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The Effects of Dipalmitoyl Phosphatidyl Choline on the Precipitation of Native Fibrils and Segment-Long-Spacing Aggregates From Collagen Solution

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The effect of dipalmitoyl phosphatidyl choline (DPPC), the major phospholipid component of pulmonary surfactant, on the precipitation of collagen in the form of native fibrils and segment-long-spacing (SLS) aggregates was studied in vitro. The effects of DPPC on both phases of collagen fibrillogenesis were analyzed spectrophotometrically, and alterations in the morphology of precipitated fibrils and SLS aggregates were ascertained by transmission electron microscopy (TEM). Low concentrations of DPPC inhibited the growth phase of fibrillogenesis, while higher concentrations were required to inhibit nucleation. Both the meshwork density and mean width of precipitated fibrils were altered by DPPC, as was the size of SLS aggregates. Segment-long-spacing aggregates prepared from pepsin-treated collagen were inhibited to a greater degree than SLS aggregates prepared from untreated collagen, indicating that the pepsin-susceptible residues of the telopeptide extensions of tropocollagen molecules stabilize SLS aggregates against the effects of DPPC. Based on these results and the inhibition of the growth phase at lower concentrations than those which inhibited the nucleation phase of fibrillogenesis, it was concluded that the primary mechanism of DPPC inhibition is electrostatic interference between the positively charged phospholipid molecules and the net positive charge of collagen. It is proposed that pathological conditions involving the pulmonary epithelium may allow interaction between surfactant and collagen, which could further weaken the interstitial connective tissue.

Key words: collagen, SLS, phospholipid, surfactant, fibrillogenesis, dipalmitoyl phosphatidyl choline, electrostatic interactions

The process of collagen fibrillogenesis consists of two independent aspects, a nucleation phase and a growth phase [1, 2]. Nucleation, a temperature-dependent process, is characterized by the de novo formation of stable tropocollagen aggregates [3–6]. The growth phase, defined as longitudinal and lateral addition of tropocollagen to nuclei, is independent of temperature [4, 5]. Recent evidence suggests, however, that the process of fibrillogenesis in vitro is asymmetric [7].

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Physiological factors such as pH and ionic strength moderate the rate of collagen fibrillogenesis in vitro [8]. Many chemical agents, some of which are endogenous to the extracellular milieu, affect the rate of fibrillogenesis. These include glycosaminoglycans [9–16], glycoproteins [17, 18], citrate [19], ascorbic acid [20], neutral salts [21], DNA and RNA [9, 22], urea [23, 24], and catecholamines [25]. Nonbiological substances have also been shown to alter the rate of collagen fibrillogenesis in vitro. Both nonionic detergents such as polyoxyethylene nonyl phenyl ethers [26] and anionic detergents such as sodium dodecyl benzene sulfonate [27] accelerate the rate of fibrillogenesis. A cationic detergent, cetylpyridinium chloride, inhibits fibrillogenesis [28]. Keech [22] has shown that the same physicochemical conditions that affect the process of fibrillogenesis in vitro also affect the morphology of precipitated fibrils. The mechanisms of precipitation of collagen in forms other than the native fibril are less well known. Although glycoproteins can induce the precipitation of fiber-long-spacing (FLS) aggregates [18], agents that alter the fibrillogenesis of native fibrils have not been systematically studied with regard to their effects on the precipitation of FLS or segment-long-spacing (SLS) aggregates.

Dipalmitoyl phosphatidyl choline (DPPC) is the major phospholipid component of pulmonary surfactant [29]. Surfactant is synthesized by type II alveolar epithelial cells and increases the efficiency of respiration by moderating alveolar surface tension [30, 31]. The DPPC molecule consists of a polar "head" group (choline), which carries a net positive charge near physiological pH, and two fully saturated non-polar "tail" groups (palmitic acid). Because of this amphoteric nature, DPPC forms micellar structures in solution [32], the molecules arranged with the polar portions exposed to the water–phospholipid interface. Pulmonary surfactant forms related structures in vivo [29]. Under normal physiological conditions, pulmonary surfactant does not come into contact with components of the extracellular matrix. However, in some pathological conditions that result in disruption of the alveolar epithelium, surfactant may diffuse into the connective tissue compartment where interactions with collagen might influence the maintenance, stability, and structural organization of the fibrous matrix.

This study was designed to investigate the effects of interactions between collagen and DPPC in vitro on the precipitation of both native fibrils and SLS aggregates. Effects of DPPC on both phases of fibrillogenesis and on the morphology of precipitated native fibrils were quantitated. Effects of DPPC on the formation of SLS aggregates from both pepsin-treated and untreated collagen were analyzed to assess possible contributions of the telopeptide portions of the tropocollagen molecules to DPPC–collagen interactions.

MATERIALS AND METHODS

Collagen Extraction

Collagen was isolated from the skins of 10-day-old rat pups following the method of Honya and Mizunuma [27]. After depilating the skin and rinsing in 30% ethanol at 0°C to remove lipids, the skin was minced and washed in cold 0.02 M NaCl. Tropocollagen was extracted by 3 separate 24-h exposures to 1.0 M NaCl while stirring at 4°C. The combined extracts were centrifuged for 30 min at 30,000g and, after removing any extraneous material, precipitation was induced by adjusting the NaCl concentration to 15% (w/v) at neutral pH (adjusted with 1.0 M NaOH). The precipitate was dissolved in 0.05 M acetic acid and reprecipitated by adjusting the NaCl concentration to 5% (w/v). Dissolution and re-

precipitation was repeated three times. Following final dissolution in 0.05 M acetic acid, the purified collagen solution was dialyzed against distilled water (3 changes) for 48 h. The preparation was then lyophilized and stored at −20°C.

Collagen Solution

For spectrophotometric analysis of fibrillogenesis and for analysis of fibril width, collagen solutions were prepared by dissolving the purified collagen for 24 h in 0.03 M acetic acid at 4°C to make a 0.5% (w/v) solution.

