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PHYSICAL PROPERTIES OF BILAYER MEMBRANES FORMED FROM A SYNTHETIC SATURATED PHOSPHOLIPID IN *n*-DECANE

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SUMMARY

A pure phosphatidyl choline containing branched-chain, saturated acyl substituents has been prepared by a novel synthesis. Bilayer membranes formed from this non-autoxidizable, pure phospholipid are very stable and have an interfacial tension of 1.5 ± 0.1 dyne/cm at 24°. Both the electrical resistance and capacitance of the bilayer are pH dependent. The resistance has a maximum value of about $10^9\ \Omega/cm^2$ in the pH range 4–6. The specific capacitance has a minimum value of $0.38\ \mu F/cm^2$ at pH 4 with a limiting value of $0.43\ \mu F/cm^2$ at high pH. The basis for the pH dependence of these electrical parameters is unknown.

INTRODUCTION

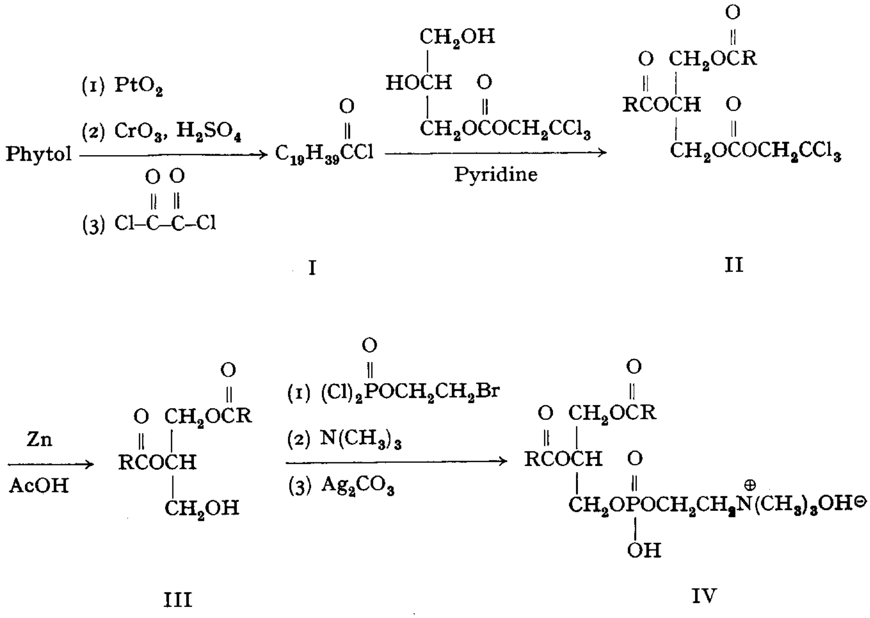
The view that a bimolecular lipid lamella constitutes the structural core of many biological membranes has provided impetus for the extensive studies of lipid bilayer systems carried out in recent years[1–3]. The bulk of this work has utilized lipids derived from natural sources. Phosphatidyl choline prepared from hen eggs, for example, has been frequently used because of the ease with which this material may be obtained. Two important disadvantages attend the use of natural products of this type. First, since these preparations invariably consist of mixtures of compounds which have similar physical properties but differ in their acyl substituents, the composition of bilayers formed from these preparations is not defined. Second, since the acyl moieties of these compounds always contain a degree of unsaturation, they are highly susceptible to autoxidation.

Chemically stable phospholipids with saturated straight-chain acyl substituents can be prepared fairly easily in pure form. However, if the fatty acid moieties are longer than palmitic acid, bilayers cannot be formed below about 50° (R. MACDONALD AND T. E. THOMPSON, unpublished data); for shorter-chain substituents, the water solubility of the phospholipid becomes appreciable[4].

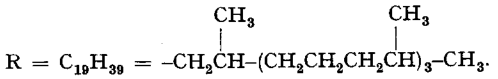
In this paper we describe some of the physical properties of bilayer membranes formed using 1,2-diphytanoyl-3-*sn*-phosphatidyl choline. This compound, prepared by a novel synthesis can be highly purified and is not autoxidizable.

MATERIALS AND METHODS

The saturated phospholipid, 1,2-diphytanoyl-3-*sn*-phosphatidyl choline, was synthesized by the following procedure (see Scheme 1).



