Biochimica et Biophysica Acta, 291 (1973) 587–591

BBA 76188

A SYNTHETIC LECITHIN CONTAINING BRANCHED-CHAIN FATTY ACIDS: PHYSICAL PROPERTIES AND MEMBRANE STUDIES

MICHAEL E. JOHNSON[a], SIDNEY SIMON[b], JOHN W. KAUFFMAN[b] and ROBERT C. MACDONALD[c]

[a]*Department of Physics,* [b]*Deparment of Materials Science and* [c]*Department of Biology, Nortwestern University, Evanston, Ill. 60201 (U.S.A.)*

(Received August 7th, 1972)

SUMMARY

A phosphatidylcholine containing branched-chain, saturated acyl moieties has been prepared by a simple and inexpensive synthesis. Bilayer membranes formed from this phospholipid are non-autoxidizable and quite stable over a wide temperature range. The resistance is typically $1 \cdot 10^8\ \Omega/\text{cm}^2$ for moderate voltages. The specific capacitance is $0.42\ \mu\text{F/cm}^2$. Calorimetric and optical studies of lipid–water dispersions showed no phase transitions over the range −30– +75 °C. A phase change from an anisotropic to an isotropic phase over the range 75–95 °C was observed optically.

INTRODUCTION

The lipid bilayer model membrane system, since its introduction in 1962[1] has been investigated in a wide variety of membrane stability and permeability studies[2–5]. This work has been done mainly with lipids from natural sources. Two disadvantages attend the use of these natural lipids. First, since the acyl moieties of these lipids always contain some unsaturated bonds they are highly autoxidizable. Second, for optimum stability of the bilayers these lipids usually must be combined with materials such as cholesterol or tocopherol. The composition of the resulting membrane is then rather poorly defined.

The use of phospholipids with saturated straight-chain acyl components has not proven effective since the materials are difficult to use experimentally. If the fatty acid moieties are as long or longer than palmitic acid, bilayers cannot be formed below 40–50 °C[6–8]; for shorter-chain components the water solubility becomes appreciable for the phospholipid[9]. A phospholipid with saturated branched-chain acyl components, 1,2-diphytanoyl-3-*sn*-phosphatidylcholine, has been synthesized and shown to produce stable membranes at room temperature[7]. The procedure for synthesis, however, is relatively long, complex, and expensive.

In this paper we describe some of the physical properties of bilayer membranes consisting predominantly of 1,2-diisostearoyl-3-*sn*-phosphatidylcholine. This phospholipid is synthesized by a relatively simple and inexpensive procedure, is not autoxidizable, and forms stable bilayers without addition of other stabilizers.

MATERIALS AND METHODS

Glycerylphosphorylcholine-$CdCl_2$ complex was purchased from Nutritional Biochemical Corp. Isostearic acid was generously provided by Emergy Industries, Inc. (No. 975-D). The composition of this acid was specified to be: 70–76% isostearic acid, 6–7% isopalmitic acid, 7–11% myristic acid, 4–5% palmitic acid, 1–2% stearic acid, and 1–2% oleic acid. Methanol was chromatographic grade. Chloroform and decane were freed from polar impurities by passage over alumina. Water was twice distilled, the second time from glass. Other chemicals were reagent grade whenever possible.

The synthesis of the phospholipid 1,2-diisostearoyl-3-*sn*-phosphatidylcholine followed the general procedure outlined by Robles and Van Den Berg[10]. The details are as follows.

Isostearic anhydride was produced by the reaction between isostearic acid and dicyclohexylcarbodiimide in chloroform[11]. Sufficient chloroform was added to form a 1 M solution of the anhydride. Trimethylammonium isostearate was obtained by adding a methanol solution of trimethylammonium hydroxide to a methanol solution of the isostearic acid. This solution was then dried in a rotary evaporator and redissolved in sufficient chloroform to form a 1 M solution.