Gelation Buffer

The buffer vehicle for DPPC in both the fibrillogenesis and SLS aggregation experiments contained 0.20 M NaCl, 0.025 M TRIZMA pH 7–9 (Sigma Chemical Company, St. Louis, MO), and various concentrations of DPPC (Sigma). The powdered DPPC was mixed with the buffer by sonication for 5 min at 50°C. The buffer was sonicated again for 5 min at the experimental temperature immediately prior to use.

Spectrophotometry

Changes in optical density during fibrillogenesis were determined at 430_u in a Bausch and Lomb Spectronic 70 spectrophotometer fitted with a 3-cell, water-jacketed, constant temperature cuvette holder and a chart recorder. Proper temperature in the cuvette holder was maintained by circulating water from a large-volume, thermostatically controlled water bath with a Polystaltic pump. During each experiment, one cell of the cuvette holder was occupied by the sample being analyzed, another by the collagen solution equilibrating for the next run, and the remaining cell by an H_2O reference. Temperature was measured from the reference cell before and after each experimental trial. Gelation buffer was equilibrated in a separate water bath at the experimental temperature. At the beginning of each experiment, four volumes of the gelation buffer were added to one volume of collagen solution in the cuvette. The solution was mixed rapidly by drawing it into a syringe and expelling the mixture into the cuvette. Recording began immediately following mixture. Opacity of the forming gel was recorded continuously as change in % transmission and later was converted to change in absorbance ($A = 2 - \log T$) at 1-min intervals. E_{60} was defined as the absorbance value at 60 min, and the duration of the nucleation phase was calculated as the time required for absorbance to reach a value of 0.005 E_{60}. Growth rate was defined as the slope of a plotted regression line using 5 points in the center of the curve.

Fibril Width Analysis

Samples for fibril width measurement by EM were prepared by the method of Wood and Keech [8]. The collagen and buffer solutions were prepared as for spectrophotometry, equilibrated, and mixed in the same manner. Immediately following mixing, 0.5 ml of each solution was transferred to a previously equilibrated (32°C) glass slide and spread to make a pool 1–2 mm deep. Each slide was placed in a Petri dish with a small foil cup containing control buffer, and the dishes were transferred to an oven at 32°C for 24 h. After removal from the oven, the slides were dried by exposure to a stream of air at room temperature, gently washed with distilled H_2O, and redried. The dry slides were shadowed with carbon-platinum at an angle of 5° in vacuo. Following application of a thin coating of collodion, the slides were scored into 2–3-mm squares and soaked in trypsin solution (0.5 mg/ml)

for 18 h. The loosened squares were then peeled from the slides, floated on distilled H_2O, and picked up on 200-mesh copper grids. The samples were viewed in a Hitachi HS-8 electron microscope. Photomicrographs were made at several magnifications from several areas of each replica. A random grid overlay was used to select fibrils for measurement, and the selected fibrils were grouped into size categories by comparison to standards of known dimension.

Pepsin Treatment

Some of the collagen used for the precipitation of SLS aggregates was pretreated with pepsin following the procedure of Drake et al [33] to remove portions of the telopeptide termini. Separate solutions of 0.02% (w/v) collagen and 0.1% (w/v) pepsin (Sigma) in 0.5% (w/v) acetic acid were dialyzed against the same concentration of acetic acid for 48 h. Pepsin was then added to the collagen solution in a 1:100 (pepsin:collagen) weight ratio, and the mixture dialyzed against fresh 0.05% (w/v) acetic acid for 24 h at 20°C followed by 48 h at 4°C. Pepsin was inactivated by dialysis against 15% (w/v) KCl–0.02 M Na_2HPO_4. The precipitated collagen was dissolved in 0.05% (w/v) acetic acid and reprecipitated with KCl–Na_2HPO_4. This last step was repeated 3 times.

SLS Aggregation

SLS aggregates were prepared from untreated and pepsin-treated collagen by diluting the respective collagen solutions to 0.1% (w/v) with 0.03 M acetic acid. A 0.5-ml volume of the diluted collagen was pipetted into small-bore dialysis tubing and dialyzed for 24 h against 6.0 ml of 0.4% (w/v) adenosine triphosphate (ATP; Sigma) in 0.1 M acetic acid. Prior to dialysis, a concentrated DPPC-buffer solution was added to obtain the desired DPPC concentrations for each sample [0.00033, 0.0033, 0.033, and 0.33% (w/v)]. Following dialysis, a few drops of the ATP solution were added directly to each collagen solution. Drops of the various collagen solutions were placed on 300-mesh Formvar-coated copper grids, allowed to stand for 4 min, and then blotted dry with Whatman number 1 filter paper. Each sample was stained for 3 min with 0.1% (w/v) phosphotungstic acid (PTA), dipped briefly in distilled water, and dried in air before staining for 5 min in 0.01% (w/v) uranyl acetate (UA). The samples were again rinsed and air-dried. Except for air-drying, all of the above procedures were carried out at 4°C. Following drying, the grids were examined and photographed in the electron microscope.

RESULTS

Spectrophotometry

The temperature dependence of the nucleation phase of collagen fibrillogenesis in vitro is demonstrated in Figure 1 and confirms the results of other investigators [2, 4, 5]. Duration of the nucleation phase decreased with increased temperature while growth rate was not affected (Table I).

The effects of low concentrations of DPPC on collagen fibrillogenesis are shown in Figure 2 and the effects of higher concentrations in Figure 3. As summarized in Table II, low concentrations of DPPC had no consistent effect on the duration of the nucleation phase. However, low concentrations of DPPC produced a pattern of decreasing growth rate and E_{60}, with increasing DPPC concentration in this range. Higher concentrations of

DPPC resulted in marked increases in the duration of the nucleation phase and further declines in growth rate and E_{60}. While both the nucleation and growth phases were affected by concentrations of DPPC above 0.02% (w/v), growth rate was inhibited by concentrations of DPPC at least 100 times lower.