Scheme 1. Synthesis of 1,2-diphytanoyl-3-*sn*-phosphatidyl choline (IV).



$$R = C_{19}H_{39} = -CH_2\overset{CH_3}{\overset{|}{C}}H-(CH_2CH_2CH_2\overset{CH_3}{\overset{|}{C}}H)_3-CH_3.$$

3,7,11,15-Tetramethylhexadecanoic (phytanic) acid chloride (I)

Phytol (25 g, George Uhe Co., New York, N.Y.) was reduced in a Parr shaker with a mixture of 600 mg of PtO_2 and 300 ml of methanol at an initial pressure of 50 lb/inch^2. After 2 h the catalyst was filtered and the filtrate concentrated to give the homogeneous, yellow, oily dihydrophytol. This was dissolved in 250 ml of acetone and treated dropwise at 0° with 60 ml of Jones reagent[5]. After stirring at 0° for 1 h, 20 ml of 2-propanol were added followed by about 500 ml of ice water, and the product was extracted into ether (5 × 200 ml). The combined ether extracts were washed with water, dried (Na_2SO_4) and concentrated to give about 25 g of crude phytanic acid[6]. The acid was of sufficient purity (thin-layer chromatography on silic gel G with cyclohexane–ethyl acetate (3:2, by vol.)) to carry on to the next stage. It could be purified by column chromatography on Woelm alumina (neutral grade, Activity III) using 2–5 % acetic acid in methanol as the eluent.

Phytanic acid (20 g) and oxalyl chloride (25 ml) were allowed to stand overnight protected from moisture and the phytanoyl chloride was isolated by a reported procedure[7].

1,2-Diphytanoyl-sn-glycerol-3-(β,β,β-trichloroethylcarbonate) (II)

The acid chloride (13.9 g, 0.0442 mole) was added dropwise at 0° to a solution of *sn*-glycerol-3-*β,β,β*-trichloroethylcarbonate[8], 20 ml of chloroform (distilled from P_2O_5) and 6.5 ml of anhydrous pyridine. The solution was then stirred at 25° for 18 h, and worked up and chromatographed by the method previously described[8] to afford 6.0 g (33 %) of the oily II; $[\alpha]_D^{25°} = -1.42°$ (*c* 5.0, chloroform).

Anal.: Calcd. for $C_{46}H_{85}Cl_3O_7$ (856.557): C, 64.50; H, 10.00; Cl, 12.42. Found: C, 64.45; H, 10.11; Cl, 12.42.

1,2-Diphytanoyl-sn-glycerol (III)

The carbonate II (5.3 g) was dissolved in 95 % acetic acid (15 ml) and ether (30 ml), the solution cooled to 20° and activated zinc (10 g) was added with vigorous stirring. The suspension was stirred at 20–25° for 1 h or until thin-layer chromatography indicated complete conversion of II to III. The 1,2-diglyceride was isolated by the reported procedure[8] and was of sufficient purity if used immediately in the phosphorylation step. An analytical sample was obtained by column chromatography on acid-washed Florisil impregnated with 10 % (w/w) of boric acid[9]. III is a colorless oil, $[\alpha]_D^{25°} = -0.6°$ (*c* 1.2, chloroform).

Anal.: Calcd. for $C_{43}H_{84}O_5$ (681.145): C, 75.82; H, 12.43. Found: C, 75.57; H, 12.38.

1,2-Diphytanoyl-3-sn-phosphatidyl choline (IV)

The method of EIBL *et al.*[10] was used to prepare the lecithin from the 1,2-diglyceride. From 11 g (0.016 mole) of III (freshly prepared from pure II and used without purification), 35 ml of anhydrous chloroform and 20 ml of anhydrous triethylamine, there was obtained 13.2 g of *O*-[1,2-diphytanoyl-*sn*-glycero-3-phosphoryl]-*β*-bromoethanol. This material was dissolved in 150 ml of dry 2-butanone containing 10 g of anhydrous trimethylamine and kept at 50° for 18 h. The reaction mixture was concentrated *in vacuo*, dissolved in methanol and filtered through a mixed bed of 300 g of Amberlite IR 45 (OH^- form) and IR 50 (H^+ form) resins. The column washings were concentrated to a glass which was dissolved in 100 ml of 90 % methanol and stirred with 10 g of Ag_2CO_3 for 1 h. The filtered solution was concentrated and chromatographed on 1.4 kg of a mixture of 2:1 (w/w) silica gel (Mallinckrodt, 100 mesh)–Supercel. The column was developed with 10–50 % of methanol in chloroform to give the white, waxy lecithin IV; $[\alpha]_D^{25°} = +7.8°$ (*c* 0.8, chloroform), R_F 0.83 on 0.25 mm silica gel GF plates with chloroform–methanol–water (65:25:4, by vol.).

