

THE PRESERVATION OF ULTRASTRUCTURE IN SATURATED PHOSPHATIDYL CHOLINES BY TANNIC ACID IN MODEL SYSTEMS AND TYPE II PNEUMOCYTES

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ABSTRACT

The preservation for electron microscopy of saturated phospholipids in general, and phosphatidyl choline (PC) in particular, remains an unsolved problem since OsO_4 and glutaraldehyde are incapable of interacting with PC directly. However, by introducing tannic acid preceding osmication, we were able to demonstrate highly ordered, preserved lamellar structures in model experiments with saturated PC, and in vivo experiments with type II pneumocytes of lung tissue. The secretory bodies of the latter are known to contain a high proportion of these saturated phospholipids. In both cases, the repeating periodicity approximated 45 Å.

It was determined that tannic acid interacts with the choline component of PC to form a "complex," which then could be stabilized by treatment with OsO_4 . In the absence of osmication, the PC-tannic acid complex did not survive conventional dehydration techniques, but osmication permitted conventional Epon embedment. Sphingomyelin (SPH), which contains choline, behaved similarly in model experiments. But there was no evidence of a comparable reaction with tannic acid using phosphatidyl ethanolamine (PEA), phosphatidyl serine (PS), or phosphatidyl inositol (PI).

Chemical studies indicated a high pH dependency for the formation of the PC-tannic acid complex. Also, experiments demonstrated its dissociation in various organic solvents. Sharp delineation and great contrast of the polar zones in the ordered lamellar structures was achieved by additional staining with lead citrate, thus leading to the conclusion that tannic acid serves as a multivalent agent, capable of simultaneous interaction with saturated PC, OsO_4 , and lead citrate stains.

KEY WORDS lecithin ultrastructure · phosphatidyl choline ultrastructure · tannic acid · type II pneumocytes

The preservation of phospholipids in as nearly a native form as possible for fine-structural study continues to present serious problems. Difficulties

partly involve their retention during embedding procedures. These are compounded when saturated phospholipids are major components, for then double bonds are unavailable for OsO_4 reduction and cross-linking (4, 5, 28, 38, 42). Also, the polar heads of phosphatidyl cholines (PCs) (lecithins) lack primary amines found in such

phospholipids as phosphatidyl ethanolamine (PEA) and phosphatidyl serine (PS), and so are unable to react directly either with OsO_4 (3, 38) or with glutaraldehyde (14, 27).

The general problem has particular pertinence for studies of the surfactant system of the lung. There the secretory bodies of the type II cells of alveoli (great alveolar cells) constitute a biological system with an extraordinarily high percentage of saturated PCs. Recent determinations demonstrate that these constitute >70% of the polar lipids present in the rat lung (8, 16, 19). This no doubt is a major reason why the preservation of the secretory bodies for electron microscopy without evident artifact has rarely been achieved.

Recently, tannic acid was introduced as a "fixative" (13, 24, 25). In a number of reports since then (e.g., 1, 26, 29, 31, 32, 33, 40), it has been clearly established that under some circumstances it enhances the ultimate contrast obtained, and so yields a particularly fine delineation of certain tissue systems. However, Simionescu and Simionescu (33, 34) have emphasized that it functions primarily as a mordant for lead salt staining, although they agree that it "concomitantly stabilizes some tissue components." Doubts concerning its mode of action specifically with saturated phospholipid systems have led us to explore this in a series of model experiments, using tannic acid alone, as well as concurrently with, or sequentially to, glutaraldehyde and/or OsO_4 fixatives. It was also determined how tannic acid might be used advantageously in preserving the contents of the secretory bodies of type II pneumocytes. The model experiments indicate that tannic acid reacts primarily with the choline "base" of (PC). This "complex", then, is readily reactive with OsO_4 . In turn, this renders the phospholipids insoluble, preserves a highly ordered structure, and finally acts as a mordant to produce enhanced contrast with lead salt stains.