2.75 g glycerylphosphoryl-$CdCl_2$ (equivalent to 5.0 mmoles glycerophosphorylcholine) and approximately 50 ml glass beads (2 mm diameter) were put together in a 250-ml glass bottle. To this were subsequently added 80 ml of the anhydride and 30 ml of the trimethylammonium isostearate. The mixture was then rotated in a water bath kept in the temperature range 55–60 °C. Periodically, samples of the mixture were analyzed by thin-layer chromatography. A gradual increase in the amount of lecithin was observed, coinciding with a decrease in lysolecithin content. The reaction took approximately two weeks to go to completion. The increase in reaction time over that reported by Robles and Van Den Berg[10] may have been due to the substitution of the trimethylammonium salt of the fatty acid for the tetraethylammonium salt.

At the end of the reaction period the solution was washed five times in 200 ml of ethanol–water (1:1, v/v) to remove the $CdCl_2$. Upon addition of acetone, a preciptitate was obtained which was cooled to −20 °C overnight. The precipitate was washed with acetone and redissolved in chloroform. This solution was then loaded onto a silica gel column and the latter eluted with chloroform–methanol mixtures of increasing polarity. The lecithin was recovered as a homogeneous fraction according to thin-layer chromatographic analysis. After removal of solvent, the product weighed 1.6 g (40% of the theoretical amount).

For bilayer work, a 2% solution of this lecithin in *n*-decane was used. Membranes were formed over a circular hole in a vertical plexiglass wall between two aqueous chambers. The brush technique[11,12] was used.

Resistance was calculated from the current measured by a Kiethley 610C electrometer in series with the membrane and a constant voltage source. Capacitance was measured by a d.c. transient method described elsewhere[6]. Bilayer area was determined using a grid reticule in the eyepiece of the microscope.

Phase transitions were investigated on a Perkin-Elmer Differential Scanning Calorimeter, DSC-1, modified to give the characteristics of a DSC-1B. The temperature scale was calibrated from melting point standards obtained from James Hinton (Columbia, S.C.), while the ordinate was calibrated using Indium (99.999% pure, A. D. MacKay, Inc.) and Gallium (99.9999% pure, Fisher Scientific Company).

The lipid, stored in a chloroform–methanol solution (10:1, v/v) was placed in a volatile sample holder and dried under vacuum until the weight was constant. Water, 3 M aqueous $CaCl_2$, and ethylene glycol–water (1:1, v/v) solutions were then added to the lipid to give a weight ratio aqueous solution–lipid of 2:1. The lid was hermetically sealed to the sample holder, and the sample held at 60 °C for 2 h. The reference sample holder contained an amount of the water solution equivalent to that in the lipid–water dispersion. During the measurements the samples were heated or cooled at either 2.5 or 5 °C per min. Each sample was cycled three times to ensure equilibrium conditions and each experiment was done with three different samples. The temperature range −30–+80 °C was scanned.

RESULTS AND DISCUSSION

The drainage behavior of the diisostearoylphosphatidylcholine membranes followed the pattern described by previous bilayer membrane investigators[2,5,12]. The stability of membranes formed from this synthetic lecithin exceeds that of egg lecithin membranes. Membrane lifetime was typically several hours. The ability of the lecithin solution to form membranes between 6 and 40 °C was investigated and it was found that stable membranes could easily be obtained over the entire temperature range. As expected, the time required for thinning increased at low temperatures, but otherwise the behavior was the same over the entire temperature range. The range of stability may extend considerably beyond that which we have investigated.

The membranes have a high d.c. resistance (typically on the order of $1 \cdot 10^8\, \Omega/cm^2$ in the linear region) which decreases slightly during the first few minutes and then becomes constant for hours. A typical current–voltage curve is shown in Fig. 1. This curve has the sigmoidal shape expected where the conduction process involves an activation energy (E) modified by the energy due to the imposed potential (eV for monovalent cations). A simple derivation shows the current across a symmetrical barrier to be $I = P\exp(-E/kT)\sinh(\alpha eV/kT)$ where P is a proportionality factor. For a central barrier $\alpha = 0.5$; a least squares fit to the data of Fig. 1 gave $\alpha = 0.49$, implying good agreement with the model (see Kauffman and Meade[13] for further discussion). The current followed approximately the predicted temperature dependence. The central portion of the curve (± 50 mV) is essentially ohmic.

