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THE PHYSICAL PROPERTIES AND PHOTOPOLYMERIZATION OF DIACETYLENE-CONTAINING PHOSPHOLIPID LIPOSOMES

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(a) The physical properties and photopolymerization of diacetylene-containing phosphatidylcholines with acyl chains of different length and in liposome form have been studied. (b) The structure of these liposomes and their stability during polymerization have been examined using electron microscopy and glucose trapping. (c) Photopolymerization of the diacetylene groupings has been followed by monitoring the conversion of monomer and the formation of coloured polymer and the optimum conditions for polymerization have been established. (d) Changes induced by irradiation on the phase transition behaviour of these lipids were determined by differential scanning calorimetry. Polymerization decreases both the transition temperature and the enthalpy of the main endothermic transition. (e) The permeability of liposomes to glycerol is changed as a result of the polymerization process.

Introduction

When arranged in crystalline arrays, compounds which contain diacetylene groups crosslink upon exposure to ultraviolet light to form polymers [1]. The resultant polymers are highly coloured due to the conjugated nature of the crosslinked backbone. We have recently synthesized phosphatidylcholine molecules containing such groupings and these have been polymerized in monolayers and multilayers as well as in liposome form [2–4].

In the present paper we report further studies of the physical properties of these systems. The structure of the liposomes formed from these molecules and their stability during polymerization are examined by electron microscopy. By measuring both the conversion of monomeric lipid (using a chromatographic approach) and the formation of coloured polymeric material, the extent of photopolymerization has been determined. The effect of polymerization on the main endotherm transition of the lipids, the transition temperature and enthalpy, as measured by differential scanning calorimetry, is studied. Changes in the permeability of the liposomes to glycerol have been determined.

These effects of polymerization on the properties of the liposomes are important for studies of protein-lipid systems where reconstitution of purified membrane-bound enzymes into bilayers of diacetylene-containing phosphatidylcholines has been accomplished and also in systems where cells which are fatty acid auxotrophs are grown on a medium containing diacetylenic fatty acids. We are presently engaged in such studies and have recently successfully incorporated [5] and polymerized diacetylene-containing fatty acyl chains within the membrane of *Acholeplasma laidlawii A*.

Materials and Methods

Materials

The phospholipids, 1,2-dieicosanoyl (C_{20}), 1,2-ditricosanoyl (C_{23}) and 1,2-dipentacosanoyl (C_{25}) -10,12-diyne-*sn*-glycero-3-phosphorylcholine were synthesized as described previously [3]. All lipids were at least 99% pure as determined by GLC and TLC. 1,2-Diheptacosanoyl-10,12-diyne-*sn*-glycero-phosphorylcholine (C_{27}) was a gift from Dr. D.S. Johnston.

Diagnostic kits for assaying glucose were obtained from Sigma (London) Chemical Co., Ltd.

Methods

Multilamellar liposomes were prepared by vortex mixing. The lipid was dissolved in a small volume of chloroform and dried on the walls of a glass tube under a stream of nitrogen to form a thin film. The resuspending liquid was added and the tube purged with nitrogen and sealed. It was then incubated at a temperature 10°C above the phase transition of the lipid for 2 h with occasional vortexing of the contents.

Liposomes were polymerized at temperatures below their phase transition temperature using a Mineralight R-52 lamp. This lamp has a peak radiation intensity at 254 nm and an energy output of 1200 $\mu W \cdot cm^{-2}$, 15.2 cm from its face.

The degree of crosslinking of the fatty acyl chains was determined by gas-liquid chromatography. Following irradiation of samples containing 1–2 $mg \cdot ml^{-1}$ of lipid, a standard amount of oleic acid was added to the samples and the lipids extracted and methyl esters of their component fatty acids prepared as detailed by Saito and McElhaney [6]. The methyl esters were separated on an SE-30 column using a Pye Unicam gas chromatograph. The area of the peaks of the methyl esters of the diacetylenic acids and the oleic acid were then compared, and the percentage crosslinking of the acyl chains of the monomeric phospholipid calculated.

Visible spectra of liposomes were recorded using a thermostatically controlled cell in a Pye Unicam SP8-100 spectrophotometer and circular dichroism spectra were recorded on a JASCO J40 CS instrument belonging to London University's Intercollegiate Research Service.

