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EFFECTS OF MEMBRANE COMPOSITION AND LIPID STRUCTURE ON THE PHOTOPOLYMERIZATION OF LIPID DIACETYLENES IN BILAYER MEMBRANES

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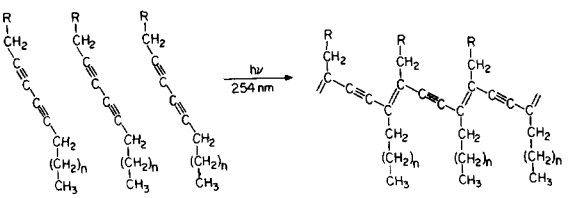
Molecules analogous to biological and synthetic lipids have been prepared with conjugated diacetylene moieties in the long alkyl chain. These lipid diacetylenes form bilayer structures when suspended in aqueous buffers. Ultraviolet light (254 nm) exposure initiates the polymerization of the diacetylenes in the lipid bilayer to give a fully conjugated, highly colored product. The reaction is topotactic, and its efficiency depends on the correct alignment of the monomeric units. Thus, the lipid diacetylenes are photopolymerizable if the hydrocarbon chains are in a regular lattice found at temperatures below the lipid transition temperature; polymerization is inhibited above this transition. The photopolymerization of a diacetylenic glycerophosphocholine in lipid bilayer membranes was observed in two-component mixtures with a nonpolymerizable lipid, either dioleoylphosphatidylcholine or distearoylphosphatidylcholine. The photochemical and thermochemical characteristics suggest that the diacetylenic glycerophosphocholine exists largely in separate domains in the mixed bilayers. Lipid diacetylenes analogous to a dialkyldimethylammonium salt and to a dialkyl phosphate have a plane of symmetry, which suggests that both chains penetrate equally into the bilayer. The photopolymerization of these symmetrical synthetic species is more than 10^3-times more efficient than that of the diacetylenic glycerophosphocholine. These differences are interpretable in terms of the expected conformational preference of the lipid molecules.

Introduction

The ultraviolet-light-initiated polymerization of suitable diacetylene-containing compounds has been demonstrated recently in lipid bilayer membranes [1–4]. The reaction, a 1,4-addition [5], occurs in the solid state [5], monolayers (Thomas, H.T., Drexhage, K.H. and O'Brien, D.F., unpublished observations) [6] and multilayers [7], as well as in lipid bilayers [1–4]. In each case the photoproduct is intensely colored. Frequently, the initial product is blue and relaxes to a red form under appropriate conditions. The reaction is shown below for long-chain fatty acid diacetylenes [R = $(CH_2)_nCO_2H$]. The polymer structure is from Wegner [5].



Bilayer membranes can be prepared from amphipathic molecules such as phospholipids, di-

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methyldidodecylammonium halides [8,9] and dihexadecyl phosphate [8,9]. Such molecules with diacetylene groups incorporated into the long alkyl chains are suitable for the formation of polymerizable lipid vesicles. The photopolymerization of diacetylene is topotactic, and its efficiency depends on the correct alignment of the monomeric units. Diacetylenic glycerophosphocholines are readily photopolymerized if the hydrocarbon chains are in a crystal-like lattice found at temperatures below the lipid transition temperature, and the light-induced polymerization is inhibited when the hydrocarbon chains are disordered as they are at temperatures above this transition [4].

We describe here some effects of membrane composition and of lipid structure on the photopolymerization of lipid diacetylenes. The rate of photopolymerization for two-component mixtures of lipid diacetylenes and nonpolymerizable lipids was determined. The efficiency of the photopolymerization was estimated for different lipid diacetylenes.

Experimental Procedure

Materials

L-α-Dioleoylphosphatidylcholine (DOPC) was obtained from Sigma Chemical Co. (chloroform solution, 20 mg/ml). L-α-Distearoylphosphatidylcholine (DSPC) was purchased from Calbiochem. The phospholipid purity was evaluated by thin-layer chromatography. Dimethyldioctadecylammonium bromide, obtained from Kodak Laboratory Chemicals, was recrystallized from acetone.

The buffers were prepared from Hepes (Calbiochem) and EDTA (Sigma), pH 7.0.

Diacetylenic glycerophosphocholine (1) was synthesized as described previously [3].