The capacitance was $0.42 \pm 0.01\ \mu F/cm^2$ (standard error of the mean for five measurements on each of five membranes), a value which is close to those reported for egg lecithin[2,14]. The capacitance was found to increase by approximatly 10% when the impressed voltage was increased from 25 to 75 mV. This increase is probably due to compression of the bilayer as suggested by White[15], though our measurement of the bilayer area is not sufficiently precise to rule out a contribution from an increase in the area.

With the exception of the water peak at 0 °C, no thermal transitions were observed by differential scanning calorimetry either for the monohydrate form of the lipid or for dispersions of the lipid in water, 50% ethylene glycol, or 3 M $CaCl_2$.

The purpose of the differential scanning calorimetry experiments in ethylene

glycol and $CdCl_2$ solutions was in the former case to eliminate the ice–water transition in order to check the possibility that the lipid transition occurred near the freezing point of water and in the latter case to raise the transition temperature in the event that the transition occurs below the lowest temperature of our measurements.

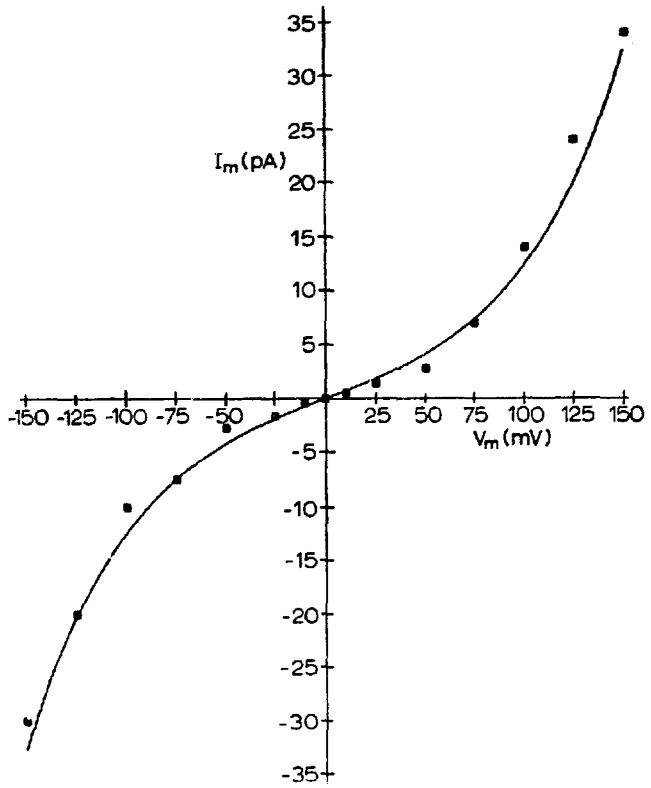

Fig. 1. Current-voltage characteristics of a diisostearoylphosphatidylcholine membrane. Membrane area $1.54 \cdot 10^{-2}$ cm^2. Aqueous phase: $1 \cdot 10^{-1}$ M KCl, $1 \cdot 10^{-5}$ M EDTA. Temperature 23 °C. The smooth curve is a computer drawn least squares fit of the relation $I = A \sinh(\alpha eV/kT)$ to the experimental data (□).

It has been shown (Simon, S., unpublished) and we have confirmed that the transition of dipalmitolyl-L-α-lecithin is unaffected by 50% aqueous glycol although the freezing point of the solution is shifted to very much lower temperatures. No transition whatsoever was observed in the glycol solution hence the lipid transition could not have been obscured by an ice peak. Differential scanning calorimetry of dipalmitoyllecithin has revealed that the transtion temperatire of this lipid is raised 25 °C by 3 M $CaCl_2$. Thus, if the diisostearoyl derivative is influenced in a comparable way, it means that the transition of the latter probably does not occur in the region of −30 to about −50 °C.