Differential scanning calorimetry (DCS) of polymerized and non-polymerized lipids was performed using a Perkin-Elmer DSC 2 instrument. After irradiation, a volume containing about 2 mg of lipid was centrifuged for 30 min at 150000 × *g* and the pellet was placed in a 20 μl pan for DSC analysis. The sensitivity varied between 2 and 0.2 $mcal \cdot s^{-1}$ (0.84 $mJ \cdot s^{-1}$), the scan rate being 5 $K \cdot min^{-1}$. Data from the first heating and cooling curves were discarded. Reproducible traces were obtained in subsequent runs. After analysis, the phospholipid concentration in each pan was determined by measuring the phosphate concentration [7]. Enthalpies were calculated by comparison with standards of dimyristoyl- and dipalmitoylphosphatidylcholine.

Freeze-fracture and negative-staining electron microscopies were performed using the transmission electron microscopes of Chelsea College and the Royal Free Hospital, London. Potassium phosphotungstate (1% in water) was used for the negative staining at room temperature. Liposome preparations used for freeze-fracture were prepared in 30% glycerol. After equilibration at the requisite temperature, droplets were transferred to gold/nickel specimen holder plates resting on a metal block at the desired quenching temperature. After equilibration for 10 min, the samples were frozen rapidly in liquid nitrogen and freeze-fractured.

Permeability of liposomes to glycerol at various temperatures was measured as detailed by De Gier et al. [8].

Solubility of polymer in organic solvents was determined by adding 5 vol. of chloroform/methanol (2 : 1, v/v) to 1 vol. of irradiated liposomes. The mixture was shaken and, after standing, separated into two phases, with soluble material being in the $CHCl_3$ phase and insoluble polymer at the interphase.

Results

Liposome structure

Examination of negatively stained preparations of vortexed liposomes by electron microscopy shows that multilamellar spherical vesicles are formed by the C_{20}-, C_{23}- and C_{25}-diacetylene phospholipids and these retain their shape after

