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PHOSPHOLIPID POLYMERS - - SYNTHESIS AND SPECTRAL CHARACTERISTICS

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Summary

A new approach has been developed for the study of model and natural biomembranes. This involves the cross-linking of diacetylene groups after ultraviolet irradiation. For the study of model biomembranes, pure phospholipids (phosphatidylcholines) have been synthesized containing diacetylene groups in each acyl chain. The physical properties of these lipids have been examined and the conditions under which they polymerise have been determined. Polymerisation occurs when the lipid is in a crystalline phase, either compressed in KBr, dispersed in water (liposomes) or deposited on a suitable support (multilayers). The resultant polymer contains a conjugated backbone and is coloured. The visible spectrum of the phospholipid polymer is sensitive to its environment. Preliminary experiments show that similar polymerisation can be induced in *Acholeplasma laidlawii* cells grown on diacetylenic fatty acid.

Introduction

In recent years there has been considerable interest in biomembrane structure and function. The arrangement of lipids within bilayer systems and the nature of protein-lipid interactions are two aspects of this topic which have attracted close scrutiny.

Monolayers, multilayers and liposomes have frequently been used as simple model membranes in attempts to gain insight into more complex natural structures. In addition to this, means for making well defined alterations to natural biomembranes have been developed and the consequences of these changes studied. For instance, membrane fluidity has been modified by adding

cholesterol [1] or hydrogenating the unsaturated lipid component [2].

In this paper, we wish to report an approach which may be useful for both model and natural biomembrane studies. This new approach involves the synthesis of phospholipids containing diacetylene groupings in their acyl chains by conventional organo-chemical procedures or fatty acid incorporation into micro-organisms. The phospholipids are subsequently polymerised by irradiation. Our approach is related to, but is distinct from, recent work by Khorana and co-workers [3—5]. Here we describe the synthesis, polymerisation and spectral characteristics of these phospholipids.

Materials and Methods

Materials

1-Alkenes and undecenoic acid were purchased from Aldrich Chemicals and used as supplied. 4-*N*,*N*-Dimethylaminopyridine (also Aldrich) was recrystallised from chloroform/diethyl ether and stored under N_2 in the dark.

CuCl was prepared by reducing $CuSO_4$ with $NaHSO_3$ in the presence of NaCl. It was used immediately.

Petroleum ether for bromination was treated with Br_2 until the colour of the Br_2 persisted for several hours. After washing successively with $NaHCO_3$ solution and water, it was dried over Na_2SO_4 and distilled.

Purified egg lecithin (B.D.H.) was treated according to the method of Chadha [6] to obtain glycerophosphoryl choline. Water was removed prior to use by evaporation of (CaH_2-dried) benzene.

Methods

Calorimetry. The transition temperatures and transition enthalpies of saturated lipid dispersions were measured with a Perkin Elmer scanning calorimeter. The rate of temperature increase was 10°C/min and calibration was made against *n*-octadecane, naphthalene and indium.

Surface pressure measurements. Surface pressure measurements were made on a Teflon trough fitted with a driven Teflon barrier and Wilhelmy balance. Monolayers were spread from chloroform solutions all having a concentration of approx. 1 mg/cm^3. The water substrate was triple-distilled, initially from alkaline permanganate. A glass heat-exchange coil was submerged in the substrate. Water from a constant-temperature bath could be pumped through the coil and in this way it was possible to maintain the substrate temperature constant at values as low as 7°C.

Liposomes. Large liposomes were prepared in uni- and multilammelar form. Diethyl ether injection, according to the method of Deamer and Bangham [7], yielded the first type and vortex mixing above the lipid transition temperature the second. Very small unilammelar liposomes were formed by sonicating multilammelar dispersions for 30 min above the lipid transition temperature.

Polymerisation. Attempts were made to polymerise the phospholipids in four different states.

(i) As monolayers on water at 7°C. The film balance cabinet was purged with N_2 during irradiation.

(ii) As multilayers on a hydrophobic support. Teflon was usually employed as the support.

