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PROPERTIES OF LIPID BILAYER MEMBRANES MADE FROM LIPIDS CONTAINING PHYTANIC ACID

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Summary

Besides the preparation of phytanic acid (3,7,11,15-tetramethylhexadecylic acid) according to the Dumas-Stass reaction, the synthesis of four different lipids containing phytanic acid residues is described. Diphytanoyl phosphatidylcholine was synthesized beginning from glycerylphosphorylcholine, whereas the other lipids, diphytanoyl phosphatidylethanolamine, diphytanoyl phosphatidylserine and monophytanoyl glyceride were prepared by total synthesis.

Some properties of lipid bilayer membranes made from the lipids containing phytanic acid were investigated. The specific capacity of these membranes was measured. Its value of approximately 400 nF cm^{-2} was found to be similar to the value of membranes from lipids with unbranched fatty acid residues. Charge pulse experiments were performed using dipicrylamine as a molecular probe of membrane structure. The results were discussed on the basis of a higher viscosity of the membranes from lipids containing phytanic acid residues compared with unbranched fatty acid residues.

Introduction

Black lipid membranes as obtained by two different methods [1,2] have been used in the past as models for biological membranes and as tools for physico-chemical studies. The study of transport systems like ion carriers or lipophilic ions determined the structural properties of these membranes [3—7]. For these investigations in most cases lipids with unsaturated fatty acid residues have been used because it is only possible to form membranes with lipids in the liquid cristalline state, i.e. above the phase transition temperature [8]. Lipids with unsaturated fatty acids are not very stable and have to be handled very carefully because of a possible oxidation of the double bonds. For the formation of membranes from lipids with saturated hydrocarbon residues (C_{16}-C_{18}-chains) high temperatures are needed (40—50°C), whereas the stability of membranes composed of lipids with shorter saturated hydrocarbon chains (C_{12}-C_{14})

which have considerably lower phase transition temperatures [9], is poor.

An alternative possibility exists in the use of saturated fatty acids with branched chains, which have a considerably lower melting point than fatty acids with a straight chain [10]. Redwood et al. [11] showed that it is possible to form black lipid membranes from a phosphatidylcholine with two phytanic acid residues at normal temperature. In this publication we describe the synthesis of different lipids with phytanic acid residues and the preparation of the fatty acid. Phytanic acid (3,7,11,15-tetramethylhexadecylic acid) is not very common in organisms. Small amounts of this fatty acid have been found in milk or in marine animals [12]. A considerably higher amount was found in human patients with Refsum's disease [13]. This disease is presumably caused by a defect in the degradation of branched-chain fatty acids [14].

It has been shown in a number of publications [3—7,15,16] that it is possible to investigate structure and dynamics of membranes by introducing transport systems. Besides carrier systems [3,4], transport properties of lipophilic ions have been used for these studies [5—7,15,16]. Based on the results of electrical relaxation studies, it has been suggested that the transport of lipophilic ions occurs in distinct steps. Adsorption and desorption between membrane-solution interface and aqueous phase and exchange of lipophilic ions between the energy minima on both sides of the membrane across the central potential barrier [17]. Whereas previous kinetic studies with hydrophobic ions have been carried out with the voltage-jump technique, the experiments with lipophilic ions reported here were performed with the more recent charge-pulse method [7]. This method has, besides a better time resolution, the additional advantage that the pertubation of the membrane is small (voltage amplitudes <10 mV). In these experiments the membrane capacity is charged up to an initial voltage by a brief current pulse of about 50 ns duration [7]. Redistribution of ions within the membrane as well as conduction processes across the membranes lead to a decay of the membrane voltage with time. This time course may give information on the transport kinetics of lipophilic ions.

Materials and Methods

Preparation of the phytanic acid

The preparation of phytanic acid was carried out starting with phytol [18] (3,7,11,15-tetramethyl-2-hexadecen-1-ol). Freshly prepared Raney nickel [19] was added to 75 ml (64 g) phytol (Roth, Karlsruhe, G.F.R.) dissolved in 500 ml ethanol. This solution was hydrogenated in a shaker using a H_2 pressure of 100 cm H_2O during 12 h at room temperature [20]. The ethanol was evaporated after filtration of the catalyst. The reaction product was distilled in vacuum (0.2 mm Hg) at 146°C. 60 g dihydrophytol (a yield of 94%) was obtained with a refraction index $n_D^{20} = 14532$ which agrees with the value given in the literature of $n_D^{20} = 14539$ [21]. Phytanic acid has been obtained by oxidation of dihydrophytol in a solution of sulfuric acid with chromium VI oxide or with potassium permanganate in alkaline solution [22,21]. Both procedures have a small efficiency and the separation of by-products which are formed during the oxidation reaction is very difficult. Therefore the method of dehydrogenation of the alcohol in a melt of potassium hydroxide was used, a pro-