MATERIALS AND METHODS

Materials

Simionescu and Simionescu (33, 34) recognized that commercial tannic acids are derived by extraction from various crude sources, and are by no means chemically pure compounds. They have performed a valuable service in emphasizing that relatively low molecular weight ($\approx 1,000$) gallotannins have superior properties. At least one established commercial source is available which is thought to consist substantially of penta- and hexagalloylglucoses (33, 34). This is the Mallinckrodt Inc. (St.

Louis, Mo.) AR product, code no. 1764. Unless otherwise noted, this was the tannic acid we used in our experiments.

Dr. N. Simionescu (Yale University) did kindly provide us with a better characterized sample of low molecular weight ($\approx 1,000$) tannic acid specifically known to consist mostly of penta- and hexagalloylglucoses. This originally had been prepared by T. H. Beasley of Mallinckrodt Inc. We used this sample to compare with the commercial Mallinckrodt product without noting significant differences. Comparative experiments also were performed with tannic acid obtained from Fischer Scientific Co. (Pittsburgh, Pa.), said to have a mol wt of $\approx 1,700$, which therefore would be expected to contain a higher proportion of hepta- to decagalloylglucoses. Occasionally, as noted in what follows, this behaved in a detectably different way than the Mallinckrodt Inc. product, but the observed differences were slight. Finally, monomeric gallic acid, obtained from Mallinckrodt Inc., was used for comparative purposes.

For the model experiments, a number of different phospholipids and related compounds were obtained as follows: Phospholipids purchased from Sigma Chemical Co. (St. Louis, Mo.) included DL- α - and L- α -phosphatidyl choline dipalmitoyl (PCDP), L- α -phosphatidyl choline distearoyl (PCDS), and -dioleoyl (PCDO), DL- α - and L- α -phosphatidyl ethanolamine dipalmitoyl (PEADP), and L- α -phosphatidic acid dipalmitoyl (PDP). Purchased from General Biochemical Co. (Cleveland, Ohio) was PS, and from Applied Science Labs., Inc. (State College, Pa.), phosphatidyl inositol (PI) and sphingomyelin (SPH).

Related compounds obtained from Sigma Chemical Co. included choline chloride, phosphorylcholine, ethanolamine, *o*-phosphorylethanolamine, L-serine, *o*-phosphoryl-L-serine, myo-inositol, and oleic, palmitic, and stearic acids. New England Nuclear (Boston, Mass.) supplied choline chloride (methyl- $^{3\text{H}}$) 1 mCi/mM.

Model Experiments

Suspensions (2-5% wt/vol) of various lipids (and related compounds) in water usually were prepared at room temperature by prolonged (2-4 h) agitation. However, in a few experiments lipids were treated above their critical temperature which allowed their immediate dispersion in water as Fluck et al. (12) have described. These include PCDP solubilized at 50°C; PCDS at 80°C; PEADP at 90°C.

1-ml suspensions of phospholipids were mixed for 60 min at room temperature with equal volumes of either tannic acid solutions or any of the fixative solutions to be tested. Since the effects of tannic acids were of particular interest, they were studied over a concentration range of 1-10% wt/vol, generally prepared in 0.1 M Na phosphate buffer, pH 7.4. Na acetate-Na veronal, HCl-Tris and HCl-Na-cacodylate buffers were occasionally used for comparative purposes. After 1-h exposure, the lipid suspensions were centrifuged, and the resulting pellets

were washed four times in fresh buffer solutions to eliminate unreacted tannic acid. The fourth wash regularly was free of tannic acid since no blackening occurred in the supernate when OsO_4 was added (OsO_4 is readily reduced by tannic and gallic acids). Suspensions of complexed phospholipid and tannic acid then either were used for chemical experiments, or were osmicated for 60 min by mixing with buffered OsO_4 to produce a 1% concentration so as to result in a pellet which could be processed for electron microscopy.