(iii) As liposomes. Dispersions were purged thoroughly with N_2 and cooled to 0°C before irradiation.

(iv) Mixed with KBr and compressed into transparent discs.

In each case, the lipid was irradiated with a Mineralight R-52 lamp. This lamp has a peak radiation intensity at 254 nm and an energy output of 1200 $\mu W/cm^2$, 15.2 cm from its face.

Significant quantities of polymer were obtained most conveniently from KBr discs. 30—40% of the 4—5 mg of lipid in each disc is converted to polymer on irradiation. KBr is removed by water wash. Unreacted monomer and polymer (100 mg) were dissolved in distilled chloroform and passed down a column (40 × 2 cm) of Sephadex LH-60 (solvent flow rate 1.0 cm^3/min). Polymer is not retained by the gel and runs with the solvent front. After the chloroform had been evaporated the polymer was freeze-dried from benzene. Pure polymer is a thermochromic wax, being deep red/purple at liquid N_2 temperature and red-yellow at room temperature. Infrared spectra of the monomer before irradiation, the monomer/polymer mixture and pure polymer are similar.

Results

Synthesis of diacetylenic acids

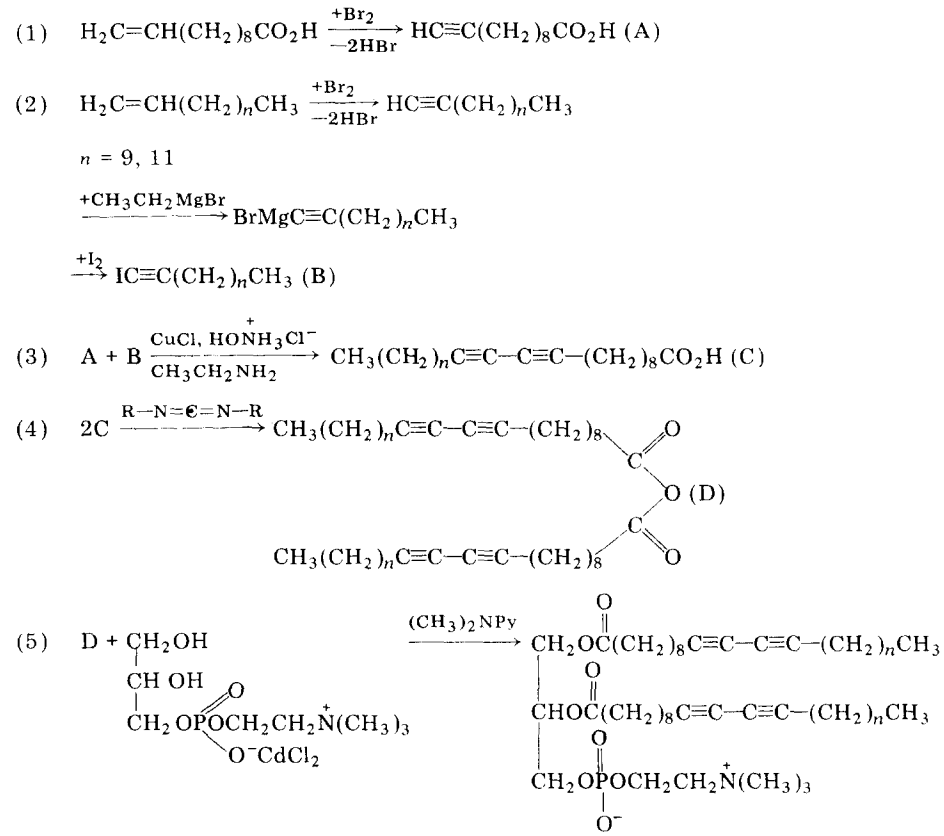
A schematic outline of the synthesis is shown in Scheme I.

The alkenes and alkenoic acid were brominated in petroleum ether by slowly adding 1 mol-equiv. of the halogen. The dibromo derivatives were immediately dehydrobrominated by refluxing them in ethanol with excess KOH. After the alcohol had been distilled off and dilute HCl added, alkyne was isolated by ether extraction and purified by vacuum distillation (10^{-3} mmHg).