Anal.: Calcd. for $C_{48}H_{98}NO_9P$ (864.259): C, 66.71; H, 11.43; N, 1.62; P, 3.58. Found: C, 66.41; H, 11.32; N, 1.70; P, 3.48.

The material used for bilayer formation was purified by repeated preparative thin-layer chromatography until it was judged pure with respect to the polar group. Brinkman Ltd. silical gel G plates were employed using the solvent system: chloroform–methanol–water (65:25:4, by vol.). Methyl esters of the acyl substituents of the purified phosphatidyl choline were prepared with boron trifluoride–methanol (14 % w/v)[11] and examined by gas–liquid chromatography using a Beckman GC4 with columns consisting of 15 % EGSSX on Gas Chrom P. The analysis of the methyl esters revealed a 99 % phytanic acid content.

The concentration of phospholipid in decane used for bilayer formation was

6 mg/ml, although this was not critical. The decane used was purchased from Lachat Chemicals and was greater than 99% pure. All other chemicals employed were analytical reagent grade.

Bilayer membranes were formed over a circular hole in a vertical wall of a Teflon pot similar to that described by HANAI *et al.*[12]. The method employed to form the membranes was a modification of the technique of VAN DEN BERG[13]. This entailed adding a small quantity of the membrane-forming solution to the aqueous electrolyte. A planar membrane could then be formed simply by raising the Teflon pot through the air–water interface and quickly returning it to a hydrostatic equilibrium position in the electrolyte. The efficient use of the membrane-forming solution made this technique preferable to the convential brush method.

Details of the apparatus used for the a.c. and d.c. electrical measurements are described elsewhere[14].

RESULTS AND DISCUSSION

The drainage behaviour of the diphytanoyl phosphatidyl choline membranes was similar to that described by HANAI *et al.*[12] for purified egg lecithin in decane membranes. The mechanical stability of the bilayers formed from the synthetic phosphatide was, however, better than that of egg lecithin bilayers and the lifetimes generally longer. This enhanced stability made it possible to determine the film tension by the hydrostatic bowing technique described by COOK *et al.*[15]. The value of the interfacial tension of the interfaces of the synthetic phospholipid bilayer in $1 \cdot 10^{-1}$ M KCl was found to be 1.5 ± 0.1 dyne/cm at 24°.

These bilayers have characteristically high d.c. specific resistances which do not vary with time over a period of several hours. In many cases it was possible to demonstrate that the measured d.c. resistance was an intrinsic property of the bilayer by bowing the membrane with a hydrostatic pressure differential and relating the d.c. conductance to the a.c. capacitance[16]. A typical current–voltage curve for the diphytanoyl phosphatidyl choline membranes is shown in Fig. 1. The curve has the

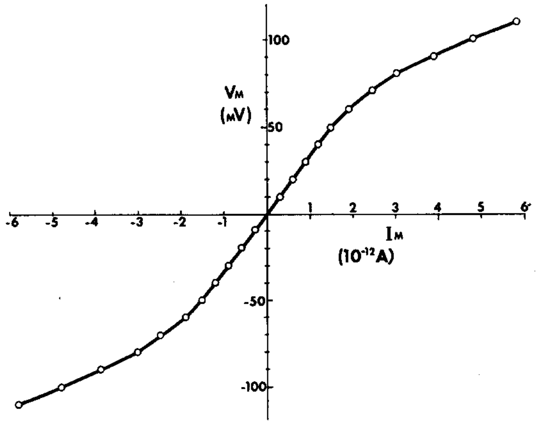


Fig. 1. Current–voltage characteristics of a diphytanoyl phosphatidyl choline membrane. Membrane area $7.9 \cdot 10^{-3}$ cm^2. Aqueous phase: $1 \cdot 10^{-2}$ M Tris–HCl (pH 7.5), $1 \cdot 10^{-1}$ M KCl, $1 \cdot 10^{-1}$ M NaCl and $2 \cdot 10^{-2}$ M $MgCl_2$. Temperature, 24°.

characteristic sigmoidal shape reported for bilayers of a wide variety of compositions. The central portion of the curve (± 40 mV) is essentially ohmic.

The Cole–Cole plot, Fig. 2, for a bilayer in $1 \cdot 10^{-2}$ M KCl is almost semicircular, illustrating that the bilayer membrane behaves as if it were a simple isotropic sheet[12, 17].