Electron Microscopy

Microscopic examination of carbon-platinum replicas of collagen fibril gels, precipitated under conditions identical to those of gels studied spectrophotometrically, revealed collagen precipitates consisting of fibrous networks whose morphological characteristics varied with DPPC concentration (Fig. 4). As demonstrated by the fibril width population of the 0.05% (w/v) DPPC gel (Fig. 5), significant differences in fibril width were noted between fibrils of control and DPPC gels. All of the precipitated fibrils, regardless of the presence or absence of DPPC, consisted of long fibrils with the 640 Å periodicity common to native collagen fibers. High concentrations of DPPC (2.0%) resulted in reduction in meshwork density of the precipitated fibers (Fig. 9).

TABLE I. Effect of Temperature on Fibrillogenesis*

Temp (°C)	Lag period (min)	Growth rate	E_{60}
36°	2.5	0.053	0.357
33°	5.0	0.0565	0.490
29°	7.1	0.0563	0.773

*Collagen concentration = 0.1% (w/v), pH = 7.8, 1 h equilibration. Lag period (duration of nucleation), growth rate, and E_{60} calculated as defined in text.

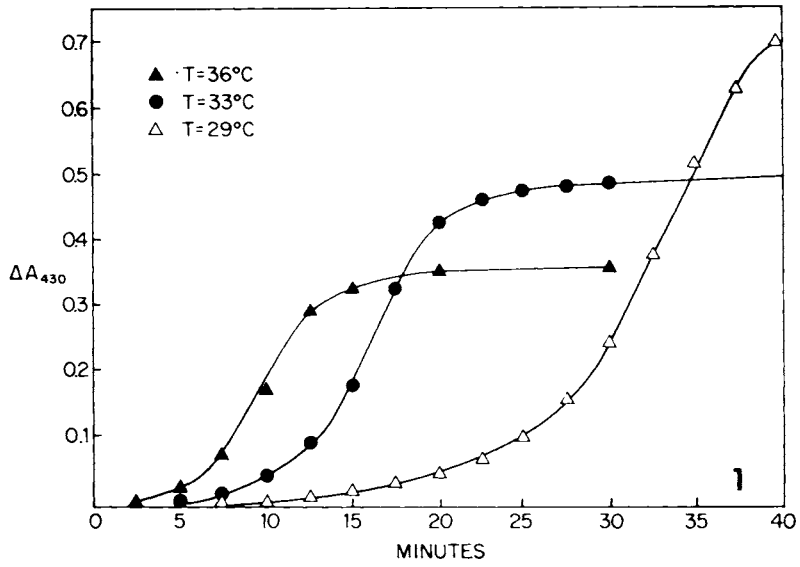

Fig. 1. Effect of temperature on fibrillogenesis. Collagen concentration = 0.1%; pH = 7.8; 1 h equilibration.

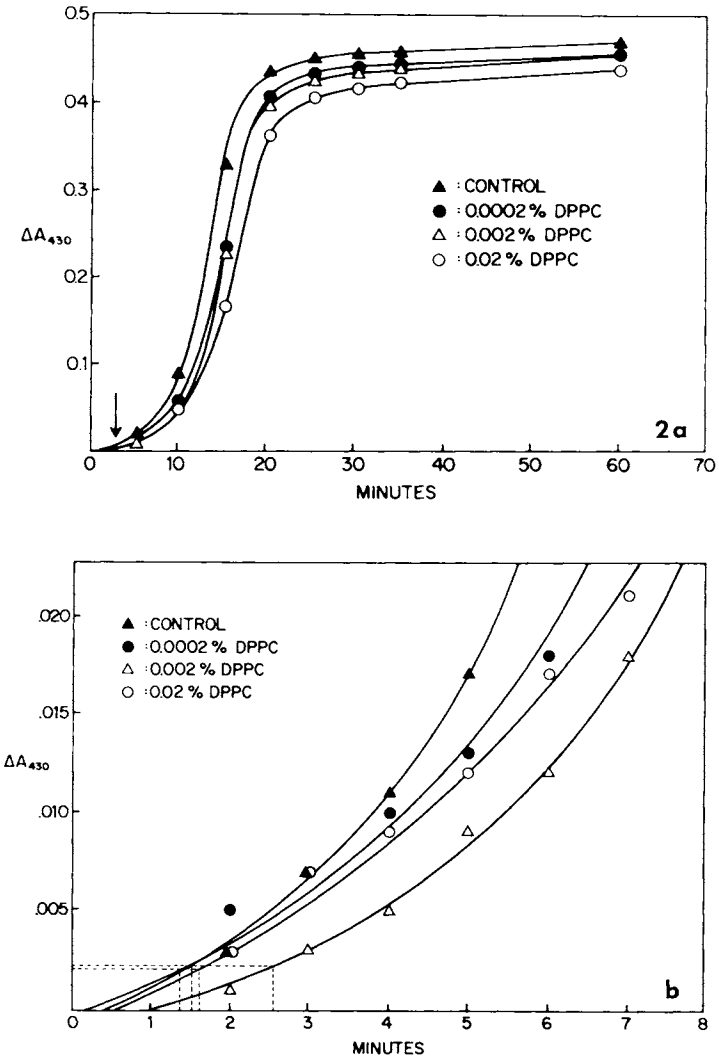

Fig. 2. a. Effect of DPPC on fibrillogenesis. Equilibration for 1 h; gelation at 32°C; pH = 7.8. b. Effect of DPPC on nucleation phase. Experimental conditions as in a.