For an asymmetrical Chodkiewicz coupling, one of the reactants had to be iodinated. The 1-alkynes were therefore added to a slight excess of ethylmagnesium bromide in dry diethyl ether (CaH_2-treated). The acetylenic Grignard reagent formed reacts directly with added I_2 to give the halo derivative desired. The contents of the reaction flask were poured into excess dilute acetic acid and the iodoacetylene extracted into diethyl ether. Unreacted I_2 was removed by a thiosulphate wash and after the diethyl ether had been evaporated purification was achieved by vacuum distillation.

The details of acetylenic-coupling reactions have been extensively reviewed [8]. To the acid dissolved in dilute KOH solution was added a trace of hydroxylamine hydrochloride and 0.25 mol-equiv. of CuCl dissolved in aqueous ethylamine. The iodoalkyne (1 mol-equiv.) was then added, a small portion at a time. The originally yellow solution turned green. The yellow colour was restored by adding a few drops of 10% hydroxylamine hydrochloride solution before the next addition of alkyne. Finally, the reaction mixture was acidified and the product extracted into diethyl ether. The diynoic acid was recrystallized from 40—60°C petroleum ether.

The acids readily polymerised. Ultraviolet and infrared spectroscopy showed



Scheme I. Schematic outline of diacetylenic phospholipid synthesis. Py, pyridine.

the presence of conjugated triple bonds and a carboxylic acid group, respectively.

Synthesis of phospholipids

The acid was dissolved in methylene chloride and converted to the anhydride by adding 0.55 mol-equiv. of dicyclohexylcarbodiimide. The anhydrides were characterised by infrared spectroscopy.

Phospholipids were synthesized by using the method of Gupta et al. [9]. Glycerophosphatidylcholine-$CdCl_2$ complex (1.0 mol-equiv.) was stirred with anhydride (2.5 mol-equiv.) and 4-*N*,*N*-dimethylaminopyridine (2.0 mol-equiv.) in dry chloroform (P_2O_5-treated) for 30 h. After removal of the solvent, the methanol/chloroform/water (5 : 4 : 1, v/v) soluble fraction was passed down a column of Rexyn I-300 resin. The product now free of $CdCl_2$ and aminopyridine was purified by chromatography on Sephadex LH-20. The overall yield based on the starting alkene is 5%.

The product and dipalmitoyl phosphatidylcholine (puriss grade, Fluka) were compared by thin-layer chromatography (Merck silica gel plates; solvent, chloroform/methanol/water (65 : 35 : 4, v/v)) and infrared spectroscopy.

TABLE I

TRANSITION TEMPERATURES AND ENTHALPIES FOR DIACETYLENIC PHOSPHOLIPIDS AND THE FOUR SATURATED PHOSPHATIDYLCHOLINES, C_{23} DIACETYLENIC, C_{25} DIACETYLENIC, DIMYRISTOYL (DML), DIPALMITOYL (DPL), DISTEAROYL (DSL) AND DIBEHENOYL (DBL) PHOSPHATIDYLCHOLINES

	Diacetylenic phospholipids					
	C_{23}	C_{25}	DML	DPL	DSL	DBL
Number of methylene units	17	19	12	14	16	20
Transition temperature (°C)	38.5	48	23	41	58	75
Transition enthalpy (kcal · mol^{-1})	10.15	13.18	6.65	8.65	10.70	14.9