Preparation of bis[2-(tricosa-10,12-diynoyloxy)-ethyl]dimethylammonium chloride (2). Tricosa-10,12-diynoic acid (1.72 g, 5 mmol) was heated under reflux with thionyl chloride (20 ml) in a dry nitrogen atmosphere for 1 h. The mixture was cooled to room temperature, the excess thionyl chloride was removed on a rotary evaporator, and the residue was evacuated overnight. This material (ν_{COCl} 1800 cm^{-1}) was dissolved in dry CH_2Cl_2 (15 ml), and 0.424 g (2.5 mmol) of bis(2-hydroxyethyl)dimethylammonium chloride and 0.610 g (5 mmol) of 4-dimethylaminopyridine were added. The resulting slurry was heated under reflux for 6 h. The CH_2Cl_2 was removed under reduced pressure, and the crude reaction mixture was triturated with ether and filtered to remove the hydrochloride salt of the catalyst (0.800 g, 101%). Evaporation of the ether from the filtrate gave 3.1 g of tan solid, which was purified by chromatography, first on a gel permeation column (CH_2Cl_2) and then on silica gel (with a CH_2Cl_2 – CH_3OH gradient), and finally by recrystallization from acetone. The solid product shows a single spot on TLC: yield 0.4 g (10%), m.p. 71–75°C; NMR (C^2HCl_3) δ 0.9 (t, 6 H, CH_3C), 1.4 (br s, 56 H, CH_2), 2.2 (m, 12 H, CH_2CO, $CH_2C\equiv$), 3.5 (s, 6 H, CH_3N), 4.2 and 4.6 (m, each 4 H, CH_2O and CH_2N); infrared (CCl_4) 1737, 1260 (CO_2R), 2300, 2400 (w, C ≡ C) cm^{-1}.

Preparation of bis(tricosa-10,12-diyn-1-yl) phosphate (3). Tricosa-10,12,diynoic acid (3.44 g, 0.01 mol) was dissolved in 20 ml of dry tetrahydrofuran and added dropwise to 300 mg (excess) of $LiAlH_4$ in 10 ml of tetrahydrofuran. The mixture was heated for 1 h at reflux, and 10% K_2CO_3 solution was added, followed by 20% HCl, until the phases separated. The organic layer was decanted from the inorganic salts, which were washed several times with ether. The combined organic phases were washed well with water, dried and evaporated. The residue was a quantitative yield of a white crystalline solid: NMR (C^2HCl_3) δ 0.9 (t, 3 H, CH_3), 1.3 (br s, 50 H, $(CH_2)_n$), 2.2 (t, 4 H, $CH_2C\equiv$), 2.5 (s, 1 H, OH), 3.6 (t, 2 H, CH_2O); infrared (film), 3360, 3410 (OH), 2140, 2160, 2180 (C ≡ C) cm^{-1}.

The acetylenic alcohol (3.30 g, 0.010 mol) was heated under reflux in 20 ml of benzene overnight with 0.48 g (0.0033 mol) of phosphoryl chloride. The residue after evaporation of solvent crystallized on standing and was recrystallized from hexane at 0°C. A single spot was found on TLC: yield 0.97 g (40%); NMR (C^2HCl_3) δ 0.9 (t, 6 H, CH_3), 1.3 (br s, 50 H, CH_2), 2.2 (br t, 6 H, $CH_2C\equiv$), 3.7 (br s, 4 H, CH_2O); infrared (film) 3400 (br, w, OH), 1230 (P(=O)OH), 1110, 1080 (CO) cm^{-1}.

Methods

Preparation of lipid diacetylene membranes. All procedures were carried out under yellow light.

The purified components of the membranes were mixed in a preestablished mole ratio and dissolved completely in $CHCl_3$. The solution was dried to a thin film on a rotary evaporator, then evacuated with a vacuum pump for 2 h. The mixture was hydrated overnight with a selected volume of 10 mM Hepes, 1 mM EDTA, pH 7.0, buffer solution, which had been saturated with argon.

When the experiment called for sonication the mixture was probe sonicated to form small vesicles. The membranes of DOPC and 1 were sonicated in an ice-water bath, whereas the membranes of DSPC and 1 were sonicated at room temperature.

Photochemistry of the lipid membranes. An aliquot of the membrane sample was irradiated at 20°C in a 1 mm quartz cell for selected times with a low-pressure Hg lamp (253.7 nm) 6.1 or 13.2 cm from the sample. Absorption spectra of the sample were taken before and after irradiation using a Cary 118 spectrophotometer. The reaction was followed by observing the appearance of the colored photoproducts, as shown in the figures. Just prior to irradiation the aqueous suspension of membranes was thoroughly flushed with argon and then capped under an argon atmosphere.