cedure which is known as Dumas-Stass reaction [23]. 20 g dihydrophytol and 30 g pulverised potassium hydroxide (Merck, Darmstadt, G.F.R., analytical grade) were heated to 250—265°C under stirring. After the H_2 evolution, the reaction mixture was cooled, acidified with hydrochloric acid and extracted with petrol ether. After evaporation of the petrol ether 18 g crude phytanic acid were obtained. The purification was carried out by vacuum destillation (b.p. 170°C at 0.1 mm Hg) or (with a higher degree of purity) by chromatography on a silica gel column (Merck, Art. No. 7734) with hexane/ether mixtures with increasing ether content (finally 85 : 15, v/v). The phytanic acid obtained in this way was a colorless viscous fluid which gave a single spot on a thin-layer chromatogramm. The refraction index n_D^{20} was 1.4518 (the literature being n_D^{20} = 1.4543 [24]). The yield as referred to phytol was between 80 and 90%.

For further characterisation of the phytanic acid, its methyl ester and the fatty acid amide were prepared. The methyl ester was found to be gas chromatographically homogeneous. Mass and infrared spectra were identical with earlier results [21]. The phytanic acid amide had a melting point of 51°C (literature: 51—53°C [24]). The fatty acid chloride was prepared using thionyl chloride in excess. The mixture was stirred over night under exclusion of humidity (from the air). After evaportation of the residual thionylchloride in vacuum, the pure phytanic acid chloride was used without further purification.

Synthesis of the lipids

L-1,2-Diphytanoyl-3-phosphatidylcholine was synthesized using the method of Baer and Buchnea [25] with some modifications introduced in order to increase the yield of the end product. 2 g L-α-Glycerylphosphorylcholine-$CdCl_2$ complex (prepared according to [26]) were added to 50 ml absolute CCl_4 together with 3 ml absolute pyridine and 6 ml phytanic acid chloride. This mixture was stirred for 3 days at room temperature. Thereafter 100 ml ether were added. The precipitated pyridine · HCl and $CdCl_2$ were separated by filtration and the supernatent was evaporated to dryness. The reaction product was dissolved in a small amount of chloroform and precipitated with acetone in ecess. The crude phosphatidylcholine was further purified by chromatography on a silica gel column with chloroform/methanol mixtures with increasing amounts of methanol. At a methanol content of 30% (v/v) chromatographically pure phosphatidylcholine was obtained (450 mg). The yield was approx. 15% as referred to glycerylphosphorylcholine.

The procedure of the lipids obtained by total synthesis differed in the phosphorylation method. Whereas in earlier published works [27,30] the reaction of diglycerides with $POCl_3$ (or derivates) have been used, the alternative possibility, the phosphorylation of protected base with phosphorus oxychloride, was carried out in this work, a method which has been described in ref. 28 for the synthesis of phosphatidylhydroxyproline. This method has the advantage that the protected base can be used in excess, thus increasing the yield.

The synthesis of D,L-1,2-diphytanoyl-3-phosphatidyl ethanolamine was carried out starting from D,L-1.2 diphytanoyl glycerol and *N*-carbobenzoxyethanolamine prepared according to refs. 29 and 30, respectively. To 1.95 g *N*-carbobenzoxyethanolamine dissolved in 30 ml absolute ether at 0°C, 4.5 ml