SLS Aggregates

Experiments with the SLS form of collagen precipitate indicated that the presence of DPPC also inhibited formation of this type of aggregate. The addition of 0.2% (w/v) DPPC to the gelation buffer prior to aggregation resulted in the formation of fewer and smaller aggregates as compared to controls (Fig. 6). DPPC, when added to a preparation of SLS aggregates at a final concentration of 0.6% (w/v), caused total dissolution of the aggregates. The inhibition of SLS formation by DPPC may be a concentration-dependent effect as the degree of inhibition increased with increasing DPPC concentration over a 1,000-fold range (0.00033, 0.0033, 0.033, 0.33% (w/v) DPPC). The density and width of

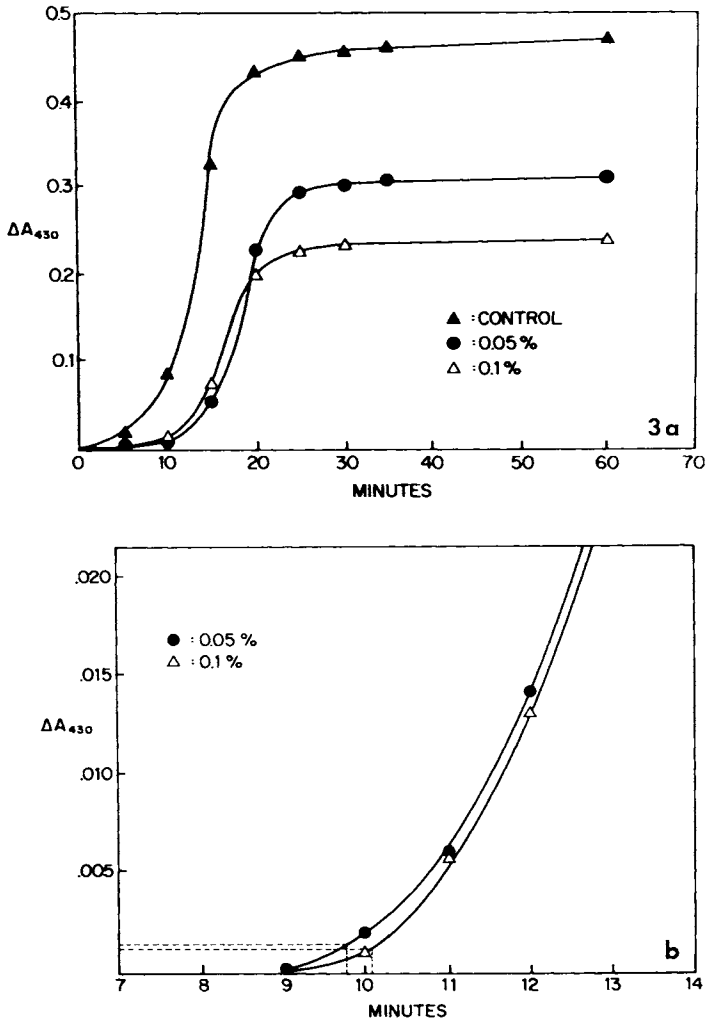

Fig. 3. a. Effect of DPPC on fibrillogenesis. Equilibration for 1 h; gelation at 32°C; pH = 7.8. b. Effect of DPPC on nucleation phase. Experimental conditions as in a.

single aggregates decreased with increasing DPPC concentration over this range, concomitant with an increase in the number and length of chain-like structures in which adjacent aggregates tended to associate only at their terminal segments (Fig. 7).

SLS aggregates were also precipitated from pepsin-treated collagen in the presence of DPPC. Aggregates precipitated from pepsin-treated collagen in the absence of DPPC served as controls and demonstrated the characteristic length (approximately 2,800 Å) of untreated SLS aggregates, but were much wider, with an estimated average of 14,000 Å (Fig. 8a). Aggregates formed from pepsin-treated collagen in the presence of 0.00033% and 0.0033% DPPC were smaller than control aggregates and formed chain-like structures, as noted previously (Fig. 8b). No chains were formed in the presence of higher concentrations of DPPC, but aggregate size continued to diminish with increasing DPPC concentration.

