Helical and Tubular Microstructures Formed by Polymerizable Phosphatidylcholines

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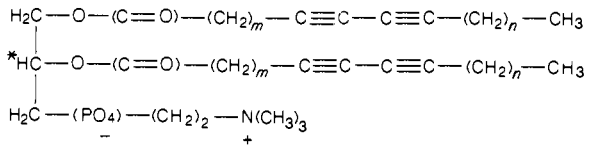
Abstract: Monomeric, but polymerizable, lecithins with diacetylenic fatty acyl chains, such as 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$), are known to form tubular microstructures when liposomes of these lipids are cooled through their chain melting transition. These lipids are soluble in alcohols and other organic solvents, but when such solutions are diluted with water at appropriate temperatures, precipitates form. From optical and electron microscopy the precipitates are seen to consist of tubules and long, open helical structures with diameters similar to those of the tubules. These helices are all right handed when made from lipid with the naturally occurring chiral head group. For an ethanol/water system the proportion of helices and tubules depends on the ratio of the solvent to nonsolvent, as does the overall length of the tubules. The temperature, lipid concentration, and specific solvent used also affect the nature of the precipitate. For $DC_{8,9}PC$ the tubular and helical microstructures vary from 0.3 to 3 μm in diameter, and from 5 to over 1000 μm in length. The width and pitch of the helical ribbons are variable, resulting in a range of structures from open helices to continuous tubules depending on the solvent system used. Upon exposure to energetic radiation such as UV rays or γ-rays, the diacetylenic units polymerize without causing loss of the helical or tubular microstructure, thereby stabilizing the microstructures. Demonstration of this formation route for tubules suggests that they are thermodynamically stable, not accidental products of deforming liposomes. The existence of this polymerizable helical microstructure that may be an intermediate in the formation of tubules supports previous indications of an underlying helical structure to tubules. This precipitation method also affords a simple method of controlling the dimensions of tubules and a screening method for the discovery of other self-organizing lipid microstructures.

Our laboratory has been synthesizing and characterizing polymerizable lipids and the microstructures they form, particularly diacetylenic lecithins such as 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine, which we have previously called $DC_{23}PC$. These lipids contain diacetylenic moieties in their two identical hydrocarbon chains whose positions can be specified by the number of methylene groups between the carboxyl and diacetylene groups (m), and between the diacetylene and terminal methyl groups (n). Hence the aforementioned compound is designated $DC_{8,9}PC$. The general formula is



The diacetylenic groups render these lipids polymerizable by ultraviolet light and other forms of radiation.[1-8] $DC_{8,9}PC$ forms liposomes in aqueous dispersion above its hydrocarbon chain melting temperature as measured in excess water (T_m of 43 °C). These liposomes are unstable at lower temperatures;[9,10] on gradual lowering of the temperature to about 38 °C, they can convert quantitatively to hollow "tubules".[9,11] Dimensions vary from 0.4 to 1 μm in diameter and tens to several hundreds of micrometers in length, with walls that vary from two to about ten bilayers in thickness, i.e., 10–50 nm. These tubules often contain one or more trapped liposomes that can allow immobilization of aqueous contents within the tubule[12] but prevent the tubule lumen from being a true open cylinder.

When examined with the electron microscope, tubules are rarely featureless cylinders. Tubule walls may have subtle regular spiral ripples and often consist of regularly wrapped helical bilayer strips of constant or varying width (see, for example, Figure 2 in ref 11). When tubules are polymerized there is an improvement in their mechanical and thermal stability,[9] although much greater reinforcement may be imparted to the microstructures by coating them with metals.[13,14] Tubules both with and without metal coatings may be oriented by both flow and magnetic fields.[13,15]

While a mechanism of tubule formation from liposomes has been proposed[16] a drawback for both study and application of tubules has been the relative lack of control over the end product of tubule formation. Tubule diameters are relatively constant regardless of the nature of the starting material, while their lengths vary widely, as do both the number of bilayers in each tubule and the efficiency of conversion. Often a sustantial proportion of the lipid material fails to form tubules,[11] and this material is difficult to separate from tubules. If the starting liposomes are too small,

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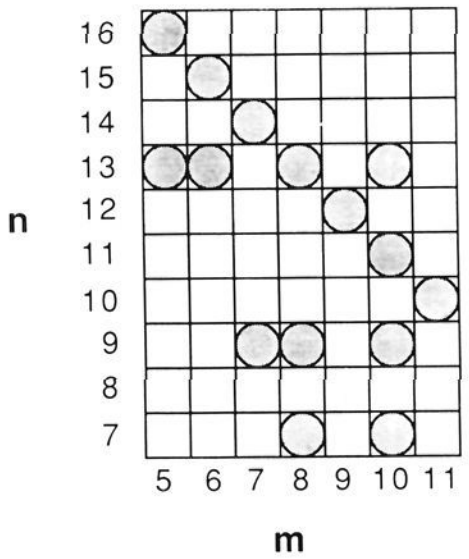

Figure 1. Methylene chain segment lengths in those diacetylence lipids that we have synthesized. The *m* segment is between the ester group and the diacetylene, and the *n* segment is between the diacetylene and the terminal methyl group.

such as those formed by sonication, little or no conversion to tubules occurs.[7,10,17] No systematic variation from lipid to lipid in the nature of the tubules formed by diacetylenic lipids has yet been found.

A method of controlling tubule characteristics that has shown some promise is manipulation of the pH and salt content of the aqueous phase. At concentrations of NaCl or $CaCl_2$ approaching 1 M there is a consistent reduction in the length of the tubules formed. Unfortunately we have found no modification of the aqueous phase that consistently increases the median tubule lengths to greater than about 50 μm. Extremes of pH seem to increase the aspect ratio of the tubules, primarily by decreasing tubule diameter. This technique has the drawback that incubation of $DC_{8,9}PC$ and other ester-containing lipids at extreme pH at the required temperatures results in appreciable hydrolysis of the ester groups and loss of the lipid. In assessing the effects of aqueous solutes on the formation of tubules it is also difficult to separate the effects of the solute on liposome formation as distinct from effects on the assembly of tubules from those liposomes.