Tissue Preparations

Lung tissue was obtained from 200–250-g Sprague-Dawley rats, ordinarily after vascular perfusion of the fixative via the abdominal vena cava. Blood was washed out of the pulmonary system first by perfusing ≈ 50 ml of a saline solution at room temperature, and draining the perfusate by an incision of the descending aorta. Primary fixation generally was based upon 1% glutaraldehyde in 0.1 M Na phosphate buffer. Approximately 300 ml of fixation was perfused before opening the thorax. Then, small pieces of lung (≈ 1 mm³) were isolated, and fixation was continued for another 2 h at room temperature before washing the blocks with buffer and continuing their processing.

In some experiments, 1% tannic acid was added to the buffered glutaraldehyde just before use, and perfused as the initial fixative. In other experiments, the glutaraldehyde-tannic acid mixture was used for simultaneous perfusion and infusion through the trachea before opening the thorax. Commonly, however, secondary treatments were delayed until isolated, glutaraldehyde-fixed, and washed tissue blocks had been obtained. This provided the opportunity not only to treat with tannic acid or to osmicate the tissue, but to do both sequentially. Secondary treatments employed 1.5% tannic acid and/or 1% OsO_4 buffered with 0.1 M Na phosphate. Notably successful experience with tannic acid led to parallel experimentation with comparably buffered gallic acid solutions. For control purposes, 2% buffered OsO_4 has also been applied by tracheal infusion as a primary fixative.

When all fixative procedures had been completed, tissue blocks were thoroughly washed with three changes of phosphate buffer. This proved to be of particular importance when tannic acid treatments preceded osmication, for then fine precipitates tended to form in the cytoplasm, probably by interaction with proteins. Even treatment with 1% Na_2SO_4 , as suggested by Simionescu and Simionescu (33), did not completely prevent or remove this precipitate. (However, these investigators employed an HCl-arsenate buffer system rather than Na phosphate.)

Electron Microscopy

Pellets and tissue blocks were dehydrated in a graded series of ethanols, transferred through propylene oxide, and finally were embedded in heat-polymerized Epon 812. Generally, sections were stained briefly, only with

lead citrate. A Siemens 1A microscope was used for electron microscopy. Measurements of spacings were performed on negative plates, originally magnified $\times 40,000$ or $\times 80,000$ (without special calibration beyond the $\pm 5\%$ inherent in the instrument). The plates were enlarged $\times 20$ for measurements, using a Nikon model 6C "profile projector" (Nikon Inc., Garden City, N. Y.) Spacings reported in the text were based on averages of over 100 individual measurements, made on various negatives.

Chemistry

Model experiments of phospholipids and phospholipid-tannic acid complexes, prepared as described above, were subjected to thin-layer chromatography (TLC), using washed silica gel H (Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.). Two different solvent systems were employed, *N*-butanol-acetic acid- H_2O (14:1:5) and chloroform-methanol- H_2O (65:25:4). TLC of choline and choline-tannic acid complexes was performed by using *N*-butanol-ethanol-acetic acid- H_2O (8:2:1:3) as the solvent system. Samples, 50–100 μg , were applied, either directly from suspensions in buffer, or after solution in 2:1 chloroform-methanol. After migration, the resultant spots were revealed by iodine vapor treatment, or by charring the chromatograph plate with dichromate-sulfuric acid. In a few experiments, spots were located by exposing chromatograph plates to OsO_4 vapor at 60°C for 2 h.

It will become evident that we believe that tannic acid particularly complexes with the choline base of lecithins. Therefore, the reaction of the various tannic acids with choline was also studied with the aid of a radioactive tracer choline. These experiments involved adding 0.1 μl of choline chloride-³H (1 mC/mM) to 1 ml of 0.075 M choline in water, plus 1 ml of various concentrations of tannic acid made up in 0.1 M buffers at different pH's. In some experiments, the choline concentration also was varied. The buffers employed covered a pH range from 4.0 to 9.5, and included phosphate, acetate, veronal-acetate, and Tris. After standing for 30 min at room temperature, precipitates were centrifuged, and the radioactivity of 0.5-ml aliquots of the supernate was determined by scintillation counting. Thus, the radioactivity of the supernate expressed the residual portion of choline which had not precipitated with the tannic acid.