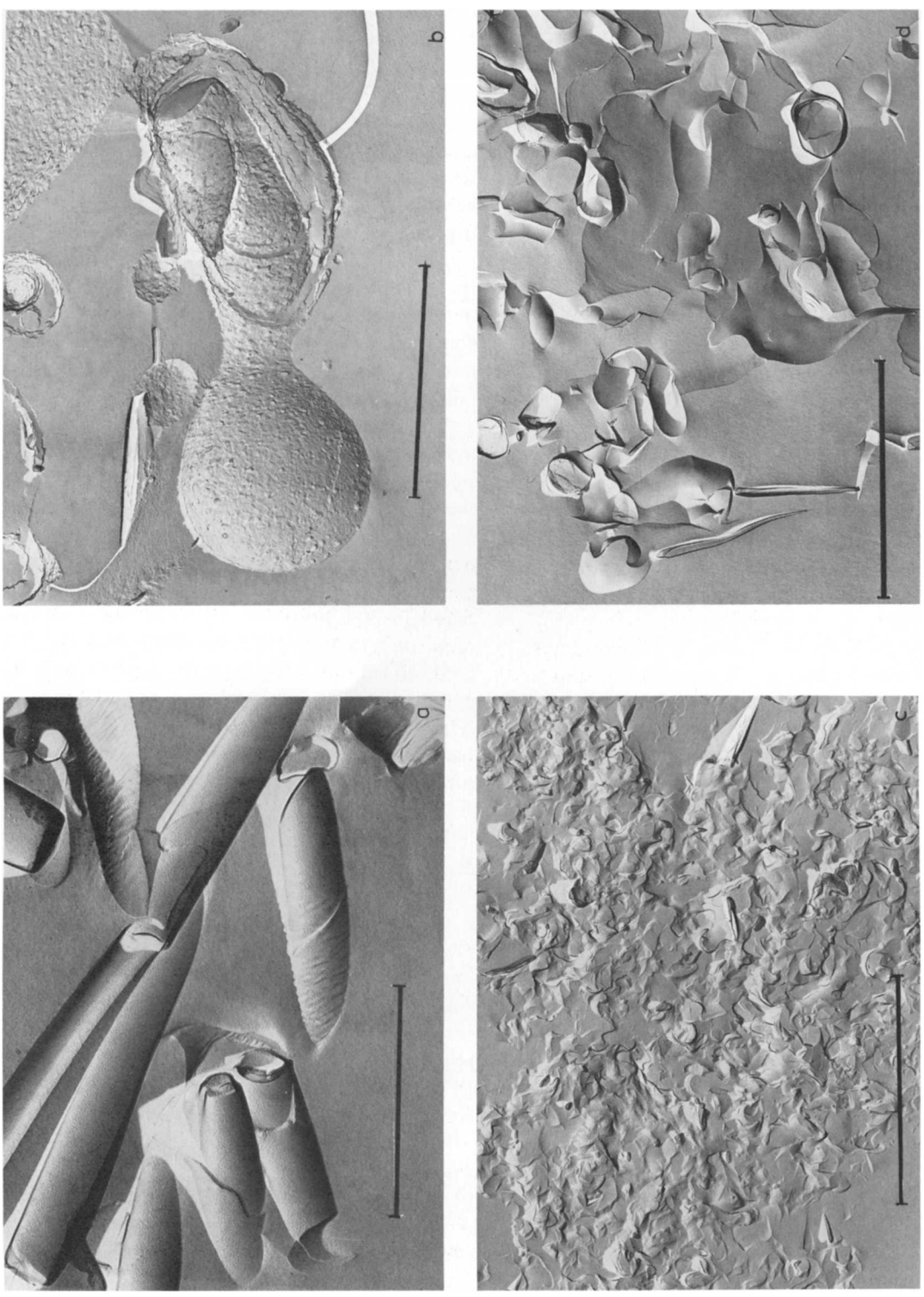


Fig. 1. Electron micrographs of freeze-fractured liposome preparations. (a) Non-irradiated C_{20}-phospholipid liposomes quenched from 4°C. (b) C_{20}-phospholipid liposomes irradiated at −15°C and quenched from 37°C. (c) Irradiated C_{20}-phospholipid liposomes quenched from 4°C. (d) Non-irradiated C_{25}-phospholipid liposomes quenched from 25°C. Liposomes formed in 30% glycerol. Bar = 1 μm.

irradiation. Occasionally long, tube-like structures are seen in non-irradiated C_{20}-phospholipid liposomes. Freeze-fractured preparations of C_{23}- and C_{25}-phospholipid liposomes also reveal the presence of spherical structures, both before and after irradiation. However, C_{20}-phospholipid liposomes quenched from 4 and 37°C appear as long, bullet-shaped bodies several micrometers long, with smooth faces (Fig. 1a). Fractures across the long axis of some of these bodies show a coil-like structure, suggesting that these may have been formed by 'rolling-up' of a flat sheet of bilayer. After irradiation for 5 min, examination of samples quenched from 4°C (the maximum temperature they were permitted to attain prior to examination) reveals aggregates, without any specific structure. Samples incubated at 37°C for 15 min and quenched from this temperature are found to contain definite liposomal structures. These are multilamellar, mainly spherical and some are dumb-bell shaped (Fig. 1b). In addition, some of the fracture faces, particularly the convex, have a very rough appearance. This is not observed on the inner faces of the liposome vacuole and appears to be restricted to the hydrophobic portion of the bilayers.

Irradiation conditions and the degree of polymerization

Irradiation with ultraviolet light results in polymerization of the diacetylenic phospholipids in liposomes. Polymerization has usually been monitored by changes in the colour of the compounds. In order to determine the optimum conditions for polymerization the effect of temperature and the duration of the irradiation were studied.

The C_{20}-, C_{23}- and C_{25}-diacetylenic phosphatidylcholines show sharp symmetrical endothermic phase transitions at 22, 39 and 50°C, respectively, as measured by calorimetry. The colour of the polymer is dependent upon the temperature both during and after irradiation. C_{20}-phospholipid liposomes form red-coloured polymer when irradiated between −15 (the minimum tested) and 4°C. From 4°C to the transition temperature, the polymer is yellow. Similar behaviour is observed with the C_{23}-phospholipid liposomes, red-coloured polymer being formed below 10°C and the highest colour yield below about 4°C. In