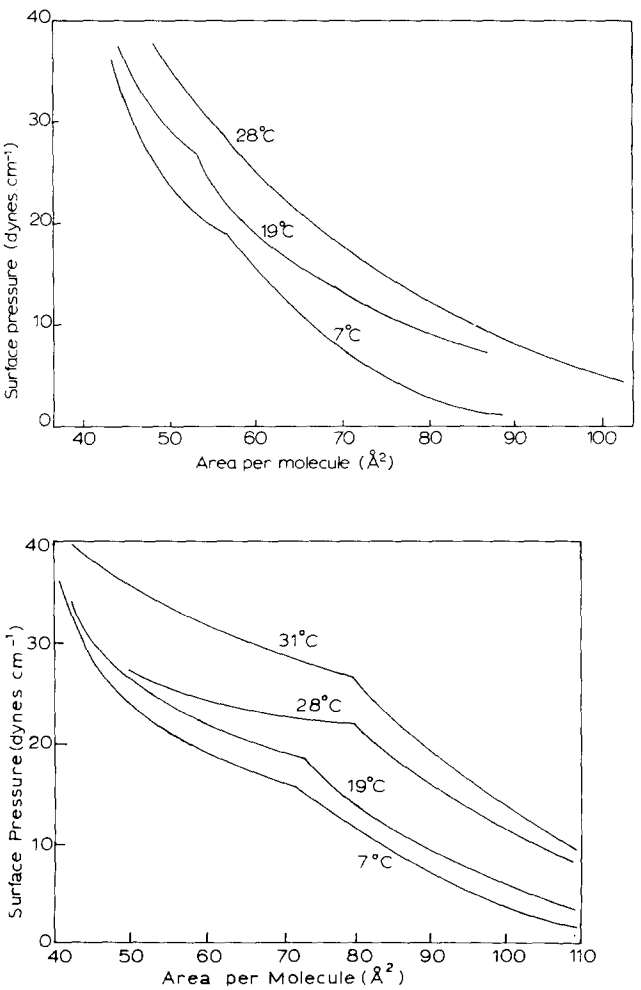


Fig. 1. Continuously recorded pressure-area isotherms of diacetylenic phospholipid monolayers. (a) C_{23} phospholipid, (b) C_{25} phospholipid.

R_f values and spectra were identical. The product gave a positive test (turned blue) when sprayed with Dittmer reagent. Examination by ultraviolet spectroscopy showed that conjugated triple bonds were present. The products are considered to be the phospholipids 1,2-ditricosanoyl (C_{23})- and 1,2-dipentacosanoyl (C_{25})-10,12-diyne-*sn*-glycero-3-phosphorylcholine.

Physical characterisation of phospholipid monomers

Calorimetry. Table I contains the transition temperatures and enthalpies of the two diacetylene phospholipids and four saturated phosphatidylcholines [10]. Compared to saturated lipids with the same number of methylene units, diacetylenic phospholipids have significantly lower transition temperatures and enthalpies.

Monolayer properties. Surface pressure-area isotherms for each phospholipid are shown in Fig. 1a and b. As observed with saturated phospholipids, the point at which the change from an expanded to a condensed phase occurs shifts to lower pressures and larger molecular areas as chain length increases. The curves for the longer lipid are somewhat unusual, the molecular areas at which the transition occurs increase with increasing temperature rather than showing the more usual decrease.

The diacetylenic phospholipid monolayers are seen to be greatly expanded for their chain lengths when compared with saturated phospholipids.

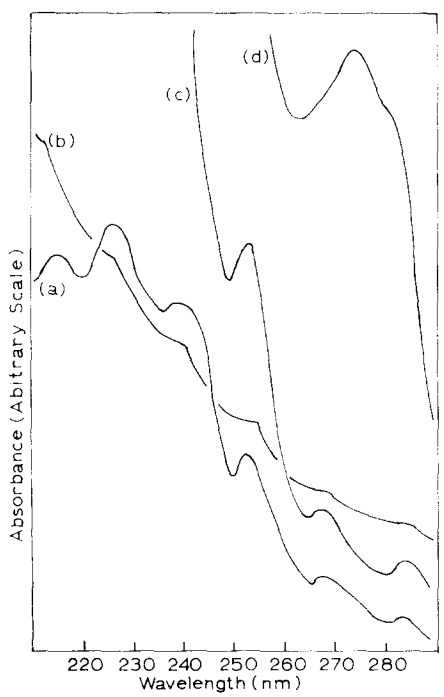


Fig. 2. Ultraviolet spectra of C_{23} fatty acid, phospholipid and phospholipid polymer. (a) Fatty acid dissolved in cyclohexane, $\epsilon = 2.95 \cdot 10^2$ l · mol^{-1} · cm^{-1} at 254 nm; (b) Phospholipid dispersed in water; (c) Phospholipid dissolved in chloroform, $\epsilon = 5.1 \cdot 10^2$ l · mol^{-1} · cm^{-1} at 254 nm; (d) Phospholipid polymer dissolved in chloroform, $\epsilon = 1.3 \cdot 10^3$ l · mol^{-1} · cm^{-1} at 275 nm.