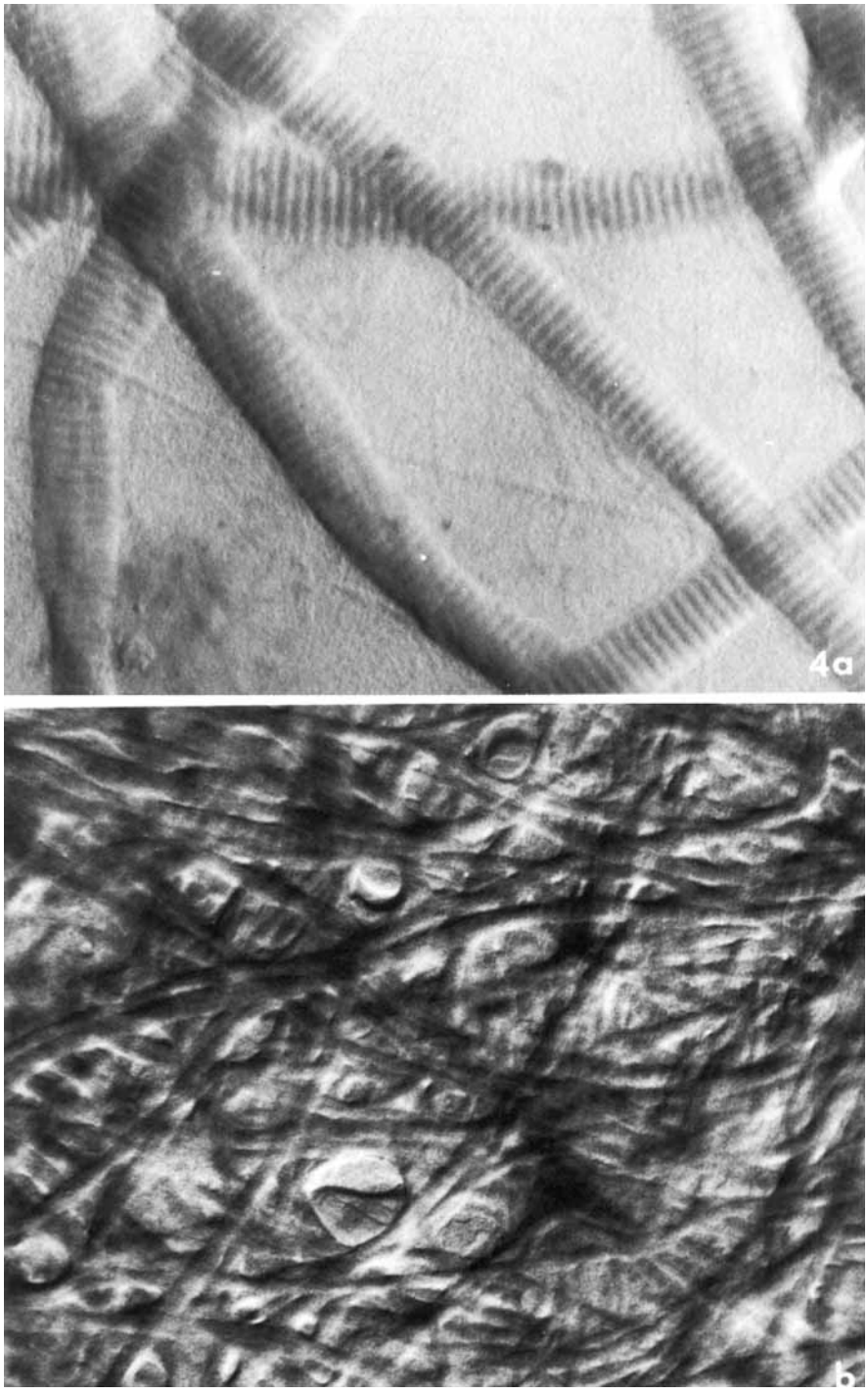

Fig 4 These electron micrographs of carbon/platinum-shadowed collagen precipitates demonstrate the changes in fibril width and meshwork density due to DPPC a) Control fibrils b) Fibrils precipitated in the presence of 0 05% (w/v) DPPC (magnification, X35,000)

TABLE II. The Effect of DPPC on Fibrillogenesis*

DPPC conc	Lag period		Growth rate		E_{60}	
	ABS[a]	Exp/ Control	ABS[b]	Exp/ Control	ABS[c]	Exp/ Control
Control	1.55	1.0	0.058	1.0	0.469	1.0
0.0002%	1.50	1.0	0.049	0.84	0.453	0.97
0.002%	2.30	1.48	0.049	0.84	0.453	0.97
0.02%	1.75	1.13	0.043	0.75	0.434	0.93
0.05%	9.60	6.19	0.039	0.66	0.319	0.68
0.1%	9.95	6.42	0.029	0.50	0.242	0.52

*Lag period, growth rate, and E_{60} as a function of DPPC concentration. Collagen concentration = 0.1% (w/v), pH 7.8, 1 h equilibration, 32°C.
[a]Minutes
[b]Δ Absorbance units (A_{430})/min.
[c]ΔA_{430} at 60 min.

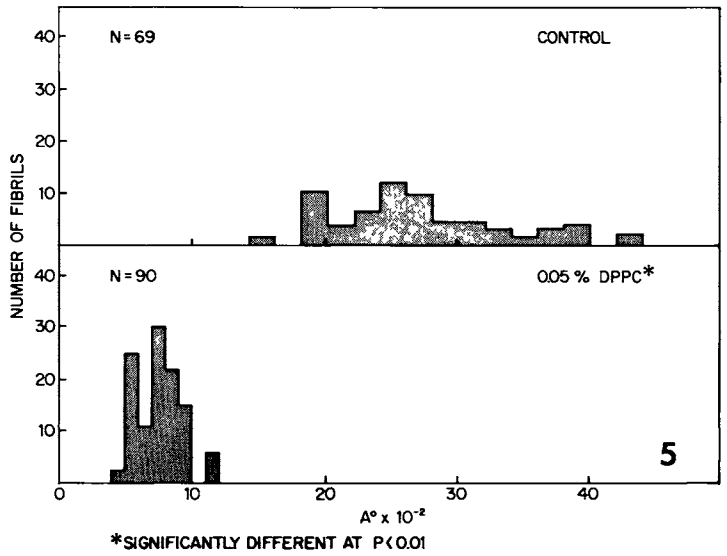

Fig. 5. Effect of DPPC on fibril width. Equilibration for 1 h; gelation at 32°C.

DISCUSSION

The results present evidence for the inhibition of both native fibril and SLS aggregate formation in vitro by DPPC. The progress of fibrillogenesis in the presence of DPPC, as followed spectrophotometrically, demonstrates 2 different levels of response to DPPC. Only the growth phase of fibrillogenesis is inhibited at low concentrations of the phospholipid, while both phases are inhibited at higher concentrations. These results support the hypothesis that tropocollagen molecules possess different functional "sites" for interaction with physicochemical components of the environment [27], since the mechanisms controlling growth and nucleation were sensitive to different concentrations of DPPC. Thus,

