spin-labeled cardiolipin described here in, e.g., its associations with membrane enzymes, it should be possible to prepare the other isomer. Acylation of dilyso-

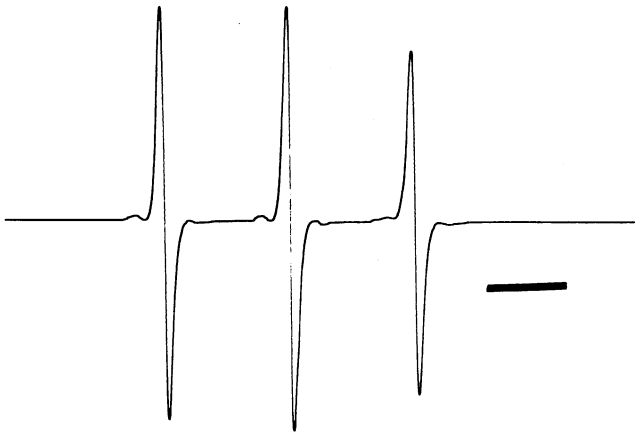

FIG. 3. Electron spin resonance spectrum of spin-labeled cardiolipin. High field is to the right. The bar indicates 10 gauss.

Table 2. Results of hydrolysis of spin-labeled cardiolipin by habu phospholipase A_2

Product	% total nitroxide radical	Phosphorus recovered	
		μg	%
Cardiolipin	18	7.0	16
Monolysocardiolipin	N.D.	5.4	12
Dilysocardiolipin	1	31	71
Fatty acid	80	0	0

The percent recovered nitroxide radical and lipid phosphorus were estimated as described in *Materials and Methods*. The digestion was carried out under routine conditions except that the incubation time was increased to 90 min. The digestion products were separated by the thin-layer chromatography in the acidic solvent system, and the zones corresponding to each compound were scraped and extracted (17). The extracts for each zone were concentrated to 0.5 ml, the spectra were recorded, and the sample was digested for phosphorus determination. Total recovery of the initial lipid phosphorus was 89%. N.D., not detectable.

Table 3. Results of hydrolysis of spin-labeled cardiolipin with the cardiolipin-specific phospholipase D

Compound	$10^6 \times$ peak height/gain	% nitroxide radical recovered
Cardiolipin	6.7	51
Phosphatidic acid	5.9	45
Phosphatidyl glycerol	0.46	3.5

The hydrolysis was carried out according to the method of Astrachan (11). The incubation mixture contained 0.5 μmol spin-labeled cardiolipin, 0.5 μmol *E. coli* cardiolipin as carrier, 2.0 ml of enzyme solution (2.0 mg protein), 2.0 ml of 10 mM $MgCl_2$ in 50 mM sodium phosphate buffer (pH 7.0), and 1.0 ml of tertiary butanol in a total volume of 5.0 ml. The mixture was incubated at 37° for 3 hr with gentle shaking and evaporated to dryness under reduced pressure with the addition of tertiary amyl alcohol to prevent foaming. The lipids were extracted into $CHCl_3$ by the method of Bligh and Dyer (17). A white precipitate (deoxycholate) was removed by centrifugation of the chilled $CHCl_3$ solution. The lipid hydrolysis products were separated by two-dimensional chromatography on silica gel H in the basic solvent system followed by the acid system. The zones corresponding to each compound (as judged using standards) were isolated and assayed for spins, and the percent label was estimated. The recovery of initial nitroxide radical was 79.4%.

cardiolipin with doxylstearic anhydride should provide cardiolipin spin labeled in both phosphatidyl groups (a biradical). Treatment with the habu phospholipase A_2 should remove the doxylstearate from the 3-(3-*sn*-phosphatidyl) group; reacylation of this spin-labeled monolysocardiolipin with an unlabeled fatty acid anhydride would provide the diastereomer to 3, 3′ (Fig. 2).

In summary, we have synthesized a spin-labeled cardiolipin stereospecifically labeled in the 3-(3-*sn*-phosphatidyl) end of the molecule. This synthesis and the characterizations were possible because of the stereospecificity of the phospholipase A_2 from habu and of the cardiolipin-specific phospholipase D for the 3-(3-*sn*-phosphatidyl) group of cardiolipin. We have also demonstrated the specificity (previously unknown) of the *B. cereus* phospholipase C for this same portion of the molecule. This preference results in the formation of the natural stereoisomer phosphatidylglycerol or phosphatidylglycerol phosphate. The biochemical significance is unclear. *In vivo* synthesis of spin-labeled cardiolipin from spin-labeled fatty acids has been observed (28), suggesting the similarity of spin-labeled fatty acids to the natural ones. We expect our synthesis to provide useful quantities of spin-labeled cardiolipin, which, together with spin-labeled phosphatidylcholine (7) and spin-labeled phosphatidylethanolamine (29), completes the availability of spin-labeled analogues of the major lipids of mitochondria and provides another important probe for the study of lipid–protein interactions. The availability of spin-labeled cardiolipin, and the recently synthesized chemically defined molecular species (2, 30) should facilitate further studies of the function of this unique phospholipid.

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