Differential scanning calorimetry (DSC). The transition temperatures of the lipid dispersions (15–50 mg lipid/ml) were determined with a Du Pont 990 thermal analyzer. The sample size used was 10 μl, and the rate of temperature increase was 5°C/min.

Actinometry. The actinometer was 0.006 M potassium ferrioxalate, described by Hatchard and Parker [10].

Results and Discussion

The stereochemical requirements of the topotactic polymerization of diacetylenes suggest that the reaction of lipid diacetylenes may be sensitive to the incorporation of nonpolymerizable lipids into the bilayer membranes. Two-component mixtures of 1 with DOPC or DSPC were prepared in ratios of 1 : 2 and 1 : 5. If the lipid molecules are miscible and the lifetime of the reactive state is shorter than the lateral and rotational diffusion time of the lipid diacetylene, then the increase in DOPC or DSPC content will inhibit the photopolymerization of the lipid diacetylene in the mixtures. Since the photopolymerization of the 1 bilayers occurs below the ordered fluid phase transition (T_c) but not above it [4], we can estimate the lateral and rotational diffusion times from previous studies. The long-axis rotation of PC in oriented bilayers below the T_c has an effective correlation time greater than 10^{-4} s [11]. The lateral diffusion coefficient, D, for dipalmitoylphosphatidylcholine (DPPC) is less than 10^{-11} cm^2/s at 21°C [12]. The lifetime of the photochemically produced excited state of *p*-toluenesulfonate diacetylenes has been reported to be about 10^{-6} s [13]. For $\tau = 10^{-6}$ s, the mean distance diffused, $(2D\tau)^{1/2}$, during the excited-state lifetime is less than 1 Å. In the absence of significant diffusion or rotation to facilitate the reaction of lipid diacetylenes, an ultraviolet-light-excited diacetylene can react only with a nearest neighbor in the proper orientation. Thus, with complete miscibility and hexagonal packing in the gel phase, a sample with a 1 mole fraction of 0.33 should have a significantly reduced rate of photopolymer formation.

The two-component mixtures were prepared with the same molar concentration of 1, and each sample mixture showed absorption maxima at 253, 239, 225 and 214 nm and had the same absorbance at the exciting wavelength of 254 nm (±10%). The photopolymer formation was monitored by an increase in absorbance at the λ_{max} of the product, 520 nm [4]. The data in Table I show that the absorbance at this wavelength increases linearly with time of irradiation for lipid bilayers of pure 1 (Fig. 1). The data are for 10–20% polymer formation. Two separate preparations were examined with very similar results. The rate of photopolymer formation is sensitive to the degree of argon purging of the membranes, and these data were the maximum rates of photoproduct formation observed with current techniques of oxygen exclusion. Inspection of the data for mixed bilayers of 1/DSPC (1 : 2) Fig. 2) shows the same degree of polymer formation per unit time as observed with pure 1 bilayers (Table I). DSC of 1/DSPC (1 : 2) bilayers from 25°C showed two endothermic transitions: a transition at 40°C, which is also observed for pure 1 bilayers [4], and a second at 55°C, which is very near to that reported for pure DSPC bilayers [14]. Both the

TABLE I

PHOTOPOLYMER FORMATION AS A FUNCTION OF EXPOSURE TIME FOR MEMBRANES OF 1 AND PHOSPHOLIPID IRRADIATED WITH 254 nm LIGHT AT 20°C

Exposure time (s)	Photopolymer absorbance at 520 nm			
	1	1/DSPC (1:2)	1/DOPC (1:2)	1/DOPC (1:4.8)
300	0.03	0.03	0.03	0.02
600	0.06	0.06	0.07	0.05
1200	0.12	0.11	0.12	0.09
1800	0.18	0.18	0.16	0.10
3000	0.29	0.29	0.23	0.17

rate of photopolymer formation and the thermal behavior indicate that the 1 and DSPC molecules are poorly miscible in bilayers and exist to a large extent in separate domains. The different chain lengths of the two lipids may partly account for their low mutual miscibility in the bilayer.