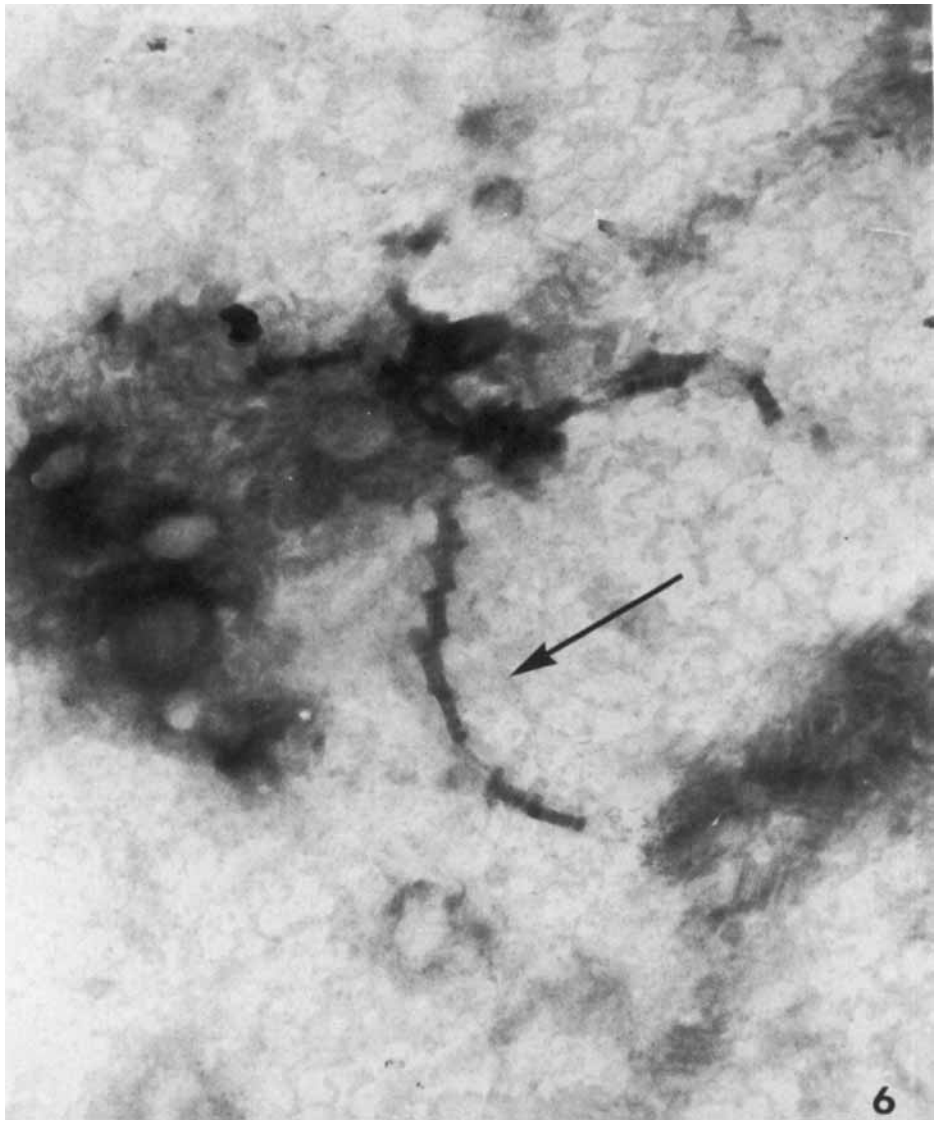

Fig. 6. The SLS aggregates seen in this electron micrograph were precipitated in the presence of 0.2% (w/v) DPPC. The aggregates are much smaller than control aggregates (no DPPC) and tend to form chain-like structures by overlapping at terminal regions (arrow) (magnification, ×35,000).

the "site" that controls nucleation may be functionally more resistant to the effects of DPPC than the "site" that controls growth. Differences in fibril width and meshwork density of precipitated fibrils between control and DPPC gels support this conclusion. The thick meshwork of narrow fibrils found in 0.05% (w/v) DPPC gels may reflect changes in the mechanism of nucleation by DPPC such that subnuclear fibril aggregates remain independent and grow slowly to become nuclei. Continued inhibition of growth at this DPPC concentration results in a large number of smaller fibrils compared to controls. It seems reasonable to predict that at concentrations of inhibitor that affect only the growth phase, meshwork density should approach that of controls, while fibril width would be decreased, depending on the degree of inhibition. To the extent that meshwork density reflects the