RESULTS

Electron Microscopy of Phospholipids:

Model Experiments

Aqueous dispersions of the various phospholipids listed, prepared as indicated in the previous section, were exposed to solutions of tannic acid. Of the various saturated phospholipids tested, PC, PEA, PI, and PS, as well as SPH and phosphatidic acid, only PC and SPH reacted with the tannic

acid so that a complex was formed, which then in turn was readily reactive with OsO_4 . In the absence of pretreatment with tannic acid, none of the fully saturated phospholipids other than PS showed any evidence of reaction with OsO_4 . PS osmicated very slowly, either with or without pretreatment with tannic acid, as has already been described for this group of phospholipids (21, 38).

Saturated PC and SPH suspensions which were treated with both tannic acid and OsO_4 were rendered insoluble, so that pellets so treated could be conventionally dehydrated and embedded for ultrathin sectioning. This was not the case after tannic acid treatment alone, without the secondary osmication. The other tannic acid-treated saturated phospholipids, which did not blacken when exposed to OsO_4 , remained sufficiently soluble so that they did not survive dehydration for embedment. The substitution of freshly buffered gallic acid instead of tannic acid did not permit secondary reactions with OsO_4 , even in the case of PC. Neither glutaraldehyde, nor glutaraldehyde followed by exposure to OsO_4 , "fixed" saturated PC so that it could survive embedment.

Micrographs are presented in Figs. 1-5 of embedded and stained PC and SPH, pretreated with low molecular weight tannic acid, followed by exposure to buffered osmium tetroxide. Figs. 1-3 are of PCDP, Fig. 4 of PCDS, and Fig. 5 of SPH. It was clear that most of the phospholipid which had been rendered insoluble by these treatments was preserved with a lamellar organization similar to that previously described for OsO_4 -treated unsaturated phospholipids (36, 37). Thus, the repeating period of lamellae approximated 45 Å, of which the hydrophobic (electron lucent) layer occupied 25-30 Å, and the hydrophilic (electron dense) zone measured 15-20 Å.

The temperature at which PCDP was originally dispersed did not affect its fundamental lamellar organization. However, dispersion at high temperature favored the formation of small aggregates of only a few lamellae (Fig. 3), as compared with relatively large masses after dispersion at room temperature (Fig. 1).

Identical results were obtained with all of the three preparations of tannic acid tested, and were essentially independent of its concentration (1-5% was used in this series of experiments).

Electron Microscopy: Lung Tissue

Principles established in the model experiments as described above were successfully employed to

preserve and demonstrate the contents of the secretory bodies of type II pneumocytes. These bodies, known to contain a high proportion of PCDP (8, 16, 19), generally have defied adequate preservation of their contents for electron microscopy.

In general, to prepare lung tissue, glutaraldehyde was used as a primary fixative simply to preserve overall cytological detail. We came to appreciate that this had no effect in rendering the lecithin content of the secretory bodies insoluble. Tannic acid, used either simultaneously with, or secondarily after, glutaraldehyde, surely did penetrate to reach the secretory bodies. It was thought that the "low molecular weight" tannic acid (Mallinckrodt Inc.) probably demonstrated more superior properties in this respect than the Fisher Scientific Co. product, as Simionescu and Simionescu have suggested (33). Tannic acid, however, did not prove to be in itself an adequate fixative as the model experiments demonstrated. It had to be used with OsO_4 to achieve saturated PC insolubility. In our experience, to demonstrate ordered structure, it was far preferable to treat lung tissue with tannic acid in advance of osmication, rather than reversing the order of this sequence.

When lung tissue was adequately treated with tannic acid before osmication, secretory bodies exhibited highly ordered lamellar arrays which nearly filled the entire contents of each body (Figs. 6-8). Obviously, in areas where the plane of section corresponded to the plane of the lamellae, or where there was decided obliquity, the lamellar structure could not be expected to be evident. However, so many bodies have been examined, exhibiting so much evidence of precise patterns, that there is no doubt in our minds but that ordered lamellar arrays were the rule.