In studying the properties of monolayers of $DC_{8,9}PC$ formed at the air–water interface, it was noticed that tubules were occasionally observed when excess lipid was added to the surface in solvents such as hexane and chloroform. It was also found that tubule formation from liposomes was unhindered by the presence of concentrations of glycols such as glycerol as high as 90%.[11] We then examined the effects of other alcohols on the formation of tubules. This led to the discovery described in this report that tubules may be formed by precipitation from solution in organic solvent—a route that involves no liposomes and allows far greater control over the end product. A brief preliminary report of this work has appeared elsewhere.[18]

Experimental Section

The syntheses of the diacetylenic lipids used in these experiments were reported elsewhere. These lipids include $DC_{8,9}PC$[19] and other homologues of the form $DC_{m,n}PC$ in which $C_{m,n}$ is $C_{10,13}$,[20] $C_{5,13}$, $C_{6,13}$, $C_{7,9}$, $C_{10,9}$, $C_{10,7}$,[21] and $C_{8,7}$. We have also synthesized a series of lipids with a total of 27 carbons in their hydrocarbon chains, including $C_{5,16}$, $C_{6,15}$, $C_{7,14}$, $C_{8,13}$, $C_{9,12}$, $C_{10,11}$, and $C_{11,10}$.[22] These are shown in Figure 1. Furthermore, two heterobifunctional lipids have also been synthesized that have a polymerizable olefin or methacrylate unit at the base of each alkyl chain, in addition to the diacetylenic unit in the middle of each chain. We refer to these as the $DC_{8,8}PC$ terminal olefin and the $DC_{8,11}PC$ terminal methacrylate, respectively.[23] Diacetylenic lipids were purified on silica gel and gave single spots when chromatographed on silica gel TLC plates. 1,2-bis(hexadecanoyl)-*sn*-glycero-3-phosphocholine (dipalmitoylphosphatidylcholine, or DPPC) was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification.

Organic solvents including methanol, ethanol, 2-propanol, and propylene glycol were all Fisher HPLC grade and were used as received. Water was distilled first in a Corning deionizing still, followed by double distillation in a quartz still. To form microstructures, dry lipid was weighed on a Cahn microbalance in a class 100 clean room under amber light and placed in a "critically cleaned" Series 300 amber borosilicate vial used as received from I-Chem Research, Inc. (Hayward, CA). Alcohol or another organic solvent was then added to the vial and the lipid was allowed to dissolve at 20 °C to a final concentration of between 0.2 and 3 mg/mL. Water was then added in one of three ways with different rates of mixing. It was either (a) added directly to the vial followed by stirring by repeated inversion, (b) slowly dripped into the vial during stirring, or (c) dialyzed in over a period of tens of hours by placing the solution in a dialysis bag in a large stirred volume of solvent, into which water was dripped. After incubation for an appropriate length of time, organic solvents were removed by dialysis against distilled water, filtration, or repeated centrifugation with exchange of the decanted supernatant. When desired, the resultant microstructures were polymerized either with a low-pressure mercury lamp (254 nm) or with between 1 and 9 Mrad doses of γ-radiation from a 1.33 MeV ^{60}Co source.

Optical microscopy was performed at room temperature with a Leitz Ortholux I (E. Leitz, Inc., Rockleigh, NJ) with use of phase contrast and darkfield illumination. Transmission electron microscopy of samples air dried on carbon films and of freeze-fracture replicas was performed as previously described on a Zeiss EM-10 (Carl Zeiss Inc., Thornwood, NY).[11] For freeze-fracture samples, glycerol (10% (v/v)) was added to samples immediately prior to freezing from room temperature by plunging them into melting nitrogen. Samples were transferred to a Balzers BAF 400D freeze-fracture unit (Balzers Union, Hudson, NH), fractured, deeply etched, and then replicated at −110 °C and 2×10^{-6} Torr. Replicas, which were made with 2.5 nm of Pt–C and 15 nm of C, were cleaned with ethanol and chloroform and transferred to the electron microscope. To facilitate visualization of the fragile helical structures in a manner that allowed the handedness of the helices to be observed, samples in water were coated with a thin layer (20–30 nm) of copper metal by solution processing techniques described elsewhere.[13,14] They were then dried and imaged in a ISI DSM-130 scanning electron microscope (International Scientific Instruments, Inc., Santa Clara, CA) in the secondary electron mode.

Tubule lengths were measured directly from optical micrographs of wet samples. Diameters were measured from transmission electron micrographs of air-dried tubules, taking into account the increase in apparent diameter by a factor of $\pi d/2$ that occurs on flattening of a cylinder.

Results

When a nonsolvent, particularly water, was added to a solution of diacetylenic lipid at a temperature below T_m of the lipid, the mixture became cloudy within seconds or minutes, depending on concentration. Optical microscopy revealed that the cloudiness was due to the formation of a precipitate of tubules and helices with a distribution of lengths. When substantial amounts of water were added so that the final concentration of solvent was relatively low (40–50% (v/v)), the precipitate formed immediately and consisted almost entirely of smooth tubules with little evidence of open helical structures. In this case the tubules were short, on the order of 10–20 μm, with a relatively broad distribution of lengths. If, on the other hand, the final concentration of solvent was relatively high (70–80%), the precipitate was a heterogeneous mix of tubules (that were in general longer than those found at lower solvent concentrations), helices, and tangles of helices of various lengths.

The range of tubule sizes that can be obtained under different conditions of solvent concentration and incubation time is illustrated in Figure 2. Here $DC_{8,9}PC$ in ethanol was mixed with water to produce final concentrations of lipid of 0.5 mg/mL and concentrations of solvent of 50, 55, and 70%. With 50 and 55% solvent, parts a and b in Figure 2, respectively, microscopic observations were performed after an incubation time of 10 h, revealing separate short tubules. The precipitate from 70% ethanol

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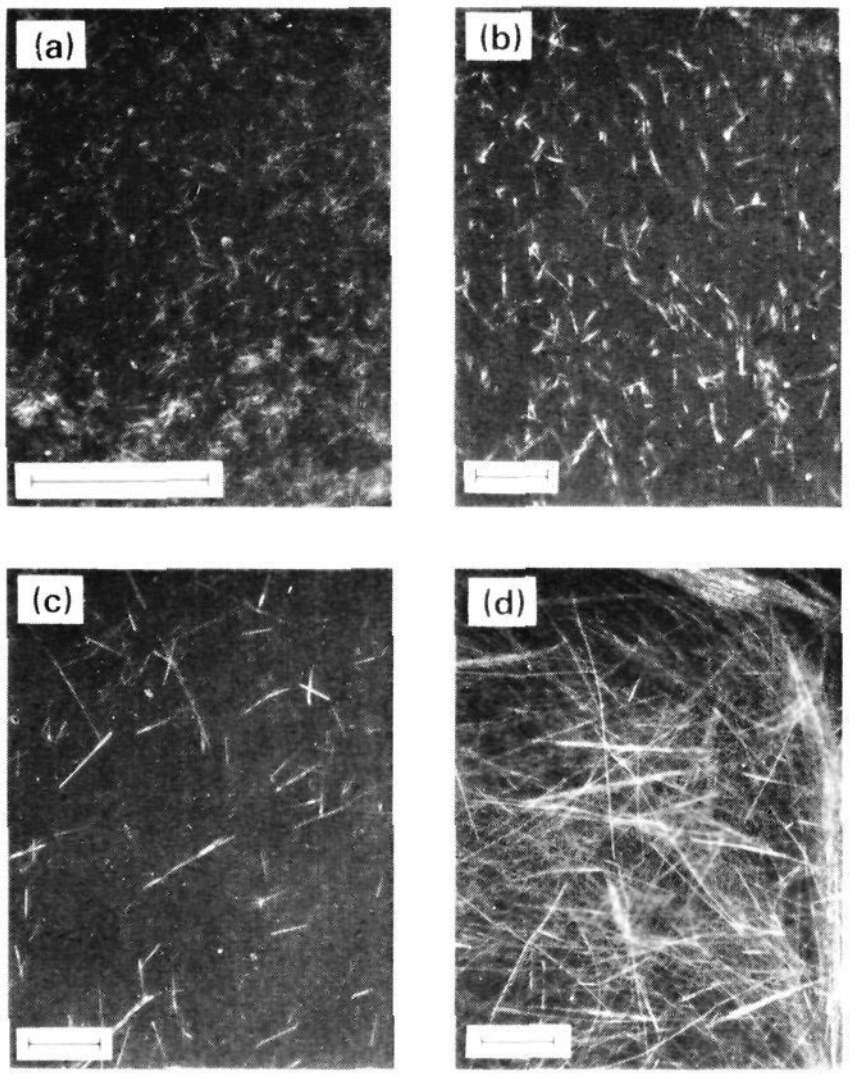