The two-component mixtures of 1 and DOPC (Fig. 3) have initial rates of photpolymer formation (Table I) similar to those of the 1/DSPC mixture and pure 1. However, the rate of photopolymer formation slows with time after an estimated 10% conversion to product. The photochemistry was performed at 20°C, which is below the 40°C transition of 1 bilayers [4] and above the −22°C transition of pure DOPC bilayers [14]. DSC from −35 to 60°C of the (1:2) mixture showed two endothermic transitions at −18 and 39°C. A smaller transition at 29°C was detectable in some DSC traces. This signal could be due to a mixed phase of 1 and DOPC, while the major transitions are due to the individual lipid domains.

The photochemistry and thermal behavior indicate that mixtures of 1 and DOPC form some independent domains which allow the photopolymerization of part of the 1 below its phase transition even in the presence of DOPC in the liquid crystalline state. The diminished photochemical conversion observed with 1/DOPC mixtures compared to 1 and 1/DSPC is likely due to some mixing of 1 with DOPC. These data suggest that

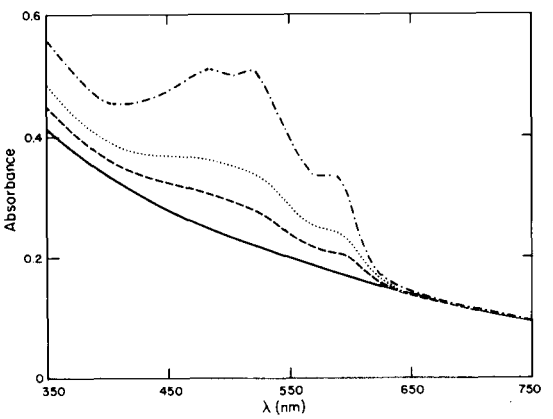


Fig. 1. Absorption spectra of membranes of 1 in aqueous buffer (pH 7.0) before exposure to 254 nm light (———); and after exposure of 600 s (---), 1200 s (· · ·), and 3000 s (-·-·-·-).

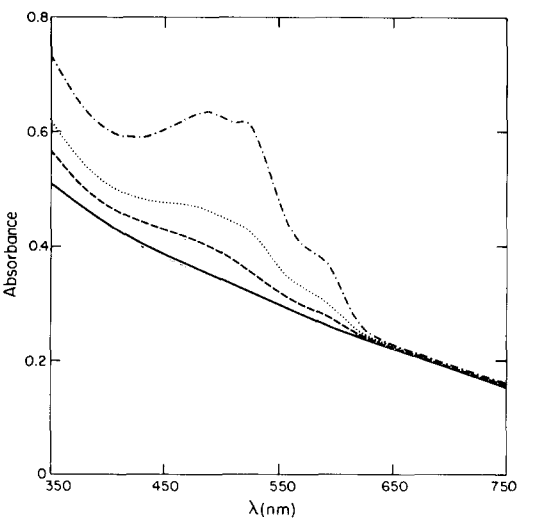


Fig. 2. Absorption spectra of membranes of 1/DSPC (1:2) before exposure to 254 nm light (———); and after exposures of 600 s (---), 1200 s (· · · ·), and 3000 s (-·-·-·).

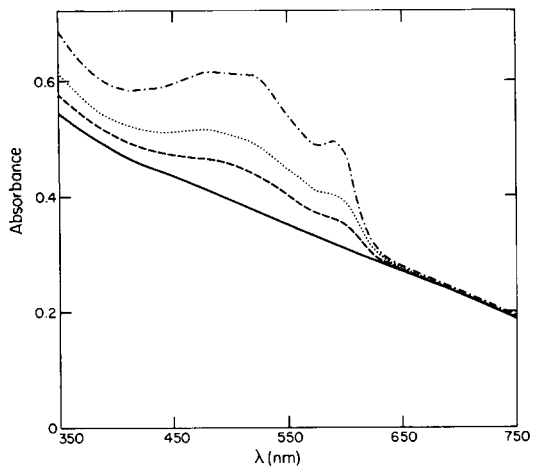


Fig. 3. Absorption spectra of membranes of 1/DOPC (1:2) before exposure to 254 nm light (———); and after exposures of 600 s (---), 1200 s (· · ·), and 3000 s (-·-·-·).

DOPC is a better solvent for 1 than DSPC. The differences in miscibility may result from the fact that the DOPC is in the liquid crystalline state at 20°C, whereas DSPC is in the gel state. In addition, the double bond in the fatty acid chain of DOPC may increase the miscibility of 1. We have observed in other vesicle systems that the presence of unsaturation in the fatty acid chains results in a small increase in the solubility of phenanthrenequinone in lipid bilayers [15].