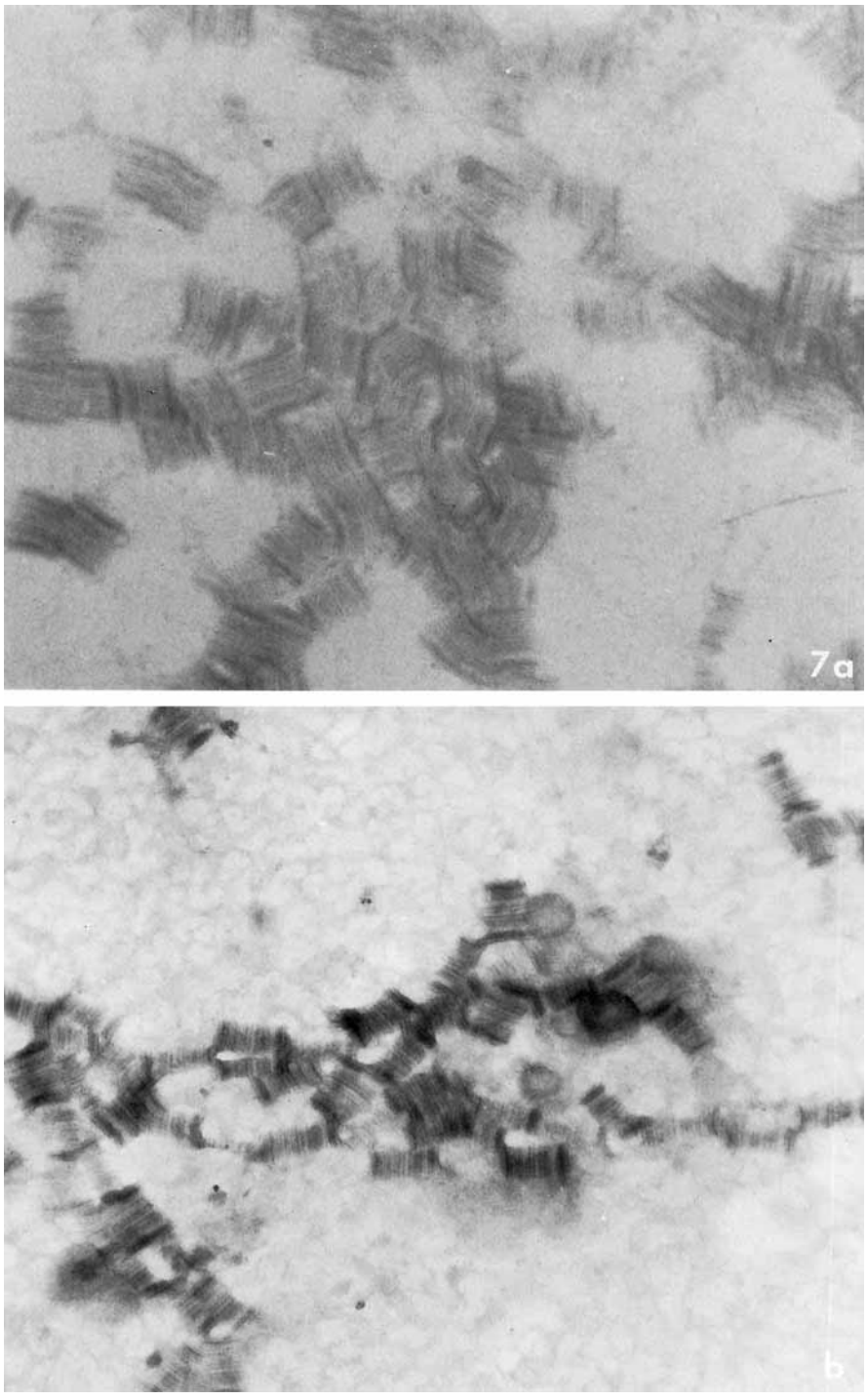

Fig 7 These electron micrographs of SLS aggregates demonstrate qualitative and quantitative differences between aggregates precipitated in the presence and absence of DPPC a) Control aggregates Aggregates tend to be of uniform size and associate randomly b) Aggregates precipitated in the presence of 0 0033% (w/v) DPPC The aggregates are smaller in width than controls and tend to associate with other aggregates only at terminal regions (magnification, ×46,500)

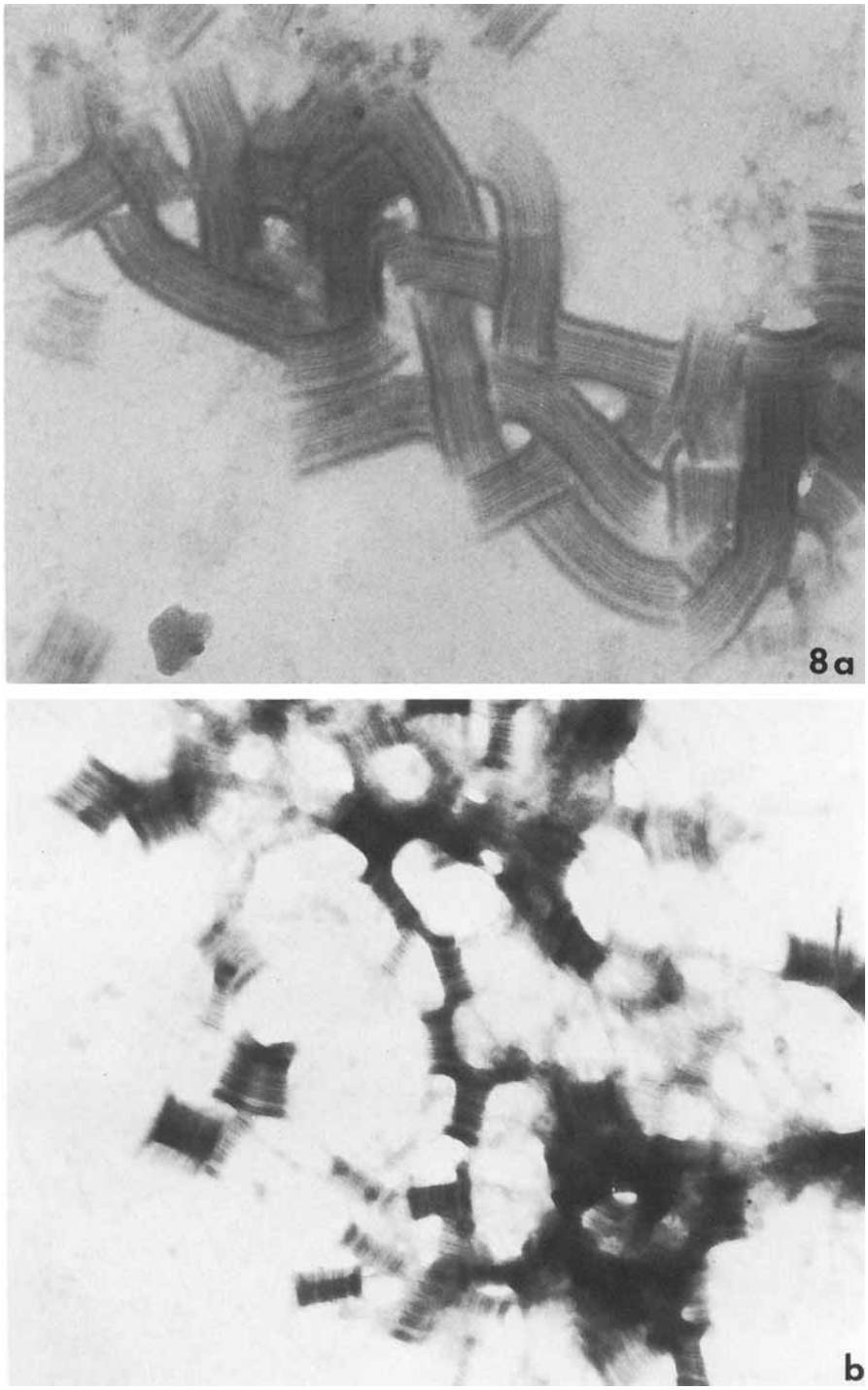

Fig 8 SLS aggregates precipitated from pepsin treated collagen a) Controls Reduction of the telo peptide termini has resulted in a greater degree of lateral aggregation than in untreated controls, b) 0 0033% (w/v) DPPC Relative reduction in aggregate width is greater compared with fibrils from untreated collagen Overlapping associations of aggregates from pepsin treated collagen are completely inhibited at higher concentrations of DPPC (magnification, ×46,500)

number of growing aggregates that must compete for available soluble collagen, these results indirectly support the statement by Wood [2] that, ultimately, fibril width is determined during the nucleation phase. Severe reduction in meshwork density at a higher (2.0% (w/v) concentration of DPPC (Fig. 9) is further evidence for substantial inhibition of nucleation.