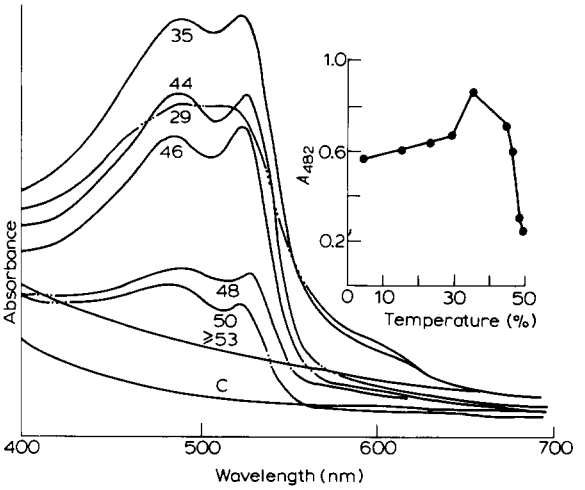
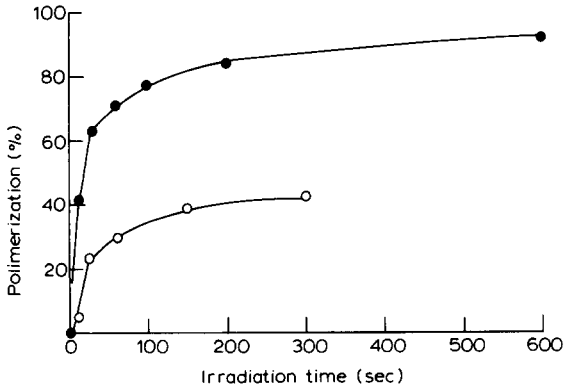


Fig. 2. Visible spectra of suspensions of C_{25}-diacetylenic phosphatidylcholine liposomes irradiated at various temperatures. Insert shows the effect of temperature of irradiation on the absorbance at 482 nm. Irradiation was for 5 min. Lipid concentration was 1 mg·ml^{-1}; light path = 0.2 cm. C, non-irradiated suspension.

contrast, a deep red polymer is formed when C_{25}-phospholipid liposomes are irradiated between 32 and 41°C. At temperatures above and below this optimum the polymer becomes progressively more yellow and the absorbance decreases (Fig. 2). The circular dichroism spectra show a temperature dependence similar to that observed in the visible spectra. With all lipids, the red form of the polymer can be converted to the yellow by heating. As a result of these observations, irradiation of C_{20}-, C_{23}- and C_{25}-phospholipids was performed at 0, 4 and 35°C unless otherwise stated.

The degree of C_{20}- and C_{25}-lipid polymerization was measured by quantitative GLC of the non-polymerized fatty acyl chains. The effect of irradiation time on the degree of polymerization of both lipids is shown in Fig. 3. With both lipids, polymerization occurs rapidly at short irradiation periods, the rate decreasing at longer periods. The C_{25}-phospholipid is much more reactive than the C_{20}, over 80% of the monomeric acyl chains having polymerized after 300 s compared with about 40% of the C_{20} acyl chains. If the absorbance of the liposome suspensions at 480 nm is plotted against the amount of crosslinked acyl chains (Fig. 4), biphasic curves are obtained for both lipids. In the case of the C_{20}-lipid, little coloured polymer is



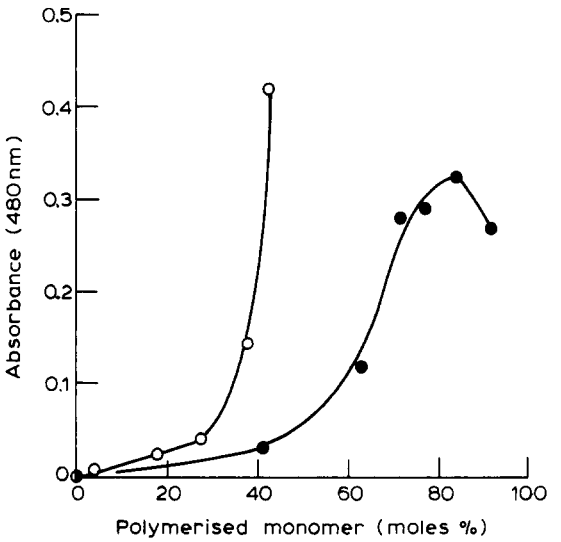


Fig. 3. The effect of the irradiation period on the polymerization of acyl chains in C_{20}- and C_{25}-diacetylenic phospholipid liposomes. Acyl chain polymerization measured as detailed in the text. Lipid concentration = 1 mg·ml^{-1}. C_{20}-liposomes irradiated at − 15°C in 30% glycerol, C_{25}-liposomes irradiated at 35°C in water. ●, C_{25} lipid; ○, C_{20} lipid.

Fig. 4. The absorbance of liposome suspensions containing various amounts of polymerized monomer. Conditions as in Fig. 3. ●, C_{25}; ○, C_{20}.

formed up to about 30% polymerization. Beyond this point, even slight increases in the amount of polymerization results in large increases in the absorbance of the suspension. The behaviour of the C_{25}-phospholipid is similar, highly coloured polymer being formed from about 50% crosslinking. At long irradiation times the C_{25}-phospholipid polymer is bleached, the absorbance decreasing.