Figure 2. Darkfield optical micrographs of tubules formed by addition of water to ethanol solutions of $DC_{8,9}PC$. The micrographs were taken at room temperature. With little water the tubules were uniformly long—some in Figure 2d are longer than 300 μm. The final concentration of the lipid was 0.5 mg/mL in all cases. The final concentrations (v/v) of ethanol were (a) 50%, (b) 55%, and (c and d) 70%. The incubation times in the ethanol–water mixtures were (a and b) 10 h, (c) 144 h, and (d) 6 months. Scale bars = 100 μm.

Table I. Tubule Dimensions as a Function of Solvent Concentration and Incubation Time (Final Lipid Concentration Was 0.5 mg/mL)

ethanol concn (%)	incubation time (h)	tubule lengths (μm)	tubule diameters (μm)
50	10	12 ± 6	
55	10	23 ± 11	
70	144	50 ± 31	0.47 ± 0.1
70	4400	170 ± 92	

after 6 days of incubation is shown in Figure 2c, which consists of substantially longer tubules. Figure 2d is of a sample similar to that of Figure 2c but incubated for 6 months, in which the tubules have grown to lengths of hundreds of micrometers. The observations of Figure 2 are quantified in Figure 3 and Table I, giving the length distributions of tubules as a function of final solvent concentration and incubation time. Samples containing solvent and left undisturbed continue to lengthen. Samples incubated for 6 months have contained tubules as long as 1200 μm. Sample flow on the microscope stage subjects tubules to bending forces, but even the longest structures appear to be rigid and straight. Despite dense packing, the tubules are generally not stuck together.

Freeze-fracture electron micrographs of precipitated tubules were obtained to compare their morphology with that of liposome-derived tubules. Tubules formed from $DC_{8,9}PC$ in 55% ethanol in water are shown in Figure 4. The walls are often featureless, but they are usually multilayered, and occasionally they show the helical features representative of wrapping that are seen on the surfaces of liposome-derived tubules.

As the final concentration of solvent is raised the variety of precipitated structures increases. When the solvent concentration reaches 60–70%, isolated helical structures are seen in increasing numbers. A darkfield optical micrograph of several $DC_{8,9}PC$ helices and tubules is shown in Figure 5; the central helical structure appears to be associated with a tubule. From one end to the other this helix gradually converts from a nearly continuous tubule to a loosely twisted strip. Such variability in structure within a single helix is a common observation. Figure 6, which shows a transmission electron micrograph of a $DC_{8,9}PC$ sample that has been air dried after precipitation from an 80% methanol solution, shows such variability in greater detail. Here are several long continuous-walled tubules, and a profusion of helices. The helices often are more loosely coiled near their ends than in their central portions. Unlike tubules, open helices are quite flexible and have been observed to bend easily under the influence of solvent shear. However, it can be seen in several cases in Figure 6 that the helical twist can be sufficiently overlapped that the structure is effectively a continuous tubule. It can also be seen that drying in air can damage these objects—all appear to have been flattened by the process (which can be observed by stereo imaging (data not shown)) and many show fractures in their walls.

To overcome the difficulty of obtaining electron micrographs of fragile helices and tubules, the technique of depositing a thin copper coat on the microstructure surface was employed.[13,14] Figure 7 shows a scanning electron micrograph of copper-coated $DC_{8,9}PC$ tubules and helices. These more robust metal-covered objects are obviously three dimensional, and it can be seen that the helices formed from the L stereoisomer of the lipid are right handed. We refer to the collection of helical and amorphous lipid material at the upper right as a "helical tangle". At high solvent concentration helical tangles from which long tubules extend are nearly always present.

Figures 8 and 9 show further evidence of the variety of structures that are observed. The sample shown in Figure 8 was formed by precipitation of $DC_{8,9}PC$ from 70% ethanol solution while the temperature was decreasing from 40 to 22 °C. The figure shows short tubule segments connected by bent helix "hinge" regions. Other unusual structures formed when the temperature was a variable were branched helices and tubules with helical side branches. Figure 9 shows a conical tubule formed from $DC_{8,9}PC$ in 75% ethanol. Precipitation from high concentrations of solvent such as this often produces tubules with diameters as large as 3 μm, and conical tubules like the one shown. Such atypical wide structures provide the opportunity to use polarized light microscopy to study the structure of the tubules. When such wide structures are polymerized, the brightly colored polymer chains render the tubules linearly dichroic; in polarized light it was determined that in such a tubule the chromophore is oriented at an angle greater than 45° to the tubule axis (data not shown). This lends strong support to the hypothesis that the tubule walls are effectively single crystals, although the data are derived from atypical tubules.

To this point we have discussed observations only on microstructures formed by $DC_{8,9}PC$, but in fact all the diacetylenic lipids represented in Figure 1 have been observed to form tubules, either by the liposomal method, by the precipitation method, or both. $DC_{6,13}PC$, for instance, was observed to form precipitates at room temperature from ethanol/water mixture having ethanol concentrations of 60, 70, and 80%. The lipid concentration was 1 mg/mL, and the precipitate consisted of both helices and tubules—some of the tubule lengths being greater than 400 μm in the 80% ethanol sample. $DC_{10,13}PC$, which has the relatively high phase transition temperature of 64 °C, formed tubules at 40 °C by precipitation from 50% ethanol/water. The lipid concentration was 0.5 mg/mL, and structures up to 80 μm in length were seen. The heterobifunctional lipids also have been observed to form tubule precipitates from solution. Since the two lipids have their phase transitions near room temperature the precipitations were performed at 5 °C, from 50% ethanol/water solutions, and formed tubules with lengths up to 40 μm. The methacrylate and olefinic lipids formed tubules that were less stable against temperature changes than the other lipids described above. If the temperature was allowed to rise a few degrees above the formation temperature, these microstructures unwrapped and fell apart. In general it has been found that precipitation of all lipids is best accomplished at T_m −20 °C.