$POCl_3$ and 1.5 ml triethylamine were added. The mixture was warmed slowly to room temperature and then the white precipitate was filtered. The solvent was evaporated and the *N*-carbobenzoxyethanolamine phosphoryl dichloride was dried in high vacuum. The resulting colourless viscous fluid was dissolved in 20 ml CCl_4. 5 ml of this solution were added to 680 mg diphytanoylglycerol dissolved in 7 ml CCl_4 at 0°C. After addition of 0.3 ml triethylamine the mixture was stirred during 3 days. For successive precipitation of triethylamine-HCl and the remaining carbobenzoxyethanol phosphatidic acid ether and petrol ether were added. The phosphoric acid chloride was hydrolysed with an equivalent amount of H_2O. The dry phosphatidyl ethanolamine was dissolved in 40 ml glacial acetic acid and hydrogenated after addition of 800 mg palladium charcoal. The catalyst was filtered off and the crude phosphatidylethanolamine was purified by chromatography on a silica gel column with chloroform/methanol mixtures with increasing portions of methanol. At a methanol content of 20% (v/v) 300 mg chromatographically pure diphytanoyl phosphatidylethanolamine was obtained. The yield was about 37% as referred to diphytanoylglycerol.

The synthesis of D,L-1,2-diphytanoyl-3-phosphatidylserine was carried out starting from D,L-1,2 diphytanoyl glycerol and from *N*-carbobenzoxy-D,L-serine, prepared according to ref. 30. 23 g carbobenzoxyserine were dissolved in 100 ml absolute ethanol under addition of 11 g cyclohexylamine. This mixture was kept at 5—7°C for 2 h. Ether was added to the solution and the crystals of the cyclohexylammonium salt were filtered off, washed with ether and dried in an exsiccator. The salt had a melting point between 158°C and 160°C; the yield was 94%. 29 g cyclohexylammonium-salt of *N*-carbobenzoxy-D,L-serine mixed with 46 g benzylchloride and 9 g triethylamine was heated to 95°C during 5 h. After this time, the remaining benzylchloride and triethylamine were evaporated in high vacuum. The residue was dissolved in ether. The solution was successively washed with H_2O, saturated aqueous $NaHCO_3$, again with H_2O and dried with Na_2SO_4. Part of the ether was evaporated and petrol ether was added. The precipitating white needles were filtered off and washed with petrol ether. For further purification the procedure was repeated. The pure *N*-carbobenzoxy-D,L-serine benzylester (23 g) had a melting point of 74°C; the yield was approx. 80%. *N*-Carbobenzoxy-D,L-serine-benzylester-phosphatidic acid dichloride was prepared by a similar procedure as described above in the synthesis of the phosphatidylethanolamine. The protecting groups were removed by hydrogenation in the presence of palladium charcoal. The crude diphytanoyl phosphatidylserine was purified by silica-gel column chromatography with chloroform/methanol mixtures with increasing content of methanol. At a methanol content of 50% (v/v) chromatographically pure diphtanoylphosphatidylserine was obtained. The yield was approx. 30% as referred to diphytanoylglycerol.

The synthesis of D,L-1-monophytanoylglycerol was carried out according to ref. 31 starting from isopropylideneglycerol and phytanic acid chloride. The cleavage of the ketal was performed with concentrated HCl in ether using the method of ref. 32. The yield was 70% as referred to phytanic acid chloride.

Membrane experiments

Lipid bilayer membranes were formed in the usual way [33] from a 1—2% (w/v) solution of the lipids in *n*-decane (Merck, Darmstadt G.F.R., standard for gas chromatography). L-1,2 Dipalmitoleoyl 3-phosphatidylethanolamine was a gift of R.A. Demel, Utrecht. Other lipids (synthetic or natural) were prepared as described in previous publications [34,35]. The purity of all lipids was checked by thin-layer chromatography. The cell used for bilayer formation was made from Teflon. The circular hole in the wall between the two aqueous compartments had an area of about 2 mm^2. The measurements were performed 20—30 min after the membranes had turned completely black (blackening time about 5 min). This waiting period was needed in order to obtain stable values of the concentration, N_t, of dipicrylamine in the membrane because of the high partition coefficient for some systems.

Dipicrylamine (Fluka, Buchs, Switzerland, puriss) was used as a concentrated stock solution dissolved in ethanol. Small amounts of this stock solution were added to the unbuffered aqueous solutions of NaCl (Merck, analytical grade) to get a final dipicrylamine concentration of 10^{-8} M and $3 \cdot 10^{-8}$ M. These concentrations were chosen in order to obtain a linear relationship between the concentrations of dipicrylamine in the aqueous phase and in the membrane [7,17,36]. The temperature was kept at 25° C during all experiments.