Molar extinction coefficients of the polymers cannot be calculated from the data in Fig. 4 in view of the non-linearity of the plots. In addition, the molecular weight of the polymer is unknown and a mixture of different polymers probably exists. For comparative purposes, we have calculated 'apparent molar extinction coefficients' based on the molecular weight of the lipid monomer and the degree of polymerization when absorbance at 480 nm is maximum. For the C_{20}-phospholipid polymer this has a value of $4.2 \cdot 10^3$ mol$^{-1} \cdot$ cm^{-1} after 300 s irradiation and for the C_{25}-phospholipid polymer the value is $2 \cdot 10^3$ mol$^{-1} \cdot$ cm^{-1} after 200 s.

Solubility of the polymers

Extracting the polymer after various irradiation times using chloroform/methanol reveals differences in its structure. At short irradiation times (up to about 60 s) both phospholipids form polymers which are completely soluble in the chloroform phase. At longer irradiation times, insoluble polymer is also formed which remains at the interface between the chloroform and water/methanol layers. The insoluble polymer formed from the C_{20}-lipid has a dense, flake-like appearance, whereas the C_{25}-phospholipid polymer has a much more diffuse, foam-like structure.

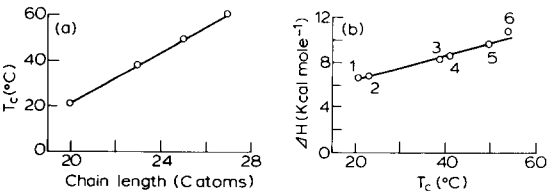


Fig. 5. (a) Variation of the t_c with chain length of diacetylenic phosphatidylcholines. (b) Change in the enthalpy of the main phase transition of various phosphatidylcholines as a function of t_c. 1 = C_{20}-; 2 = dimyristoyl-; 3 = C_{23}-; 4 = dipalmitoyl-; 5 = C_{25}-; 6 = disteoroylphosphatidylcholine.

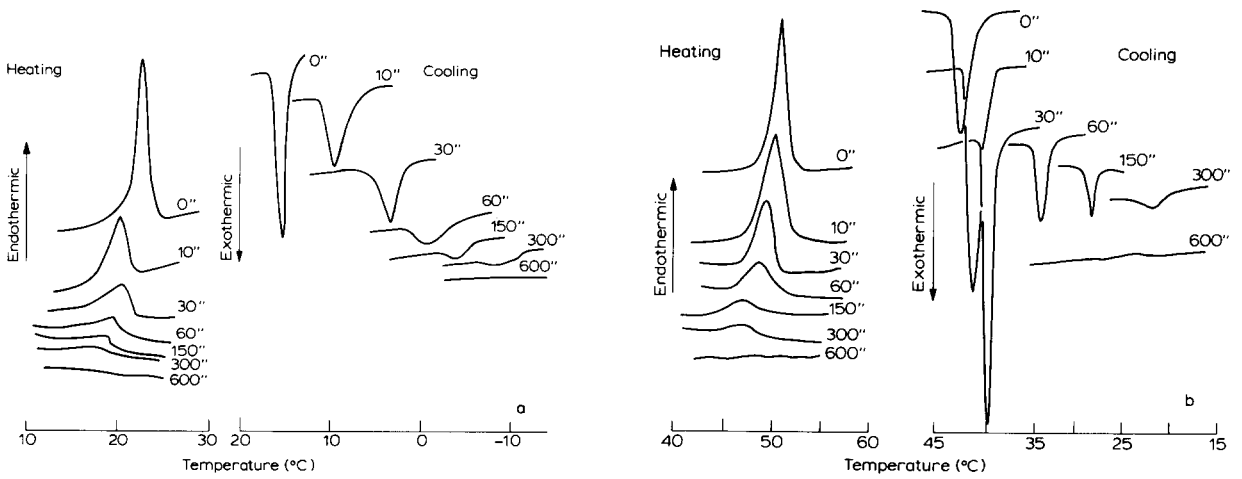


Fig. 6. Calorimetric heating and cooling curves for (a) C_{20}- and (b) C_{25}-diacetylenic phosphatidylcholine liposomes after various irradiation times. The C_{20}-phospholipid liposomes were irradiated at 0°C and the C_{25}- at 35°C.