Infrared spectra. The infrared spectra of paraffin mulls of pure phospholipid and its polymer are typical of those obtained with normal phosphatidylcholine molecules.

Ultraviolet spectra. The ultraviolet spectra of the pure phospholipid and its polymer are shown in Fig. 2.

Polymerisation characteristics

A qualitative comparison with the acids suggested that the reactivity of triple bonds in the anhydrides and phospholipids is reduced. The phospholipids could be left exposed to laboratory light without any appearance of colour whilst under similar circumstances the acids were observed to polymerise.

Monolayers show a similar difference in reactivity. Both fatty acids but neither phospholipid polymerised when irradiated at 7°C.

However, the phospholipids do polymerise in multilayer and liposome form. The details of multilayer polymerisations will be described elsewhere.

Below the lipid transition temperature, large uni- and multilammelar liposomes polymerised even if the originally slightly hazy dispersions were cleared by 2 min sonication. However, above the transition temperature, even prolonged irradiation did not cause any noticeable polymerisation.

Very small unilammelar liposomes would not polymerise at any temperature.

Physical characterisation of phospholipids polymers

Calorimetry. No sharp phase change could be detected in the temperature range 0—200°C.

Monolayer properties. Pure polymer could be spread at the water/air interface from chloroform solution. Pressure-area isotherms were measured at 7 and 32°C. No true collapse point or phase change was evident.

Visible spectra: C_{23} phospholipid. The visible spectra of polymer compressed in KBr and dissolved in chloroform are quite different. In chloroform solution

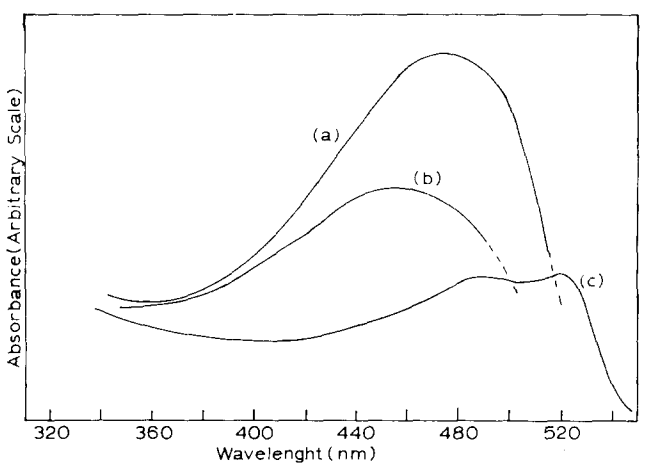


Fig. 3. Visible spectra of C_{23} phospholipid polymer (a) and (b) polymer dissolved in chloroform. Monomer had been compressed in KBr (5 mg monomer per 100 mg KBr) and irradiated. KBr was removed by water wash. The spectra show polymer from two individual discs. (c) Spectrum of a KBr disc containing polymer (1 mg monomer compressed in 100 mg KBr and irradiated).