The lack of an obvious thermal transition is not particularly surprising in view of the heterogeneity of the fatty acids of commercial isostearic acid. Lipids with a distribution of fatty acid chain lengths from *Mycoplasma laidlawii*[16] and lipids rich in branched-chain fatty acids from *Micrococcus lysodeikticus*[17] have both shown broad thermal transitions. The distribution of chain types and degrees of saturation present in this lipid suggest that any thermal transition may be too broad to observe[18].

Using the heating stage of a Mettler FP5 and FP52 temperature controller in conjunction with a Leitz microscope and crossed polaroids, samples of the lipid–water dispersion were observed over the temperature range –20 – +100 °C. The only transition observed was one from a viscous anisotropic state (presumably liquid crystal) to an isotropic liquid. This transition was quite broad, extending from 75–95 °C. The crystal to liquid crystal transition for dipalmitoylphosphatidylcholine was easily observed under similar conditions.

The presence of one or two percent of oleic acid in the fatty acid starting material means that, strictly speaking, the lecithin we have prepared is not completely non-autoxidizable. In practice, this appears not to be a serious disadvantage. The lipid is a white powder and it undergoes no obvious discoloration even after being exposed to air for long times. Should it be necessary for critical investigations to eliminate all double bonds, pure isostearic acid (available from Lachat Chemical, 20200 Ashland Avenue, Chicago Heights, Ill.) could be utilized for the synthesis.

ACKNOWLEDGEMENT

This investigation was supported in part by Public Health Service Grant NS 09448.

REFERENCES

1 Mueller, P., Rudin, D. O., Tien, T. and Wescott, W. C. (1962) *Circulation* 26, 1167–1170
2 Tien, H. T. and Diana, A. L. (1968) *Chem. Phys. Lipids*, 2, 55–101
3 Castledon, J. A. (1969) *J. Pharm. Sci.* 58, 149–165
4 Mueller, P. and Rudin, D. O. (1969) in *Current Topics in Bioenergetics* (Sanadi, D. R., ed.). Vol. 3, pp. 157–249, Academic Press, New York and London
5 Tien, H. T. and James, L. K. (1971) *Chemistry of the Cell Interface* (Brown, H. D., ed.). Part A, pp. 205–253, Academic Press, London and New York
6 MacDonald, R. C. and Thompson, T. E. (1972) *J. Membrane Biol.* 7, 54–87
7 Redwood, W. R., Pfeiffer, F. R., Weisbach, J. A. and Thompson, T. E. (1971) *Biochim. Biophys. Acta* 233, 1–6
8 Stark, G., Benz, R., Pohl, G. W. and Janko, K. (1972) *Biochim. Biophys. Acta* 266, 603–612
9 Kögl, F., Dehaas, G. H. and Van Deenen, L. L. M. (1960) *Rec. Trav. Chim. Pays-Bas* 79, 661–674
10 Robles, E. C. and Van Den Berg, D. (1969) *Biochim. Biophys. Acta* 187, 520–526
11 Selinger, Z. and Lapidot, Y. (1966) *J. Lipid Res.* 7, 174–175
12 Mueller, P. and Rudin, D. O. (1969) in *Laboratory Techniques in Membrane Biophysics* (Passow, H. and Stämpfli, R., eds), pp. 141–156, Springer-Verlag, Berlin, Heidelberg and New York
13 Kauffman, J. W. and Mead, C. A. (1970) *Biophys. J.* 10, 1084–1089
14 Hanai, T., Haydon, D. A. and Taylor, J. (1964) *Proc. R. Soc., London, Ser. A* 281, 377–391
15 White, S. H. (1970) *Biophys. J.* 10, 1127–1148
16 Steim, J. M., Tourtellotte, M. E., Reinert, J. C., McElhaney, R. N. and Rader, R. L. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 104–109
17 Ashe, G. B. and Steim, J. M. (1971) *Biochim. Biophys. Acta* 233, 810–814
18 Phillips, M. C. and Hauser, H. (1972) *Chem. Phys. Lipids* 8, 127–133