Differential scanning calorimetric studies

All the diacetylenic phospholipid monomers show narrow symmetric heating and cooling curves. As with other unsaturated phospholipids, no pretransition is observed. The t_c (temperature at which the onset of the transition occurs) of the C_{20}-, C_{23}-, C_{25}- and C_{27}-lipids is at 22, 39, 50 and 60°C, respectively and a hysteresis of about 5°C is observed. The increase in the t_c with the length of the acyl chain is linear (Fig. 5a). The t_c values of the diacetylenic phospholipids are lower than those of the corresponding saturated phosphatidylcholines but higher than that of the *cis*-unsaturated homologues. The molar transition enthalpies (ΔH) for the diacetylenic lipids fit well into a plot of ΔH vs. t_c for saturated phosphatidylcholines [9] (Fig. 5b).

The results of the DSC studies on the C_{20}- and C_{25}-lipids after various irradiation times are shown in Figs. 6–9. For both lipids, the t_c (heating) is consistently higher than the t_c (cooling) at all irradiation times (Fig. 7). The t_c (heating) of the C_{20}-phospholipid is much less sensitive to the amount of polymer than the t_c (cooling) showing a 9°C decrease compared with 26°C for the same irradiation period. The decrease in both the heating and cooling transition temperatures is linear with respect to the amount of monomer crosslinked. After an initial sharp decrease, the reduction in the enthalpy of the transition as a function of the amount of polymerization is biphasic, showing a slow decrease to 30 mol% polymerized followed by a more rapid decrease (Fig. 8).

The t_c (heating) of the C_{25}-phospholipid decreases steadily from 50 to 47°C (55% polymerized) and then decreases more rapidly to 42°C as the amount of crosslinked monomer increases to 90%. The t_c cooling shows a similar slow decrease up to 55 mol% of monomer polymerized, but beyond this point the reduction is much more pronounced (Fig. 7b). The enthalpy of the phase transition shows a similar break with a slow decrease up to about 60 mol% polymerized and a much more rapid decrease thereafter (Fig. 8).

The heating curves are consistently broader than the cooling curves at all irradiation times. Increases in the proportion of polymerized material result in a broadening of the transitions, with the exception of the C_{25}-phospholipid cooling curves,

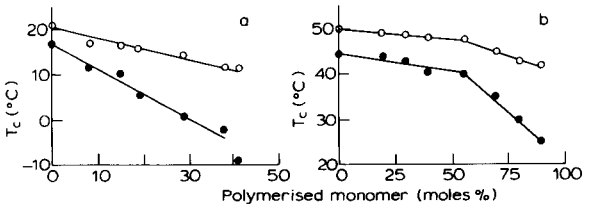
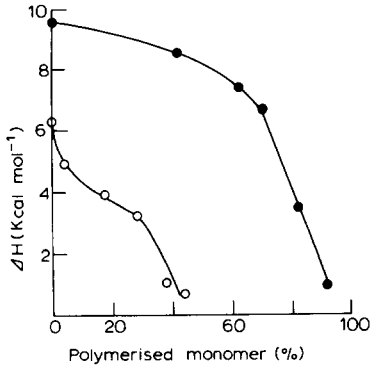


Fig. 7. The (○) heating and (●) cooling t_c of (a) C_{20}- and (b) C_{25}-diacetylenic phosphatidylcholine cxontaining various proportions of crosslinked acyl chains.



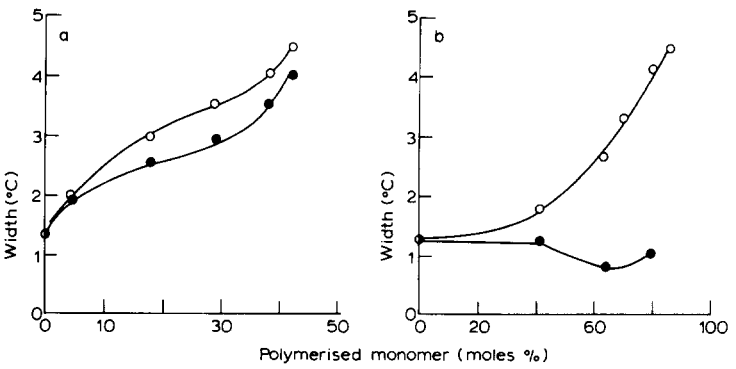


Fig. 8. (Left-hand figure.) The effect of amount of polymerized acyl chains on the enthalpies of the main phase transition. ○, C_{20}; ●, C_{25}. Heating and cooling enthalpies were very similar.