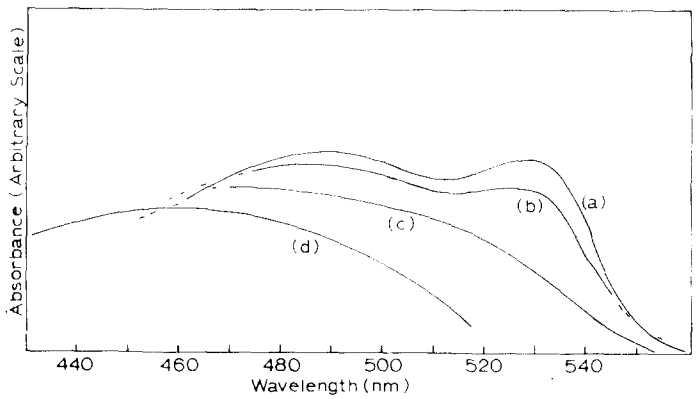


Fig. 4. Visible spectra at various temperatures of pure C_{23} phospholipid polymer dissolved in chloroform and methanol. (a), (b) and (c) polymer dissolved in methanol at temperatures 0, 18 and 50°C, respectively. At 50°C, $\epsilon = 3.8 \cdot 10^2$ l · mol^{-1} · cm^{-1} at 480 nm. (d) Polymer dissolved in chloroform. Spectrum invariant over temperature range 0—50°C, $\epsilon = 3.3 \cdot 10^2$ l · mol^{-1} · cm^{-1} at 460 nm.

there is only one peak and it occurs between 455 and 474 nm. Some disc polymers are purple in appearance but it was not possible to determine their absorption maximum. Only when a disc contained less than 1 mg of monomer could a spectrum be obtained and these discs are red. The absorption profile was bimodal with maxima at 490 and 519 nm (purple discs contained 4—5 mg of monomer) (Fig. 3).

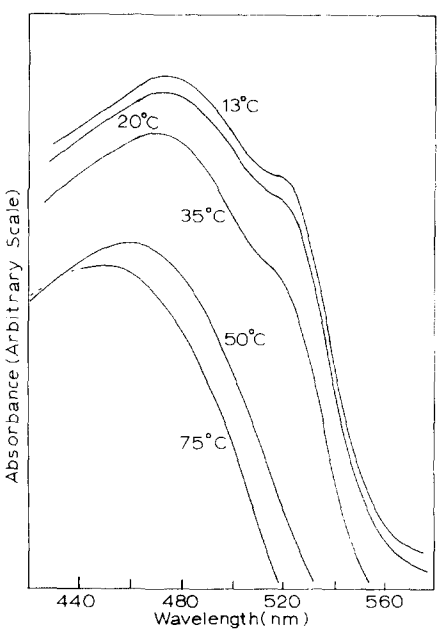


Fig. 5 Visible spectra at various temperatures of partially polymerised C_{23} phospholipid liposomes. 1.5 mg of lipid dispersed in 3 cm^3 of pure distilled water and irradiated at 0°C. Conversion to polymer approx. 25%.

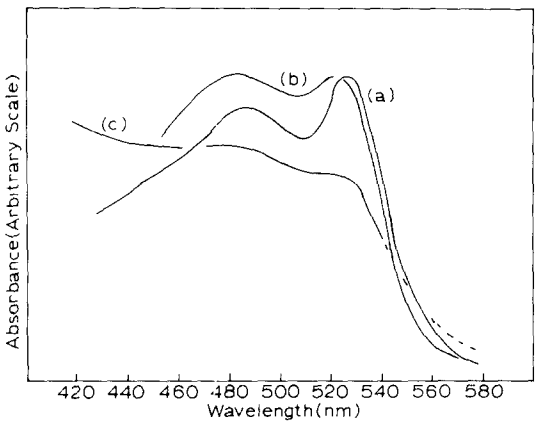


Fig. 6. Visible spectra of C_{25} phospholipid. (a and b) Polymer dissolved in chloroform/methanol (1 : 19, v/v) at 0 and 20°C, respectively. (c) Partially polymerised liposomes at 24°C.

Pure polymer is soluble in methanol (Fig. 4). At 50°C, the absorption profile is unimodal, but as temperature is reduced a second peak appears at long wavelength and the whole spectrum shifts to the red. At 0°C, the absorption maxima occur at 529 and 490 nm. At 50°C, the single peak is situated at 474 nm. In chloroform the absorption profile is unimodal and the peak , at 460 nm for this polymer preparation, is virtually unaffected by temperature changes. Polymer solutions in chloroform or warm methanol are bright yellow, in cold methanol bronze-red.