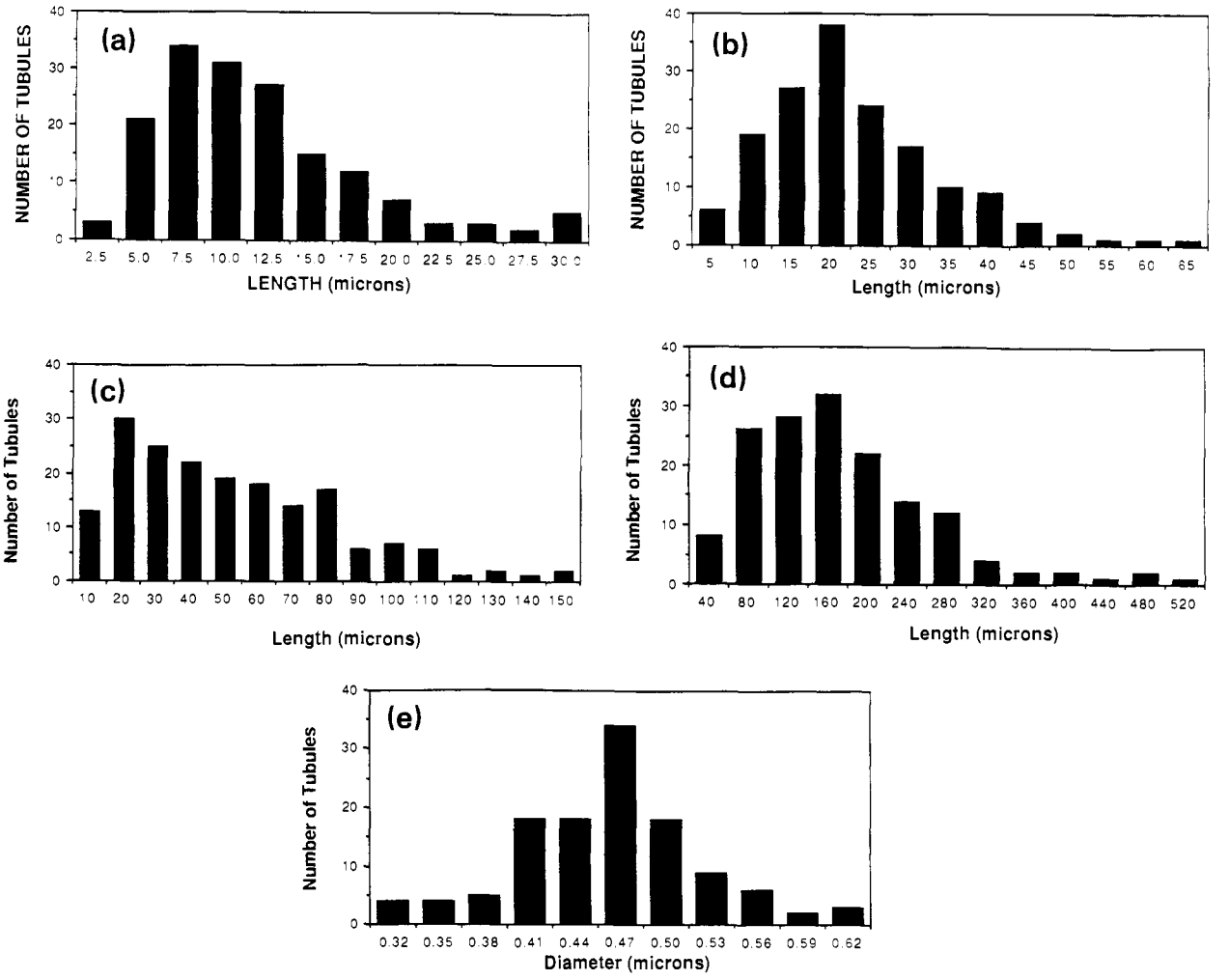

Figure 3. Five bar graphs representing diameters and lengths of tubules of $DC_{8,9}PC$ as prepared in solvent conditions described in Figure 2. Length distribution measured from optical micrographs at (a) 50% ethanol at 10 h, (b) 55% ethanol at 10 h, (c) 70% ethanol after 144 h, and (d) 70% ethanol after 6 months. Diameter distributions of the 70% ethanol sample as in Figures 2c and 3c, as measured by transmission electron microscopy after air drying, are shown in graph e. The mean diameter of 0.47 μm with a standard deviation of 0.1 μm has been corrected for the assumed increase (by a factor of $(\pi]/2)$ caused by flattening of the tubules on drying.

We checked the ethanol–water precipitation procedure described herein using DPPC and found that only small irregular crystals were produced after incubation at room temperature and at ethanol concentrations of 30, 40, 50, 60, and 70%.

Discussion

The formation of lipid microstructures by addition of water to solutions of lipids in water-miscible organic solvents is not new. It has been known for over a decade that lipid vesicles can be formed by injecting small amounts of lipid solutions in ethanol or ether into water (for a review of comparative properties and methods of preparation of lipid vesicles (liposomes) see Szoka and Papahadjopoulos[24]). However, these vesicle formation procedures require that the hydrocarbon chains of the lipid in the resultant microstructure be fluid. If the temperature is below the phase transition temperature of the lipids, liposomes are not generally produced. This is the first report of which we are aware of a phospholipid producing an organized microstructure other than amorphous material or small three-dimensional crystals by mixing solvents and nonsolvents below T_m of the lipids. The failure of DPPC (as an example of a non-diacetylenic lipid with a T_m close to that of $DC_{8,9}PC$) to produce tubules by room temperature mixing of its ethanolic solution with water is consistent with the absence of reports of tubule-like structures being formed by similar procedures with any other lipids. This particular precipitation method cannot produce tubules from all lipids.