Fig. 9. The width at half height of the calorimetric transition peaks as a function of (a) C_{20}- and (b) C_{25}-diacetylenic phospholipids; ○, heating ●, cooling.

which are reasonably constant at about 1°C (Fig. 9).

Non-polymerized liposome preparations and polymerized C_{23}- and C_{25}-phospholipid liposome preparations are reasonably stable at room temperature as measured by the lack of precipitation of material. Polymerized C_{20}-phospholipid liposomes are less stable, precipitation of polymer occurring much more readily than with C_{25}-polymerized liposomes.

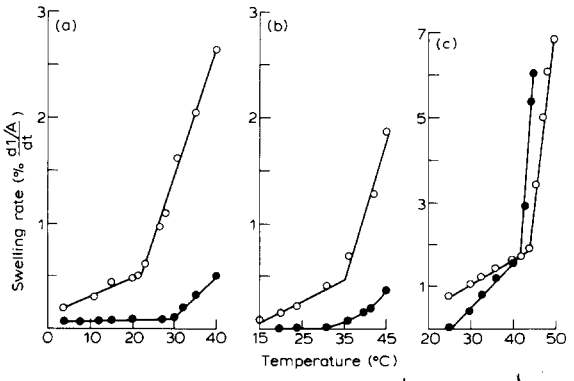


Fig. 10. Initial swelling rates in isotonic glycerol of polymerized and non-polymerized diacetylenic phospholipid liposomes as a function of temperature. (a) C_{20}-, (b) C_{23}- and (c) C_{25}-diacetylenic lipids. ○, non-irradiated; ●, irradiated for 5 min.

Permeability of liposomes to glycerol

Liposomes were found to behave as ideal osmometers when tested as detailed by De Gier et al. [8]. Comparison of the swelling rates in glycerol solution of non-irradiated C_{20}-, C_{23}- and C_{25}-diacetylenic phospholipid liposomes at various temperatures shows a discontinuity at temperatures in the region of the phase transitions of the lipids (Fig. 10). At higher temperatures, the liposomes become much more permeable. This is similar to the results obtained by various authors using other lipids and whole cells enriched with specific fatty acids [8,10]. Discontinuities are also observed in the permeability of the polymerized liposomes. In the case of the C_{23}- and C_{25}-lipids, this occurs at temperatures below those of the non-irradiated sample. The 4°C decrease in the position of the break as a result of polymerization observed with the C_{25}-lipid is in good agreement with the 5°C decrease in the t_c (heating) as measured by calorimetry for these preparations. In contrast, the irradiated C_{20}-phospholipid liposomes show a discontinuity above the temperature at which it is observed in the non-irradiated sample.

Although in general polymerization causes a significant reduction in the permeability of the liposomes to glycerol, irradiated C_{25}-diacetylenic phospholipid liposomes appear to be more permeable at temperatures above 42° than are the non-irradiated samples.

Discussion

Although the structure of the liposomes formed by the C_{25}-phospholipid is apparently not affected by polymerization (c.f., the stability of liposomes of diacetylene-containing phospholipid analogues [11], that of the C_{20}-phospholipid liposomes as seen in freeze-fractured preparations is considerably altered. The bullet-shaped structure observed in the non-irradiated preparations are similar to the 'cochleate' cylinders formed by Ca^{2+}-induced fusion of unilamellar phosphatidylserine vesicles. Fused vesicles are believed to roll-up to form cylindrical vesicles [12]. The C_{20}-phospholipid liposomes may similarly be produced by the rolling-up of planar lamellae to form bullet-shaped structures. Irradiation apparently causes these liposomes to collapse, possibly as a result of strain induced in the vesicles by crosslinking of the acyl chains. Incubation of the irradiated preparations above the transition temperature leads to the formation of another, more rounded type of liposome. These liposomes have a rough surface appearance, apparently within the hydrophobic portion of the bilayer, which may represent polymerized material. Various observations suggest differences in the type of polymer formed by these two lipids (see below).

In order to assess the various changes in the properties of the liposomes e.g., the phase transition behaviour, which occur as a result of irradiation, it is necessary to have a quantitative method of measuring the degree of polymerization. It is important to note that these particular phospholipids, with diacetylene groups in both acyl chains, may undergo both intra- and intermolecular crosslinkage.