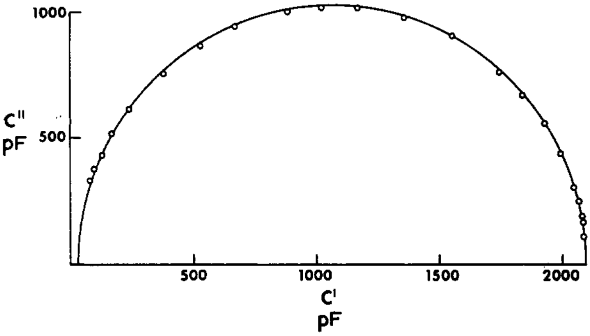


Fig. 2. Cole–Cole plot for a diphytanoyl phosphatidyl choline membrane. Membrane area $5.3 \cdot 10^{-3}$ cm². Aqueous phase: $1 \cdot 10^{-2}$ M KCl (pH 5.6). Temperature, 24°. The solid line corresponds to the true semicircle; characteristic frequency at 6.5 kHz.

A systematic investigation of the electrical properties of the bilayer as a function of the pH of the aqueous electrolyte revealed a dependence of both the specific resistance and specific capacitance on pH. The results obtained are plotted in Fig. 3. Both these curves agree qualitatively with the results obtained by OHKI AND GOLDUP[18] and OHKI[19] for egg lecithin bilayers. However, the d.c. resistances of the pure diphytanoyl phosphatidyl choline membranes are at least an order of magnitude higher than reported by OHKI AND GOLDUP[18]. In this respect the present values agree more closely with the values reported for such membranes by HANAI *et al.*[16] and ANDREOLI *et al.*[20]. Furthermore, the specific capacitance of the diphytanoyl membranes may

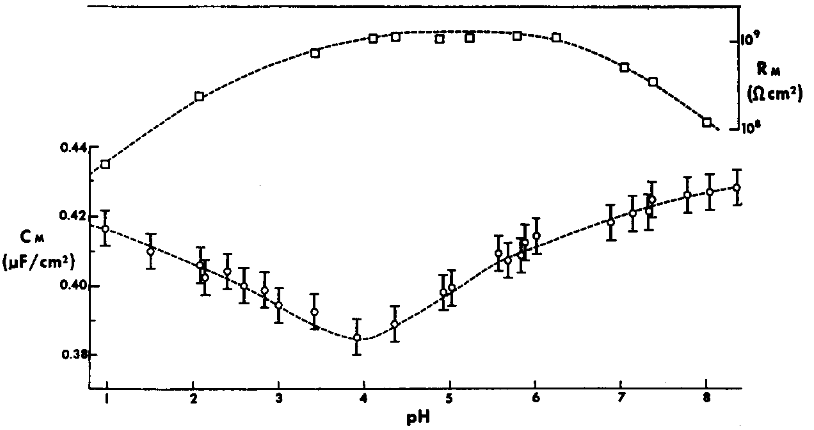


Fig. 3. Specific resistance (□) and specific capacitance (○) of diphytanoyl phosphatidyl choline membranes as a function of pH. Each point is the average of at least ten membranes. Aqueous phase: $1 \cdot 10^{-1}$ M KCl. Temperature, 24°.

reach a limiting value of 0.43 $\mu F/cm^2$ at high pH. Because of the chemical stability and purity of the synthetic phosphatide employed in these studies, it is very unlikely that the pH dependence of the electrical properties of this system is due to the presence of charged contaminants. It does not seem reasonable either to attribute the dependence to the titration of the primary phosphate or the quaternary ammonium groups of the phosphatidyl choline moiety. Certainly the p*K*'s of these groups are outside the pH range studied. The quaternary ammonium group is, in fact, a fixed charge cation behaving much like Na^+.

At the present time we have no explanation for the dependence of the electrical properties on the pH of the ambient aqueous phase. It may be significant, however, that the capacitance values obtained above pH 8 agree closely with the value reported by TAYLOR AND HAYDON[17] for a bilayer stabilized by n-C_{16} sorbitan monopalmitoleate. The linear correlation between capacitance and chain length of the stabilized molecules found by these two authors led them to conclude that the equilibrium thickness of the bilayer region was twice the fully extended chain length of the hydrocarbon regions of the stabilized molecule. This evidence suggests that the diphytanoyl phosphatide bilayers attain this limiting thickness only above pH 8. The dielectric breakdown voltage recorded for diphytanoyl phosphatidyl choline bilayers was usually in the range 150–250 mV over the complete pH range investigated.

Low cationic membrane potentials develop across the diphytanoyl lecithin membranes when an alkali metal chloride concentration difference is established across the membrane. The calculated values of the transferase number are similar to those previously reported for egg lecithin membranes[20].

The high mechanical stability and reproducible electrical properties, which do not vary with time over several hours establish this system as an attractive one for use in studies directly relevant to biological membranes[14].

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