The variable dimension of SLS aggregates decreases with increasing DPPC concentration, which indicates a progressive inhibition of lateral aggregation of tropocollagen. At higher concentrations, the tendency to form chains involving overlap of the terminal regions indicates involvement of these regions in the interaction with DPPC. Although removal of a part of the telopeptide extensions with pepsin allows a higher degree of

Fig. 9. Fibrils precipitated in the presence of 2.0% (w/v) DPPC. Fibril width is significantly less ($P < 0.01$) than that of control fibrils, and meshwork density is severely reduced (magnification, ×35,000).

lateral aggregation in control SLS precipitates (Fig. 8a), pepsin treatment results in an enhanced degree of inhibition of lateral aggregate growth in DPPC preparations. Pepsin treatment does not excise the complete nonhelical telopeptide extension. Since intrinsic viscosities of collagen solutions are not altered by limited pepsin treatment, the cross-link containing regions of the telopeptide must remain intact [4]. But the remaining amino acid residues may have considerably less hydrophobic character than the intact telopeptide. Thus, interactions between pepsin-treated collagen molecules may be more susceptible to interference by DPPC. Honya and Mizunuma [27] have reported that limited pepsin treatment increased the stimulatory effect of anionic surfactants on fibrillogenesis. Monosaccharides have been shown to inhibit fibrillogenesis in pepsin-treated collagen [28].

Of the many agents for which possible roles in the mediation of collagen fibrillogenesis in vivo have been proposed, the components of the extracollagenous connective tissue matrix, particularly the glycosaminoglycans (GAG) and proteoglycans (PG), have been the most widely studied. With some exceptions [14, 15], this class of biological material is thought to exert its effects on the fibrillogenesis of collagen in vitro by electrostatic interactions. The facilitation of fibrillogenesis by chondroitin sulfate during the nucleation phase probably involves electrostatic binding of this material to fractions of the collagen solution participating in the formation of nuclei [11, 16]. Retardation of fibrillogenesis during the growth phase by high-molecular-weight GAG (chondroitin sulfate A and chondroitin sulfate C) also involves electrostatic binding to collagen and the formation of large collagen/polyanion complexes [10]. Anderson and Jackson [17] have shown that glycoprotein fractions isolated from bovine achilles tendon bind electrostatically to collagen in vitro and may destabilize fibrils in vivo. Proteoglycans that bind electrostatically to collagen at low concentrations retard both phases of fibrillogenesis [34].

An electrostatic mechanism for the inhibition of collagen fibrillogenesis in vitro by DPPC is compatible with the results presented here. Localized densities of the positively charged phospholipid could be expected to interact with charge distributions of the collagen molecules. Since both DPPC and collagen [35] carry a net positive charge under the experimental conditions, the inhibition of the growth phase of fibrillogenesis by low concentrations of DPPC could be the result of repulsive electrostatic interactions that interfere with the forces directing lateral aggregation. Because hydrophobic forces are active in the extension peptides, which are responsible for forces directing the formation of nuclei [3, 4, 36, 37], stronger electrostatic forces would be required to overcome these forces during the nucleation phase. Our results support this supposition; concentrations of DPPC that inhibited nucleation were 100 times higher than those that minimally inhibited growth.

Electrostatic effects can also account for the inhibition of SLS formation in the presence of DPPC. The yield of SLS aggregates is very sensitive to changes in the physicochemical environment [38]. The decrease in size of aggregates precipitated in the presence of DPPC (when compared to controls) can be attributed to an increase in instability in direct proportion to aggregate size. If it can be assumed that electrostatic interactions play a larger role in the precipitation of collagen in the SLS form, then it is possible that disruption of electrostatic influences may result in an increased importance of hydrophobic forces. This may be demonstrated by the formation of chain-like structures involving the overlap of terminal, relatively hydrophobic regions of the collagen molecules. Removal of susceptible residues from the telopeptide termini of collagen lengthens nucleation time and increases the sensitivity of nucleation rate to changes in ionic strength – ie, electrostatic interactions [5, 39, 40]. However, when collagen that will be precipitated in the SLS form is treated with pepsin, the resultant aggregates are much larger (Fig. 8a) than aggre-

gates from untreated collagen (Fig. 7a). This suggests that the predominantly hydrophobic, pepsin-susceptible residues decrease the stability of SLS aggregates. It would be expected, however, that removal of these residues results in greater sensitivity to electrostatic interference in the formation of SLS aggregates from pepsin-treated collagen. This appears to be the case as DPPC causes a greater reduction in aggregate size relative to controls (no DPPC) in aggregates from pepsin-treated collagen (Fig. 8b) than in aggregates from untreated collagen (Fig. 7b). Reduction of hydrophobic residues in the extension peptides may create a situation in which the remaining hydrophobic residues are not able to overcome the repellent electrostatic forces at the higher concentrations of DPPC, while remaining competent to do so at lower DPPC concentrations.

We have demonstrated that dipalmitoyl phosphatidyl choline (DPPC), the major phospholipid component of pulmonary surfactant, inhibits the process of collagen fibrillogenesis in vitro and affects the morphology of the precipitated fibrils. The results from the studies of fibrillogenesis in the presence of DPPC and from experiments demonstrating the effects of DPPC on the precipitation of SLS aggregates from untreated and pepsin-treated collagen support an electrostatic model for the interaction of DPPC with collagen.

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