The charge-pulse experiments were performed as described earlier [7]. A voltage source was connected to the membrane during 50 ns by means of a fast FET switch. The impedance of the switch in the "open" position was larger than 10^{12} Ω. The voltage decay, V_m (t), across the membrane was measured with a voltage follower with a high input-impedance and recorded with a storage oscilloscope (Tektronix 7633/7A22). Test experiments were performed with undoped membranes in parallel to an external resistor as well as with dummy circuits replacing the membrane. The V_m (t) was purely exponential in these test experiments and no distortion of the signal down to 1,5 μs was observed.

As has been shown previously [7], the V_m (t) across a membrane in the presence of lipophilic ions is governed by two relaxation processes with $\tau_2 > \tau_1$:

$$V_m(t) = V_1 \exp(-t/\tau_1) + V_2 \exp(-t/\tau_2) \quad (1)$$

The time constant of slow aqueous diffusion, τ_D, has a value of about 100 s in the presence of dipicrylamine [7]. Because τ_D is much larger as the time constant of undoped membranes, τ_2 reflects essentially the conductivity of undoped membranes and has a value of several seconds.

The specific membrane capacity, C_m, was measured by applying rectangular voltage pulses of 10 mV to the membrane. The capacitive current was measured as a voltage drop across an external resistance with a storage oscilloscope. The specific capacity of the membranes was calculated from the data as described earlier [34].

Results and Discussion

The transport of lipophilic ions across lipid bilayer membranes occurs in different steps, namely, the adsorption-desorption reaction between the aqueous

phase and the membrane interface (rate constants k_{am} and k_{ma}) and the exchange of lipophilic ions between both membrane interfaces across the potential barrier in the middle of the membrane (rate constant k_i) [7,17]. In principle, all rate constants are voltage dependent, but we could show that it is not a serious restriction to assume only k_i to be voltage dependent [7]. For charge pulse experiments in the limit of small voltages ($|V_m| << 25$ mV), the decay of the voltage is given by ([7]):

$$V_m(t) = V_m^0 [a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)] \quad (2)$$

$$\text{with: } a_1 + a_2 = 1 \quad (3)$$

$$\tau_1 = \frac{1}{2k_i(1 + bN_t)} \quad (4)$$

$$a_1 = \frac{bN_t}{1 + bN_t} \quad (5)$$

$$b = \frac{F^2}{4RTC_m} \quad (6)$$

N_t is the total concentration of the lipophilic ions per unit area of the membrane, C_m the specific capacity of the membrane, R the gas constant, F the Faraday constant and T is the absolute temperature. As pointed out in the previous section, the slow process is governed in the presence of dipicrylamine by diffusion polarisation, which has a longer time constant than that of an undoped membrane [7].

$$\tau_2 \approx \tau_m = R_m \cdot C_m \quad (7)$$

where R_m is the specific resistance of the membrane.

The experimental data obtained in charge-pulse experiments with membranes from phytanoyl lipids are given in Table I. For comparison, the experimental results from lipids with straight C_{16} or C_{18} chains and one double bond are also included in Table I. The results for the phosphatidylcholines have been taken from ref. 7. In the case of phosphatidylserine, only a sample of natural origin (brain) was available, which presumably contains fatty acid residues with C_{16} or C_{18} chains. The τ_1 of the first relaxation process show only small variations for the different lipids. Only in the case of the phosphatidylethanolamines was τ_1 found to be much smaller.

From Eqn. 6 it is seen that the value of the specific capacity for the single systems is needed for the calculation of the N_t of dipicrylamine in the membrane. The values of the specific capacity are given in Table I. There is no large variation between the single values and the dielectric thickness of the hydrocarbon layer; for all membranes it is close to 5 nm. The influence of the branched chain on the thickness of the hydrocarbon layer may therefore be considered as small.

The results for the translocation rate constant k_i and the N_t of dipicrylamine are given in Table I together with the partition coefficient β:

$$\beta = \frac{N_t}{2c} = \frac{k_{am}}{k_{ma}} \quad (8)$$

TABLE I

KINETIC PARAMETERS OF DIPICRYLAMINE TRANSPORT THROUGH MEMBRANES FROM DIFFERENT LIPIDS DISSOLVED IN *n*-DECANE

The aqueous phase contained 0.1 M NaCl, 25°C. The results for membranes from dioleoyl- and from dipalmitoleoylphosphatidylcholine are taken from ref. 7. The values of the specific capacity are taken from refs. 34, 35 and 37 as indicated.