Hupfer et al. [16] and R. Büschl and H. Ringsdorf (unpublished data) have observed that diacetylenic phospholipids analogous to PC, phosphatidylethanolamine and phosphatidic acid, form monolayers with DSPC or DPPC. In the condensed state, the two-component mixtures of phospholipid diacetylene and nonpolymerizable lipids exist in separate domains. Our observations in vesicle bilayer membranes are consistent with their monolayer studies.

The photosensitivity of the mixed-bilayer vesicles was dependent on the thermal history of the membranes in a manner similar to that reported for 1 [4]. Membrane bilayers of 1 are light sensitive when prepared below the phase transition but become insensitive to light after the membranes are heated above the transition (40°C). The membranes remain insensitive to light even after they are cooled to 25°C, and only when they are cooled to near 0°C is the light sensitivity regained. The hysteresis of the photosensitivity is represented in Fig. 4. A membrane of pure 1 was heated to 56°C, cooled to 22°C, and irradiated for $7 \cdot 10^3$ s under the same conditions as in Fig. 1. No photoproduct was observed. A second sample of 1 was heated to 56°C, then frozen. Irradiation at room temperature produced the photoproduct, as shown in Fig. 1. If the sample was heated to 56°C and stored at room temperature for at least 7 days, it still remained insensitive to light but could be resensitized when it was cooled to near 0°C.

The 1/DSPC (1:2) bilayers show two endothermic transitions at 40 and 55°C when warmed from 25°C. After the sample was warmed above 55°C, then cooled to 20°C, the DSC was rerun. Only the transition at 55°C, which is due to the DSPC, was observed. The sample was no longer light sensitive. If the membrane was cooled to 10°C or lower, light sensitivity was restored, and the DSC showed two endothermic transitions at 40 and 55°C. The hysteresis is observed over a different temperature range for 1 bilayers than for 1/DSPC (1:2) bilayers. The low-temperature end of the cycle is between 10 and 20°C for 1/DSPC (1:2), whereas it is near 0°C for 1 membranes.

The 1/DOPC (1:2) membranes have have endothermic transitions at −18 and 39°C. After warming above 40°C and cooling to 20°C, the membranes became insensitive to light, and the endothermic transition at 39°C was not observed upon reheating the sample. If the sample was

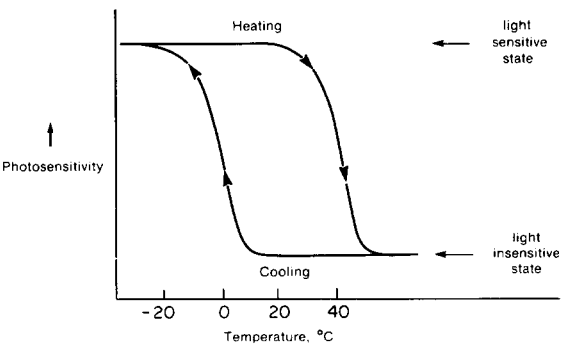


Fig. 4. Hysteresis of photosensitivity of 1 membranes upon heating and cooling. Heating a light-sensitive sample to above 40°C produces a light-insensitive state, which is retained as the sample is cooled. When the sample is cooled to near 0°C, the light sensitivity is regained.

cooled to 10°C, light sensitivity was restored and the transition at 39°C was again observed in the DSC. The behaviour is analogous to that of the 1/DSPC (1 : 2) membranes.

Kunitake and Okahata [8,9] observed that hydrated dialkyldimethylammonium salts form vesicle bilayer structures. Diacetylene groups can be incorporated into the long alkyl chains of this type of quaternary ammonium salt by the esterification of fatty acid diacetylenes with dimethyldiethanolammonium chloride. 2 was prepared by this route; in aqueous suspension it formed bilayer structures, as shown by electron microscopy. These bilayers or sonicated vesicles of 2 are very sensitive to ultraviolet light (Table II). Mixed bilayers of 2 with dimethyldioctadecylammonium bromide in a mole ratio of 1 : 2 are also very sensitive to ultraviolet light (Table II). The rate of product formation was independent of the presence of the nonpolymerizable lipid. This behavior of 2 in mixed-lipid bilayers is strikingly similar to that of the 1 described earlier and shows that these lipids coexist in separate domains in the bilayers.