The product of this precipitation process is similar but not identical with that produced by cooling liposomes. While liposomes are formed when the temperature of the solvent/water mixture is above T_m for the lipid, there is no evidence for even transient existence of liposomes on precipitation at temperatures substantially below T_m. Precipitated tubules have neither trapped liposomes nor the compartments that such trapped liposomes can create—they are true open hollow cylinders. It is possible to consistently produce tubule preparations with no non-tubular contaminants, which is extremely difficult from liposomal cooling. There is far greater control of the tubule length using this process than is possible by liposomal cooling. Tubule populations that are consistently shorter or much longer than those that can be produced by liposomal cooling have routinely been achieved. At most alcohol/water ratios precipitated tubules have walls that contain fewer bilayers than are commonly observed in liposome-derived tubules. From studies of transmission electron micrographs such as Figure 6, we have observed tubules and helices which appear to have only one bilayer per wall, which we believe cannot be formed by the wrapping process that has been proposed to form tubules from liposomes.[16] Such tubules consist of a single cylindrical bilayer without seams or overlaps and are just as straight and rigid as are those with multiple bilayer walls. Beyond these differences, the tubules differ little from those produced by liposomes, as can be seen by the strong resemblance between the freeze-fracture micrograph in Figure 4 and previously published freeze-fracture images of liposome-derived tubules.[11]

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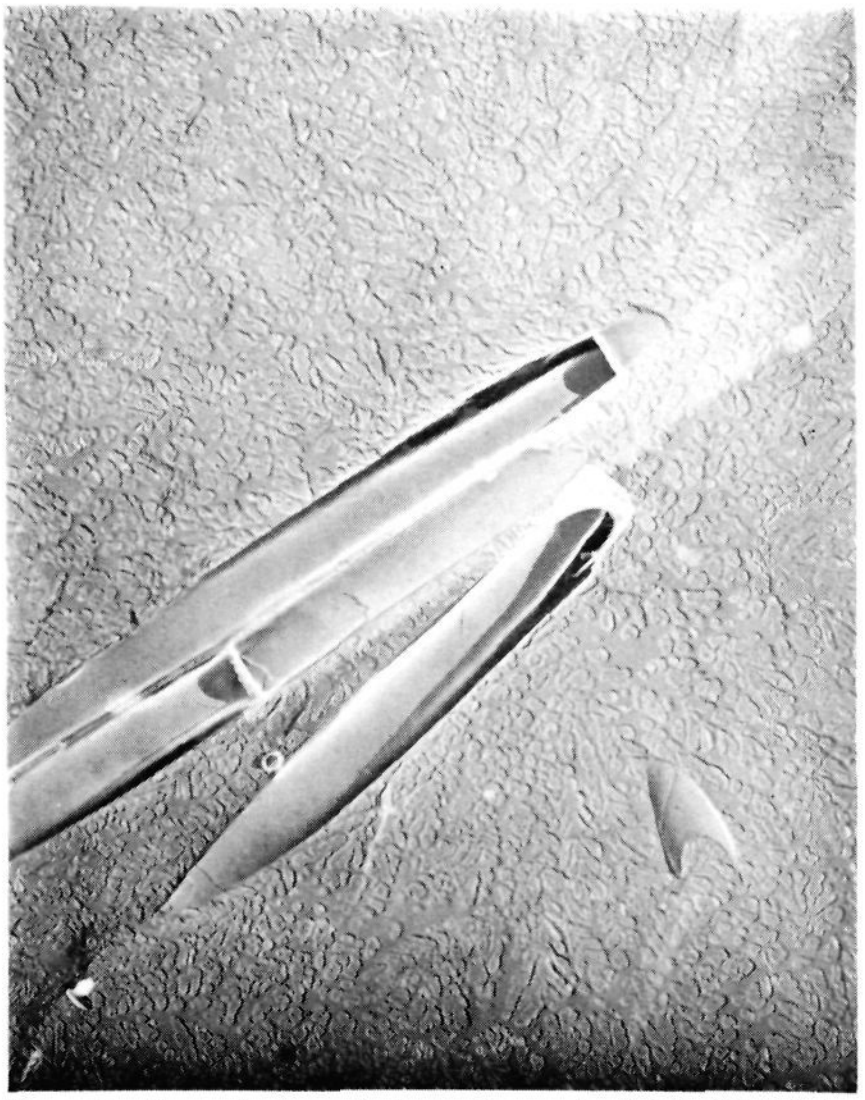

Figure 4. Freeze-fracture electron micrograph of $DC_{8,9}PC$ tubules formed from 55% ethanol in water. After addition of the water the sample was allowed to stand for 3 h and then washed three times in pure water by centrifugation. The cryoprotectant glycerol was added to the last wash to a final concentration of 10% by volume. The tubules are relatively smooth and have fewer of the diagonal wrapping marks seen in tubules formed from liposomes. Scale bar = 1 μm.

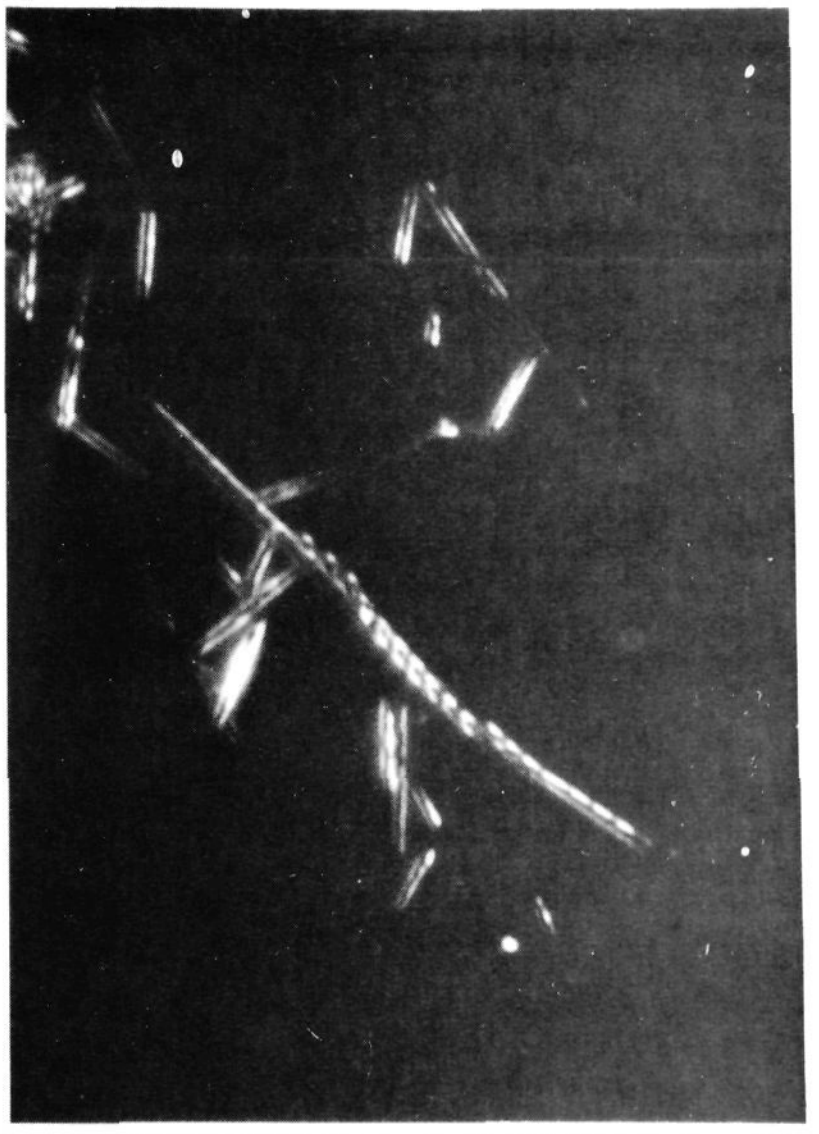

Figure 5. Darkfield optical micrograph of a $DC_{8,9}PC$ helix formed from 80% methanol in water. Note the presence of open helices, nearly continuous helices, and complete tubules. Scale bar = 20 μm.