Fig. 5 contains spectra of partially polymerised liposomes. Monomer and water had been vortex mixed above the lipid transition temperature and irradiated at 0°C for 5 min. Conversion to polymer was estimated to be about 25%. The changes in shape and position of the absorption curve with changing temperature resemble those observed with solutions of pure polymer in methanol. At 75°C, the absorption profile is unimodal with a maximum at 451 nm, at 13°C it has a pronounced shoulder, the peaks occuring at 474 and 518 nm. This shoulder first becomes apparent at approx. 35°C, the transition temperature of the lipid. Some displacement of spectra upwards occurs as temperature is reduced. The displacement is caused by a reduction in scattered light. Liposome size was such that the dispersion was not completely clear.

Visible spectra: C_{25} phospholipid. The spectra of pure polymerised phospholipid in methanol and partially polymerised liposomes are shown in Fig. 6. In both cases when compared to C_{23} polymer, the long-wavelength peak/shoulder is seen to be more pronounced.

All the spectral changes described above are completely reversible.

Discussion

There is no previous report on the synthesis and properties of phospholipids containing diacetylenic groupings. Studies of fatty acids have, however, been reported [11,12].

The presence of acetylenic groupings in the chains of the phospholipid

provides a probe which may be useful for the study of model biomembranes containing intrinsic molecules such as cholesterol or proteins. We will describe elsewhere the ^{13}C-NMR spectra of model biomembranes where clear separate signals from the acetylenic carbons are apparent.

Our present study shows that polymerisation can be induced with the phospholipid molecules in different conditions: compressed in KBr, in liposomes, or in multilayers (particularly on Teflon). The polymerisation of diacetylene groups requires the chains be in a condensed phase, i.e., it occurs readily below the lipid transition temperature (T_c) (polymerisation studies have previously been reported for fatty acids but not phospholipids; polymerisation readily occurred when the fatty acids were in a condensed phase). The polymerisation mechanism has been established [13] and is illustrated in Fig. 7. A polymer containing a conjugated backbone is produced on irradiation.

The reactive species formed on irradiation of diaceylenes is thought to be a carbene [14]. The readiness with which the crystalline monomer phase converts to a crystalline polymer phase is governed by the root mean-square displacement of atoms necessary [13]. The smaller the displacement, in other words the more alike the monomer and polymer phases, the more readily polymerisation occurs.

There are two possible modes of polymerisation with phospholipids, alternate inter, intramolecular linking or exclusively intermolecular. With either mode the headgroup covalent link between the acyl chains might be expected to hinder their rearrangement during polymerisation and make phospholipids less reactive than the corresponding fatty acids. This may explain why the fatty acids readily polymerise at an air/water interface [11] whilst the phospholipids under similar conditions do not.

Solid diacetylene polymers are highly crystalline and usually very insoluble. Solvent interactions with the phosphatidylcholine head group are, however, sufficiently strong to make phospholipid polymers soluble in most organic solvents. The only soluble diacetylene polymers previously reported are those which have an amide group attached to the polyconjugated chain [15].

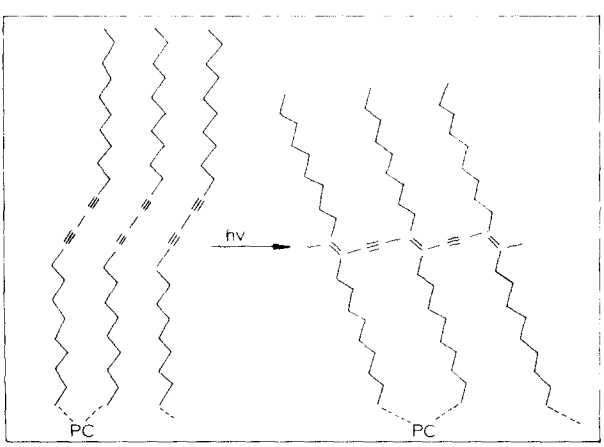


Fig. 7. Formation of polyconjugated phospholipid polymer from diacetylenic monomer. PC, phosphatidylcholine.