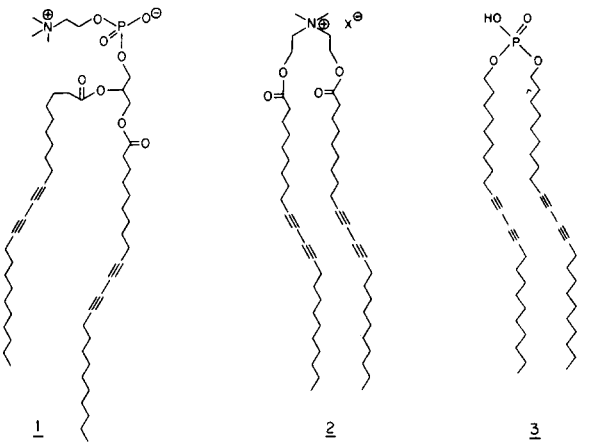
The similarity between 2 and 1 ends here, however, as the photopolymerization of 2 is much faster than that of 1 [17]. The photopolymerization of diacetylenes proceeds as a 1,4-addition to give a fully conjugated structure as shown previously. Since the reaction is topotactic [5], its efficiency depends on the correct alignment of the monomer units.

TABLE II

PHOTOPOLYMER FORMATION AS A FUNCTION OF EXPOSURE TIME OF MEMBRANES OF 2 AND DIMETHYLDIOCTADECYLAMMONIUM BROMIDE IRRADIATED WITH 254 nm LIGHT AT 20°C

DMDOA, dimethyldioctadecylammonium bromide.

Time (s)	Photopolymer absorbance			
	2		2/DMDOA (1:2)	
	650 nm	575 nm	650 nm	575 nm
5	1.12	0.56	1.09	0.55
10	1.44	0.82	1.43	0.77
30	1.75	1.16	1.82	1.08



1 2 3

The structure shown for 1 is based on the preferred conformation of PC and phosphatidylethanolamine molecules in lipid bilayers [18]. In this configuration the two fatty acid chains do not extend equally into the lipid bilayer. It is unlikely that the diacetylene groups on the α- and β-chains of the 1 molecule will be in the proper stereochemical arrangement to allow intramolecular reaction. Therefore, the diacetylene groups must polymerize with suitably oriented diacetylenes in adjacent molecules, α-chain to α-chain and β-chain to β-chain. Since polymerization is initiated by light absorption by either an α- or a β-diacetylene chromophore, the polymer formed in a given microregion will be either an α- or a β-chain polymer, respectively. The synthetic bilayer-forming molecules such as 2 and 3 have planes of symmetry. The symmetry of these structures shows that the diacetylene groups will extend to equal depths into the bilayer, and therefore the topotactic polymerization reaction should be enhanced. We have previously reported [17] that these expectations are fulfilled and that the photopolymerization of 2 and 3 are more than 1000-times as efficient as that of 1 in hydrated bilayer membranes. The photopolymer of 2 is blue (λ_{max} 644 nm), which suggests a longer and more ordered polymeric structure for poly 2 than for the less efficient formation of the red (λ_{max} 540 nm) poly 1 from 1 [11]. Patel and co-workers [19] have demonstrated that more efficient polymerization of diacetylenes is facilitated by better alignment of monomers, which leads to a longer-chain polymer with deeper absorbance. The

quantum efficiency of polymer formation from 2 in pure hydrated bilayers is 60 [17], similar to recent estimates reported for the photopolymerization of diacetylenes in the solid state [20].

The dialkyl phosphate diacetylene, 3, readily forms bilayer membranes, which are sensitive to ultraviolet light and rapidly form a deep-blue photopolymer. The spectral characteristics of the product and the speed of the polymerization are similar to those of the membranes of 2 [17].

Conclusions

The light-induced polymerization of the hydrated bilayers of the lipid diacetylenes in this report is insensitive to the presence of nonpolymerizable lipids in the bilayer but very sensitive to the structure of the lipid diacetylene. Two-component mixtures of lipid diacetylenes with nonpolymerizable phosphatidylcholines or dimethyldioctadecylammonium bromide display photochemical and thermochemical properties consistent with the formation of separate domains of the two lipids. These results show that polymerizable lipids such as lipid diacetylenes can be incoporated into bilayer membranes and cell walls and should be polymerizable even in the presence of membrane constituents other than lipids, e.g., lipid-sensitive membrane proteins and oligosaccharides. The efficiency of the polymerization reaction is substantially greater in membranes composed of symmetrical dialkyldimethylammonium diacetylenes and dialkyl phosphate diacetylenes rather than diacetylenic phosphatidylcholines. These observations are consistent with the previously determined conformational preference of phospholipids.

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