There were angular cavities to be seen regularly in tannic acid-treated and osmicated secretory bodies (Figs. 7 and 8). In the simplest patterns, elongate, lens-shaped cavities separated two lamellae (Figs. 7 and 8, arrows). Generally larger, triangular cavities often could be seen as resulting from loci where lamellar systems diverged from each other (Fig. 7, double arrows). Although the cavities appeared to be essentially empty after the final processing of the tissue, some irregular granular material was always to be found in the larger cavities, where it was adherent to adjacent lamellae. The manner in which ordered systems of lamellae regularly bordered these cavities would seem to preclude interpretation based simply on "inadequate" fixation and subsequent extraction.

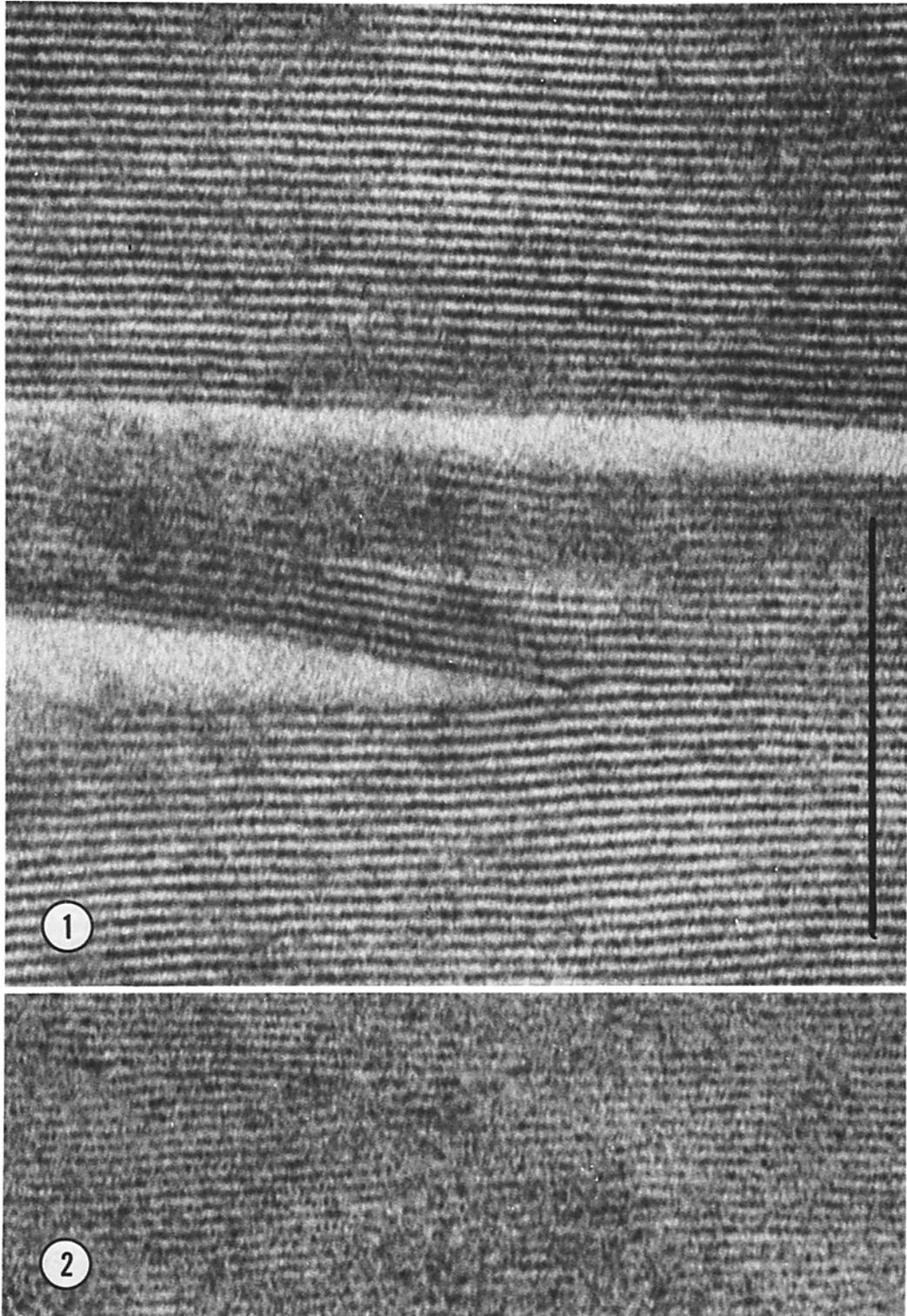


FIGURE 1 The lamellar organization of the fully saturated phospholipid, PCDP, prepared as an aqueous suspension at room temperature, and treated successively with tannic acid and OsO_4 . Bar, $0.1 \mu\text{m}$. $\times 640,000$.

FIGURE 2 A portion of the same negative used for Fig. 1, demonstrating a less well-ordered region of preserved PCDP. It is possible to appreciate that the dense bands representing the polar heads of the phospholipids appear to consist of linear arrangements of electron-dense units. $\times 640,000$.

The repeating distance between the lamellae of the secretory bodies was similar to that described above for synthetic PCDP, i.e., $\approx 45 \text{ \AA}$, consisting of 25–30 \AA for the hydrophobic, and 15–20 \AA for the hydrophilic, layers. In this respect, it made no difference whether low or high molecular weight tannic acid was used, nor did variations in the concentration of tannic acid alter the results. The substitution of neutralized gallic acid for tannic acid did not permit the preservation of any structural organization. In the case of the secretory bodies, phosphate buffers appeared to be decidedly superior to cacodylate.