Previous methods of quantifying the polymerization of diacetylene-containing compounds, e.g., fatty acids, have been based mainly on estimation of the amounts of high-molecular-weight polymer produced. Often, this has involved the separation of monomeric and polymeric material by gel permeation chromatography [13] or differential solubilization using organic solvents [14,15] followed by estimation of high-molecular-weight material on the basis of its extinction coefficient or weight. Calorimetry, to determine the enthalpy of the main phase transition, has also been used as a measurement of the amount of residual monomer [11]. Unfortunately, none of these methods is entirely satisfactory, due to the similarity in the properties of low molecular weight products and the monomer. The GLC method which we have used has the advantage of measuring only the conversion of monomer, irrespective of the molecular weight of the crosslinked product. With this method we have an easily quantifiable and sensitive measure of the rate of polymerization which, coupled with changes in the visible spectra of the samples, gives a good indication of the course of polymer formation.

The colour of the polymer is dependent upon its molecular weight and more especially upon the average conjugation length over which the π-electrons of the adjacent carbon atoms in the conjugated backbone are delocalized [16]. The red coloured polymer represents a more planar, less twisted structure containing fewer structural defects than the yellow form. The effect of the temperature of irradiation on the type of polymer produced by the C_{20}- and C_{23}-lipids is therefore as expected. At lower temperatures the packing of the acyl chains of the lipids will be more crystalline and the resultant polymer will have a more planar backbone. At higher temperatures the packing will not be as good, and consequently the polymer conjugation length is reduced. The temperature dependence of the polymerization of the C_{25}-diacetylenic phosphatidylcholine is different. Although the polymerization at 35–40°C is better than that at higher temperatures, the polymer becomes more yellow as the irradiation temperature decreases below this region. Probably at temperatures below 35°C factors other than the crystalline packing of the monomer begin to affect the structure of the polymer, e.g., extensive sidebranching of the polymer which will increase the twisting of the backbone. If this is the case then we would expect differences in the form of the C_{20}- and C_{25}-polymers and this is seen in the solvent extractions. Whereas the C_{20}-forms a very compact polymer, the C_{25} is much more diffuse, perhaps as a result of its highly branched nature. Differences are also seen in the morphology of the liposomes (see above) and in the stability of the polymerized liposomes.

Comparison of the degree of polymerization of monomer as measured by gas-liquid chromatogra-

phy with the absorbance of the polymer (Fig. 4) shows that although the C_{25}-phospholipid polymerizes to a greater degree than the C_{20}, both appear to polymerize by the same process. Shorter, poorly absorbing regions of crosslinked material appear to form initially and these are subsequently joined at longer irradiation times, resulting in a large increase in the depth of colour of the suspensions from only a small increase in the amount of crosslinking. The differences in the degree of polymerization which must occur before coloured polymers begin to be formed probably reflects differences in their structures as may the variation in the extinction coefficients of the two phospholipid polymers.

The calorimetric studies show that even in non-irradiated samples, the t_c is 5°C higher in the heating mode than in the cooling mode for both lipids. This may be due to the rigid, bulky diacetylene groups hindering crystallization of the acyl chains. Due to the non-linear nature of the reduction of the enthalpy of the phase transition of these two compounds as a function of irradiation time (Fig. 8), calorimetry alone can give only an approximate measure of the amount of polymerization which has occurred.

As the amount of polymer increases in the liposome system, so does the width of the melting transition peak (Fig. 9). Above the transition, a reasonably homogeneous mixture will exist. The transition is sharper in the cooling than in the heating mode. This is especially noticeable with the C_{25}-phospholipid. The difference in the rate of decrease of the t_c for the C_{25}- and C_{20}-lipids may reflect differences in the structures of the polymers which are formed. The point at which the t_c (cooling) of the C_{25}-lipid begins to decrease rapidly (60 mol%) is the point at which highly coloured polymer begins to form. Presumably, as irradiation of the lipid takes place a mixed phase system develops consisting of increasing proportions of polymer to monomer. The heating and cooling curves will then reflect the extent of co-crystallization and phase separation which can occur within these liposome structures (X-ray diffraction studies will be useful for examining these phase characteristics and changes of liposome dimensions and should clarify the interpretation of the calorimetric data).

The results of the glycerol permeability study of the C_{25}-phospholipid liposomes are consistent with those results obtained from the calorimetry study. The permeability of the irradiated samples is lower than that of the non-irradiated preparations, indicating a less fluid structure, and the point at which the permeability of the C_{25}-phospholipid liposomes increases dramatically is shifted to lower temperatures in the irradiated samples.

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