The most striking difference between precipitation and cooling of liposomes is the production of a large proportion of helical structures, particularly at high solvent concentrations. Helices are occasionally seen when liposomes are cooled (see, for example, ref 11, Figure 2), but rarely do they occur as open structures separate from intact tubular structures. Helices are quite similar in diameter to tubules, but they differ from tubules in that they are far more flexible and are often seen by optical microscopy to curve through large angles over the space of a few tens of micrometers. The edges of a flat bilayer strip such as those that appear to form the helix would be unstable because they would expose hydrocarbon interior to water. We believe the bilayer edges in these helices are initially stabilized by the presence of high concentrations of organic solvents like ethanol that can themselves incorporate into disordered regions of lipid bilayer. Addition of large quantities of water to the initial solution of $DC_{8,9}PC$, or allowing the initial alcohol–water solution to remain undisturbed for a longer period of time (of the order of hours), often results in the transformation of a large percentage of the helices into continuous hollow tubules. It is clear, however, that helices can be stable even when the overall concentration of organic solvent is reduced to very low levels after extensive dialysis or rinsing; whether this is because the edges of the bilayer strips are stabilized by retaining some solvent or because they are no longer stabilized but are simply trapped in this conformation is not yet clear.

We have obtained little information as yet on the nucleation of tubules as they precipitate, beyond the expected finding that when more water is added, which effectively produces greater supersaturation of the lipid solution, precipitation is more rapid and results in smaller tubules. The number of successful nuclei, then, depends on the degree of supersaturation. The helical tangles that appear at high solvent concentrations, and which seem to be the origin of most of the tubules produced in these samples, must contain nucleation sites. From the high proportion of multiple-

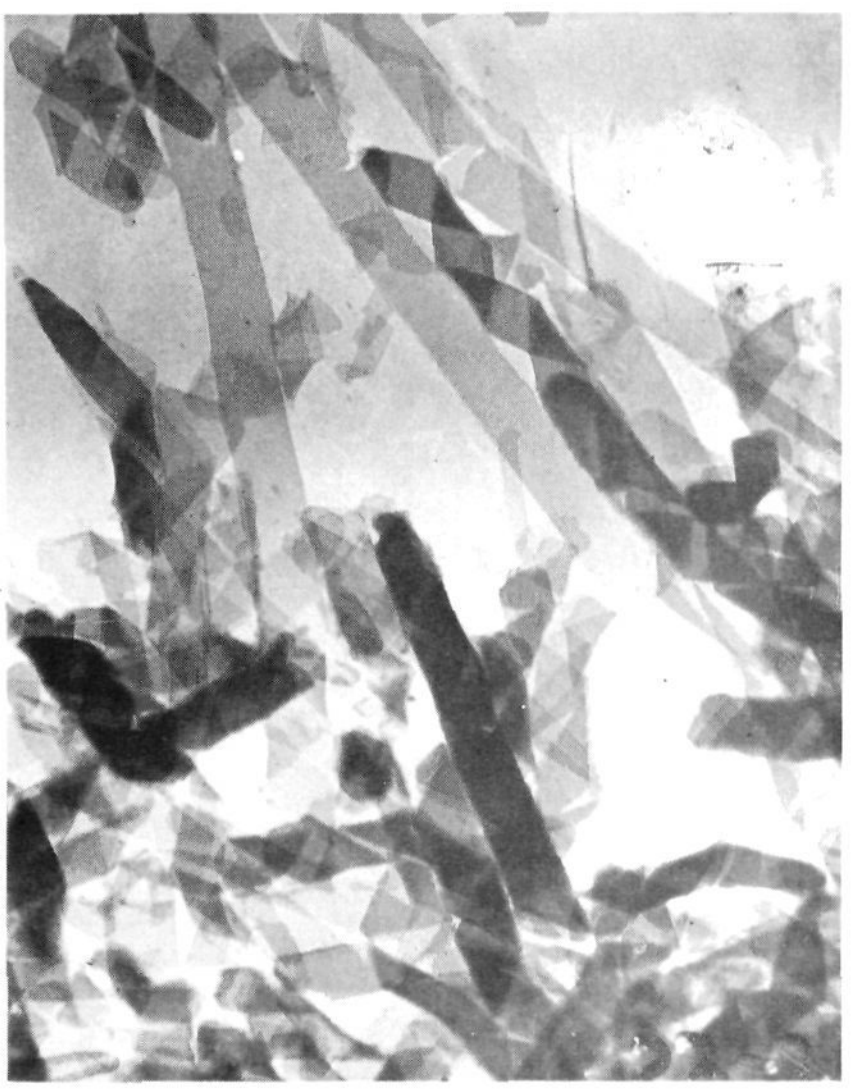

Figure 6. Transmission electron micrograph of air-dried unstained helices and tubules prepared from an 80% methanol-in-water mixture of $DC_{8,9}PC$. Samples were transferred onto carbon-coated grids, air dried, and observed without staining. Note a few possible cases of unwrapping of layers, presumably caused by the drying process, and the presence of tubules with multiple bilayers in their walls. Scale bar = 1 μm.

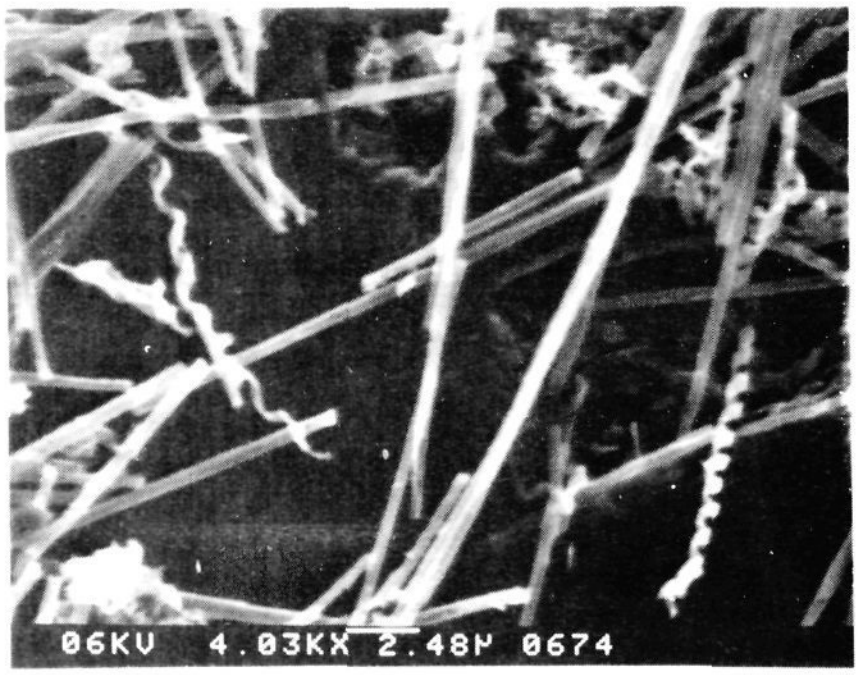

Figure 7. Scanning electron micrograph of tubules and helices formed from $DC_{8,9}PC$ at 50% 2-propanol in water that were subsequently coated with copper metal as described in the text. Note that all helical structures are right handed and that the pitch of the helices is somewhat variable. Scale bar = 2.48 μm.