When osmication preceded tannic acid treatment, the results were quite different from the reverse sequence. To be sure, a great deal of lipid was preserved which ultimately was stained with great intensity (Figs. 9 and 10). However, at best, ordered lamellae were poorly demonstrable (Fig. 10). This result contradicts the experience of Simionescu and Simionescu (33) who preferred this sequence for the preservation of cytomembranes in general. However, they did not investigate any system of tightly packed phospholipid lamellae that might be regarded as comparable to that of the secretory bodies of type II pneumocytes.

Experiments were conducted to test some of the prerequisites for the successful preservation of lamellar structure. As with the model experiments, omission of osmication resulted in an almost complete loss of the contents of the lamellar bodies in spite of glutaraldehyde fixation and tannic acid treatment. Control experiments, basically conventional in character, confirmed a poor preservation of secretory bodies after glutaraldehyde fixation, secondarily osmicated. As other investigators have generally found, most of their content was extracted, and only some residual organization could be detected in the form of relatively few stacks of partially ordered lamellae (Fig. 11). Fixation with osmium tetroxide alone, without prior fixation with glutaraldehyde, gave similar results, with only a few distorted lamellae being preserved (Fig. 12). The preservation of some lamellar structure after these conventional fixative procedures might be due to the small percentage of unsaturated phospholipids in the secretory bodies (16, 19), available for fixation with OsO_4 . Also, glutaraldehyde reacts at least with primary amines, and might fix phospholipids containing such groups, which are also present in small quantities in surfactant lipids (14, 27).