Fatty acid residues	C_m/nF cm^{-2}	Ref.	τ_1/μs	a_1	k_i/s^{-1}	N_t/pmol cm^{-2}	$\beta/10^{-2}$ cm
Phosphatidylcholines/10^{-8} M dipicrylamine							
Dipalmitoleoyl	387	[34]	320	0.45	850	0.36	1.8
Dioleoyl	374	[34]	410	0.65	430	0.74	3.7
Diphytanoyl	366	[34]	290	0.65	590	0.73	3.7
Phosphatidylethanolamines/$3 \cdot 10^{-8}$ M dipicrylamine							
Dipalmitoleoyl	425		36	0.32	9610	0.21	0.35
Dioleoyl	372	[34]	81	0.60	2470	0.59	0.98
Diphytanoyl	398		160	0.44	1810	0.33	0.55
Phosphatidylserines/$3 \cdot 10^{-8}$ M dipicrylamine							
Brain	355	[35]	670	0.091	680	0.038	0.061
Diphytanoyl	377		420	0.050	790	0.021	0.035
Monoglycerides/$3 \cdot 10^{-8}$ M dipicrylamine							
Palmitoleoyl	445	[37]	340	0.13	1280	0.069	0.12
Oleoyl	390	[37]	550	0.24	690	0.13	0.22
Phytanoyl	420		740	0.14	580	0.075	0.13

Except for the results from the phosphatidylethanolamines, the difference between the k_i values for the different lipids is relatively small. The reason for the much higher values of k_i in the case of phosphatidylethanolamine membranes is not clear. The thickness of the phosphatidylethanolamine membranes is very similar to the thickness of the other membranes and can therefore not account for the difference in the k_i [35]. Monolayers from phosphatidylethanolamine and from phosphatidylcholine show an almost equal surface potential (420 mV and 440 mV, respectively [38]), whereas the surface potential for monoolein monolayers is much smaller (320 mV, [38]). This smaller value is qualitatively reflected in the partition coefficient β which is smaller in the case of monoolein membranes compared with membranes from the other uncharged lipids. The product $k_i \cdot \beta$ may be regarded as a measure of the magnitude and the sign of the dipolar potential in the membranes surfaces. This is approximately fulfilled for phosphatidylcholine and phosphatidylethanolamine. In the case of monooleine membranes a large difference between the expected (from the difference of the surface potentials) and the observed values of $k_i \cdot \beta$ was found. From this deviation one may conclude that not only the variation of the surface potential is responsible for the differences between the single lipids. The adsorption of the dipicrylamine molecule to the zwitterionic polar headgroup or structural properties of the membranes may also play a role.

In the case of the negatively-charged lipid phosphatidylserine a strong influence of the ionic strength of the aqueous solutions on β was found. With increasing ionic strength β increased from $3.9 \cdot 10^{-5}$ cm (10^{-2} M NaCl) to

$3.3 \cdot 10^{-3}$ cm (1 M NaCl). The concommittant decrease of the surface potential was fitted with a surface charge density of one elementary charge per 0.55 nm^2 and a partition coefficient β at infinite ionic strength of $8.8 \cdot 10^{-2}$ cm. Similar results have been obtained for membranes from phosphatidylserine of natural origin in the presence of dipicrylamine [35] and for negatively charged membranes in the presence of other charged lipophilic molecules [3]. Divalent cations like Ca^{2+} showed in concentrations $\leqslant 10$ mM almost no influence on the transport kinetics (0.1 M NaCl $3 \cdot 10^{-8}$ M dipicrylamine).

The phase transition temperature of a lipid may be regarded as a measure for the fluidity of the membranes [9]. It has been shown that the series of phase transition temperatures of lipids with the same polar headgroup but different fatty acid residues closely agree with the series of the melting points of the fatty acids [39]. Therefore the phase transition temperature of the lipids containing phytanic acid should be relatively low because of the low melting point of phytanic acid (phytanic acid, −7°C, palmitoleic acid, 1°C, oleic acid, 13°C [10]). Membranes from lipids containing phytanic acid residues should therefore be more fluid than, for instance, the corresponding oleoyl lipids. The values for k_i given in Table I do not support this hypothesis. The translocation k_i has similar values for phytanic and oleic acid residues, whereas the values for palmitoleic acid are much higher. A possible interpretation of these findings is that bulky methyl side groups of the phytanic acid chain impede the movement of the dipicrylamine ion, thus reducing the translocation rate constant k_i.

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