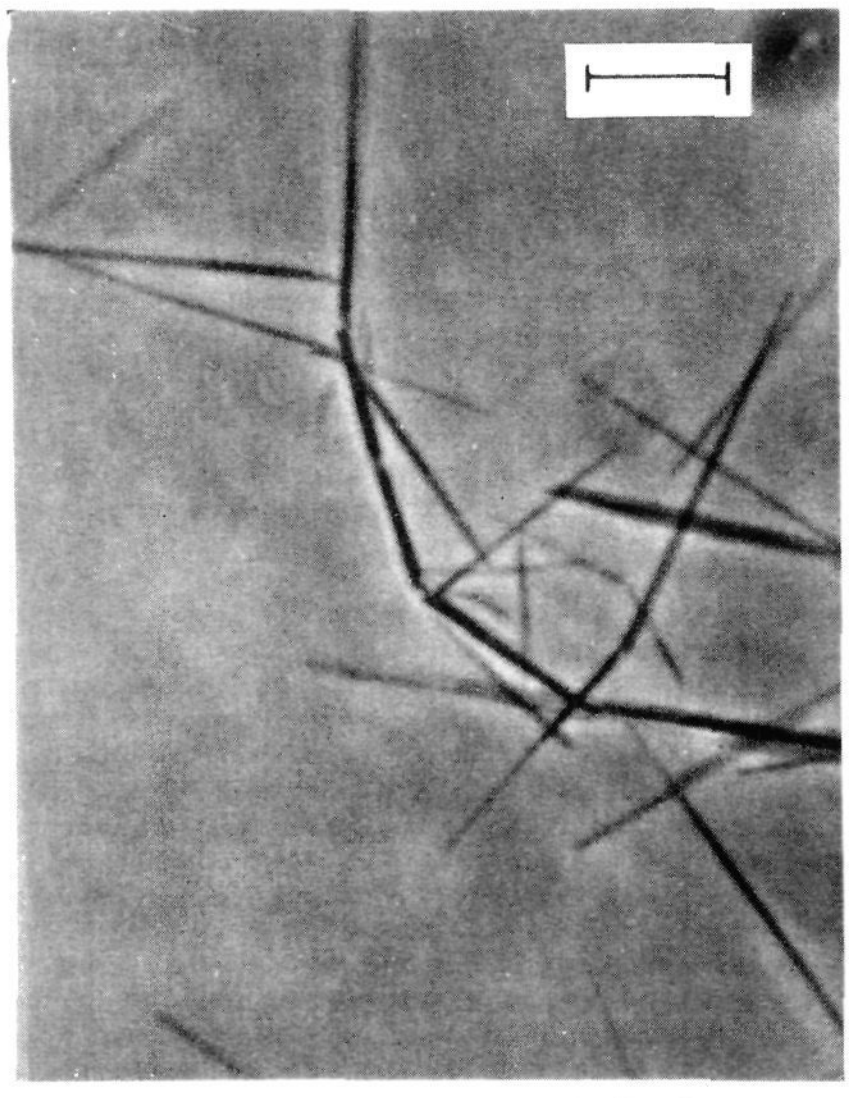

Figure 8. Phase contrast optical micrograph of $DC_{8,9}PC$ tubules with helical hinge regions. The sample was formed in a 70% ethanol–water mixture at a lipid concentration of 1 mg/mL. It was cooled from 40 to 22 °C at a rate of 1.5 °C per h during the course of precipitation. Tubules hinged by helical ribbons, branched tubules, tubules with helical branching, and cone shaped tubules are often seen under these formation conditions. These structures are often observed when the temperature of the sample is varied during precipitation. Scale bar = 10 μm.

walled tubules found, it is clear that the surface of a tubule is a good nucleus for the precipitation of additional helical or tubular material. The initial nucleus could be dust, but the initial efforts to keep the solutions as free of dust particles as possible have had no observed effects on the precipitation process. Oligomeric strings of lipid molecules might be excellent nucleation sites whose presence would be hard both to detect and to remove. We will explore this possibility in future studies.