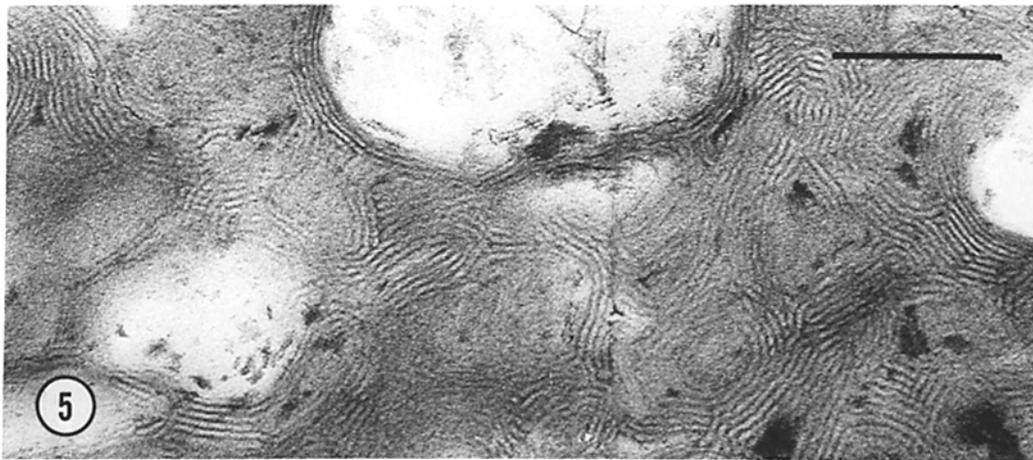
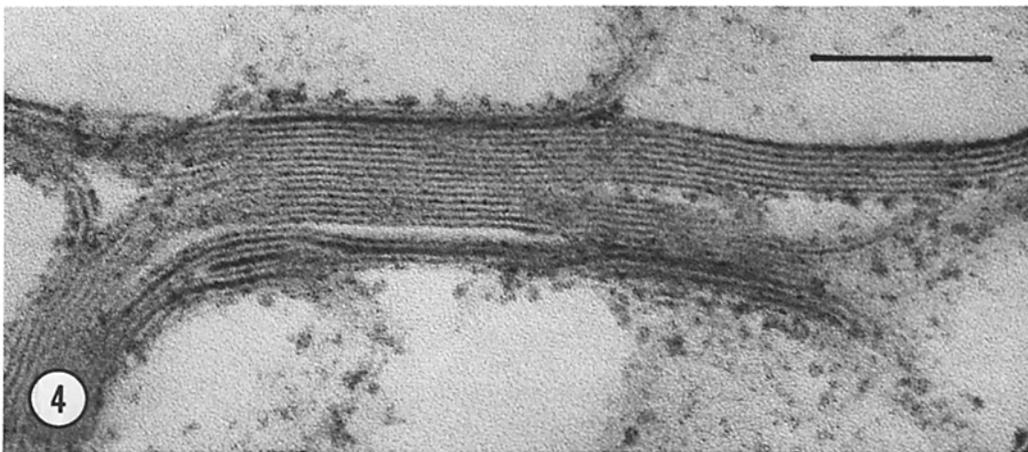
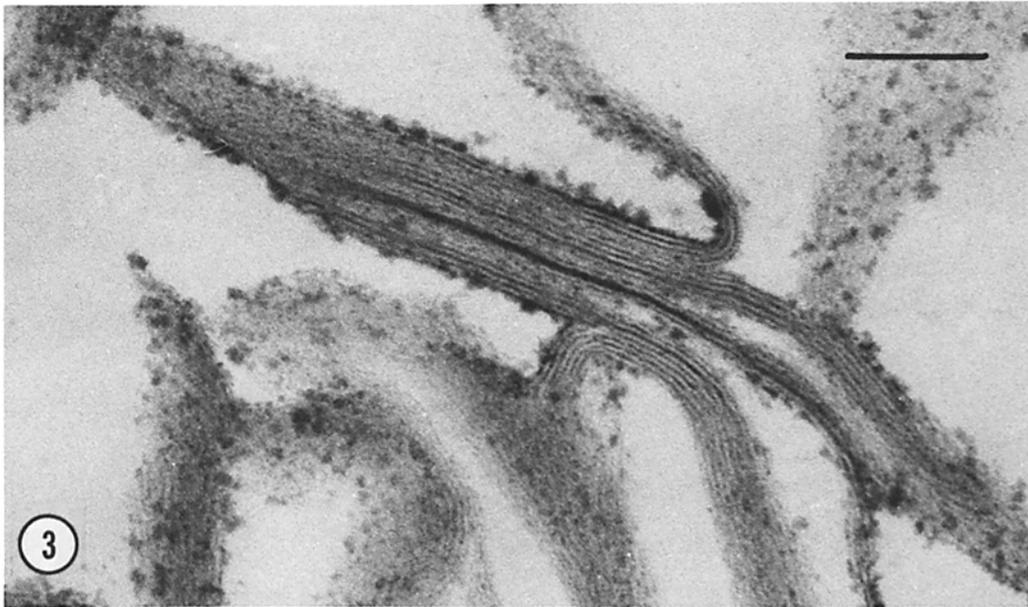
Chemistry