Because the polymerisation process leads to a conjugated backbone the visible spectrum of the phospholipid polymer may be useful as an indication of the extent to which polymerisation has occurred within both model and cell biomembranes. The fact that the spectrum of the polymer is sensitive to its local environment may also provide a useful probe for future studies of environmental changes in lipid bilayers.

Our present results show that shifts to longer wavelength occur when:
(i) the polymer is compressed in a KBr matrix;
(ii) transferred from a good solvent for the acyl chains, chloroform, to a poor one, methanol;
(iii) methanol solutions or aqueous dispersions are cooled.

Conjugation of multiple bonds decreases the energy of their first photo-excited state. Overlap of p-orbitals and delocalisation of the promoted electron over many nuclei are thought to be responsible. Efficient p-orbital overlap can only occur when there is no rotation about chain single bonds. Coplanarity leads to a fall in entropy and for polyenes in solution, 'effective conjugation blocks' cannot contain more than 30—33 carbon atoms [16].

Very similar absorption spectra have been reported by Patel and Chance [17] for soluble polydiacetylenes with amide groupings. In this instance, shifts to longer wavelenght were observed when chloroform polymer solutions were diluted with hexane. Studies using infrared spectroscopy showed that there was an increased interaction between the amide side groups as the proportion of hexane in the solvent was increased. The appearance of a second peak as the spectra are red-shifted suggests that another chromophore is formed. Exciton coupling has been observed in polyene solutions [18,19] and the second peak could indicate the formation of aggregated polymer chains. The results of Patel and Chance seem to rule out this possibility (the colour changes are quite independent of the polymer concentration, the solution viscosity does not change as hexane is added and ultracentrifugation does not precipitate polymer from hexane-rich solutions).

Another explanation for the bimodel absorption spectra is that chain crystallisation increases the energy of excited vibrational and rotational levels of the multiple-bond system. The number of such levels that can be populated in the first photo-excited state may then be reduced. This could lead to a selective narrowing of the long-wavelength absorption band.

A marked change in the spectrum of polymer dispersed in monomer occurs at the monomer transition temperature. The motions of the acyl chains of the polymer are probably influenced by the surrounding monomer. Thus, the polymer may serve as a probe of its environment within the lamellar phospholipid structure. Hochstrasser and Kasha [20], in 1964, suggested that 'the molecular ultra-structure of lammelar systems might be deduced by spectrophotometric observation' if they contained suitable chromophores. Kuhn et al. [21] have used spectra to study the structure of multilayers built up from surface-active dyes.

In addition to model biomembranes prepared from pure phospholipids, we have also carried out preliminary studies on *Acholeplasma laidlawii* cells grown in the presence of diacetylenic fatty acid. It was found that the fatty acid was biosynthetically incorporated into the cells. Brief irradiation of the

cells causes visible spectral changes similar to those observed when synthetic lipid liposomes are irradiated. This indicates that a similar polymerisation process occurs within the biomembranes of these cells (this work will be published in detail elsewhere).

Conclusions

Phospholipids (phosphatidylcholines) have been synthesized which contain diacetylene groups in their acyl chains. These conjugated acetylene groups may provide a useful probe for model biomembrane studies using ^{13}C-NMR spectroscopy. Upon ultraviolet irradiation the phospholipids polymerise, cross-linking the acyl chains via a conjugated backbone. Polymerisation readily occurs when the lipids are in a crystalline phase, either compressed in KBr, dispersed in water as liposomes and cooled below the T_c or deposited in multilayer form. The conjugated backbone has spectral properties which enable the production of polymer to be detected. The spectral characteristics of the polymer have been investigated. Very small concentrations of phospholipid polymer can be dispersed and detected in other phospholipid lamellar structures. The nature and state of this lipid strongly influences the polymer's absorption spectrum. The changes in the visible spectra appear to be related to the arrangement of the polymer's acyl side chains.

Preliminary experiments indicate that *A. laidlawii* cells can incorporate the diacetylenic fatty acids into their biomembranes and upon ultraviolet irradiation they undergo cross-linking and polymerisation. The spectral behaviour is similar to that observed with model biomembranes.

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