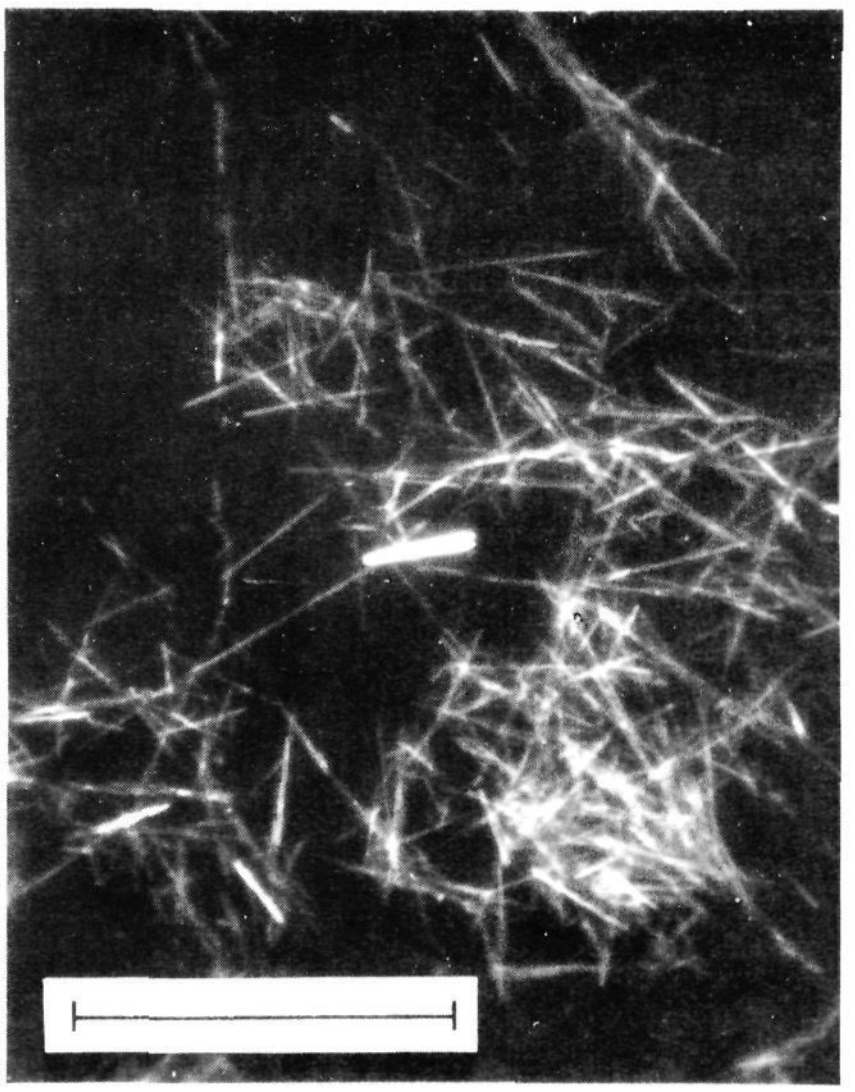

Figure 9. Darkfield optical micrograph of a conical tubule. The sample was formed by precipitation of a 0.6-mg/mL sample of $DC_{8,9}PC$ from 75% ethanol in water, followed by 90 h of incubation. Scale bar = 100 μm.

We have found that all diacetylenic lipids with identical hydrocarbon chains that we have synthesized form tubules under the appropriate conditions,[19-23] but it is often more difficult to form tubules from liposomes than by precipitation. In every case for which we initially could form tubules only from solvent it was subsequently found that after careful lipid purification tubules could be formed by both routes. The precipitation process itself purifies the lipids to a certain extent, which may account for its greater success. The optimal conditions for tubule formation by precipitation include a requirement that the formation temperature be 10–30 °C below the phase transition temperature of the lipid as measured in excess water. The reason for being a few degress below T_m is obvious; if the lipids are fluid the precipitation produces liposomes, not tubules, and as the organic solvent depresses the phase transition of the lipids, a margin of a few degrees is necessary. That tubules do not form efficiently from ethanol at more than 30 °C below that temperature may be a result of the formation of too many nuclei at excessive supercooling, or because of the existence of another stable crystal form for the lipid at low temperatures.

The molecular structure underlying the microstructures observed here is a matter of substantial interest. Several techniques have been employed, including vibrational spectroscopy,[7,8,10,25] low-angle X-ray scattering,[16] birefringence of tubules oriented by the diamagnetic susceptibility of the $DC_{8,9}PC$ hydrocarbon chains,[14] thermodynamic studies indicating complete phase separation of enantiomers of the chiral $DC_{8,9}PC$ molecule,[26] preliminary electron diffraction studies, as well as the aforementioned polarized light microscopy of wide polymerized tubules. Furthermore, polymerization occurs in diacetylenic systems only when the reactive groups are in a crystalline array with a particular range

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of spacing and orientation.[27] Since ultraviolet irradiation of the tubules[9] and helices produces colored polydiacetylenic chains, the lipid hydrocarbon chains in the tubule phase must have high local order and appropriate relative chain positions. The straightness of tubules probably also reflects ordered molecular packing. The evidence points to highly ordered lipid molecules with an unusual packing; the polarized light measurement suggests that tubules may have sufficient long-range correlation to be considered single crystals.

The production of tubular microstructures by precipitation suggests a solution to the major problem in the understanding of the formation of tubules from liposomes. The process of tubule formation by cooling of liposomes is topologically and mechanically complex, requiring, we suggest, the pulling of liposomal bilayers from one hydrated microstructure onto the growing tubules, combined with rotation of the growing tubules.[16] That such a process should occur at all is remarkable, but it raises the possibility that the existence of tubules is an artifact of the way in which an unusual lipid crystal forms on the surface of a liposome. The large flat arrays of sheets[10] that form from supercooled small vesicles that have thermal properties identical with those of tubules might be the true minimum energy form of the $DC_{8,9}PC$ system below its T_m. However, the fact that tubules can be formed by an entirely different route as demonstrated here suggests that the tubule structure and the helical precursor structures that also form are the thermodynamically favored structures. The curvature and helicity shown in these microstructures, unusual though they may be, are intrinsic to the hydrated diacetylenic phosphatidylcholine, and any modeling of tubule structure at the molecular level will have to take the curvature into account. We are actively pursuing such modeling.

The analogies between the behavior of the diacetylenic phosphatidylcholines and that of glutamate-based chiral lipids synthesized by Kunitake's group[28] are strong. The glutamate-based lipids form helical structures as intermediates to complete cylindrical structures, and the overall dimensions of these structures are nearly identical with those of tubules produced by diacetylenic lipids. However, the glutamate-based helices do not appear to be particularly flexible, and the conversion process from helix to tubule appears to occur within individual microstructures over a long time period in the absence of organic solvent. In our hands helices have been observed to persist in samples for months with or without removal of organic solvent.

Our view of the nature of the precipitation process is as follows. When a solution of $DC_{8,9}PC$, or similar diacetylenic lipid, below its effective hydrocarbon chain melting point is rendered unstable by addition of a nonsolvent such as water, crystallization begins. The initial crystals are quasi-two-dimensional in that they are no thicker than two bilayers, but they have an intrinsic helical curvature. These crystals can grow along two axes at relative rates that depend on conditions of temperature, concentrations, and the like. Growth under high solvent conditions produces a helix with a pitch of about 1 μm and a diameter of about 0.4 μm. Such structures may be produced first if the concentration of solvent is sufficient to support the unstable bilayer edges. Growth along the other axis widens the helix into a spiral ribbon that may extend in width to the point of fusing to a seamless tubule. Once fusion has occurred, growth may proceed at both circular ends of the tubule. The instability of the ribbon edges is such that, over time, cylindrical bilayers, which have only a small amount of exposed edge material at either end, become the dominant structure as long as sufficient solvent remains to allow intermicrostructural diffusion. In this scheme helical structures are required precursors to the formation of tubules.

Conclusions

Helical and tubular lipid microstructures are formed by addition of water to a solvent solution of the diacetylenic lecithin $DC_{8,9}PC$ or any of 16 other related diacetylenic lipids. The tubules are qualitatively similar to those previously observed to form from liposomes, with the exception that they do not contain trapped liposomes. The fact of their formation suggests that they are the thermodynamically stable crystal for these hydrated lipids in the reported temperature ranges. Variation of temperature, concentrations, and the particular solvents used in the precipitation process affords some control over the diameters and substantial control of the lengths of these structures. The helical structures produced by precipitation at high solvent concentration, while not unique, are the only such structures that can be polymerized and thereby rendered rugged. To better understand the formation of these ususual microstructures, we are currently synthesizing and characterizing lipids that vary in selected details from those described above. This technique proved a quick and reliable screening method for the formation of stable microstructures by lipid samples that would not easily form them from pre-existing liposomal suspensions or other aggregated phases.

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Registry No. $DC_{8,9}PC$, 109150-63-2; $DC_{6,13}PC$, 109123-89-9; $D\text{-}C_{10,13}PC$, 109123-90-2.

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