The data from the model experiments indicated that of the various saturated phospholipids tested, only PC and SPH were capable of interaction with tannic acid so that subsequently OsO_4 would be reduced. This led to experiments to determine whether phospholipid bases were capable of forming precipitates with tannic acid. The following compounds were examined: choline and phosphorylcholine, serine and phosphorylserine, ethanolamine and phosphorylethanolamine, and inositol. Palmitic, stearic, and oleic acids also were tested. Of all of these compounds, it was only phosphorylcholine and choline that did form precipitates, which then could be readily osmicated. There was no evidence of reaction with gallic acid.

Some of the parameters of the choline-tannic acid interaction to form complexes were tested by using radioactive choline. It became apparent that the reaction was highly pH dependent, as Fig. 13 demonstrates. No choline-tannic acid precipitate was formed below a pH of 4.3 or above pH 8.5. Maximum precipitation occurred at pH 7.5, with a very sharp decline in the basic range. This effect was not due to decomposition of the tannic acid at high pH, for the pH reaction was reversible.

The precipitation of choline by tannic acid was substantially dependent upon the latter's concentration as seen in Fig. 14. Even in the presence of 10% tannic acid, which must be regarded as a great excess, only 70% of the choline was precipitated. (As indicated by the experiments described previously, even 1% tannic acid proved sufficient to help preserve optimally the structural organization of model PC systems, as well as ordered structure in type II pneumocyte secretory bodies). Varying the concentration of choline made little difference. Even when this was reduced to 10^{-4} M , still only 50% was precipitated by 5% tannic acid. In this series of experiments a small difference could be demonstrated between high and low molecular weight tannic acids (from different sources, as indicated earlier), with the former being slightly more effective (Fig. 14).

Finally, TLC was used to demonstrate certain aspects of the interactions between choline, tannic acid, and OsO_4 . It was established that the choline-tannic acid complex appeared as a distinctive spot, with an R_f value quite different than that of choline itself (Fig. 15). The position of the complex was easily determined by exposure of the



TLC plates to OsO_4 vapor, which the tannic acid then reduced to show a blackened spot. We did not detect any differences when the several different tannic acids were employed. However, no evidence of any complex at all was observed in comparable experiments when gallic acid was substituted for tannic acid.

An effort also was made to characterize the PC-tannic acid complex. The reaction product proved to be unstable in the chloroform-methanol-water solvent systems usually employed for the separation of phospholipids, so that the PC and the tannic acid separated from each other, as is seen in Fig. 16. However, a relatively successful separation was achieved using a solvent system, (*N*-butanol-acetic acid- H_2O), commonly employed for the separation of tannic acids (20). Then, the R_f value of the movement of the complex was similar to that of the phospholipid by itself (Fig. 17). Once again, the position of the tannic acid was demonstrated by its capacity to reduce OsO_4 . The complexes formed by the various tannic acids had the same R_f values, and reacted similarly with OsO_4 .

DISCUSSION

The present study clearly indicates that tannic acid has a stabilizing effect on ordered lamellar structure of PCs in general, and saturated ones in particular, as well as of SPH. We conclude that this is achieved primarily by an interaction of tannic acid with choline bases to form complexes, which then enable the tannic acid to interact secondarily with OsO_4 . Osmication after the tannic acid treatment is a prerequisite for the retention of the structural organization through conventional embedding procedures. In this respect, tannic acid alone cannot be said to have a true fixative effect on saturated PC, for without osmication the complex readily dissociates in organic solvents unless the latter are decidedly polar. This

is in general agreement with the conclusions of Simionescu and Simionescu (33, 34), that tannic acid is not a true fixative in a commonly accepted sense, although the latter investigators were not particularly or specifically concerned with the preservation of PC. They did emphasize that, when it is bound in tissue, tannic acid then fulfills the function of being a mordant between "osmium-treated substructures and lead." This concept was later supported by Wagner (41).

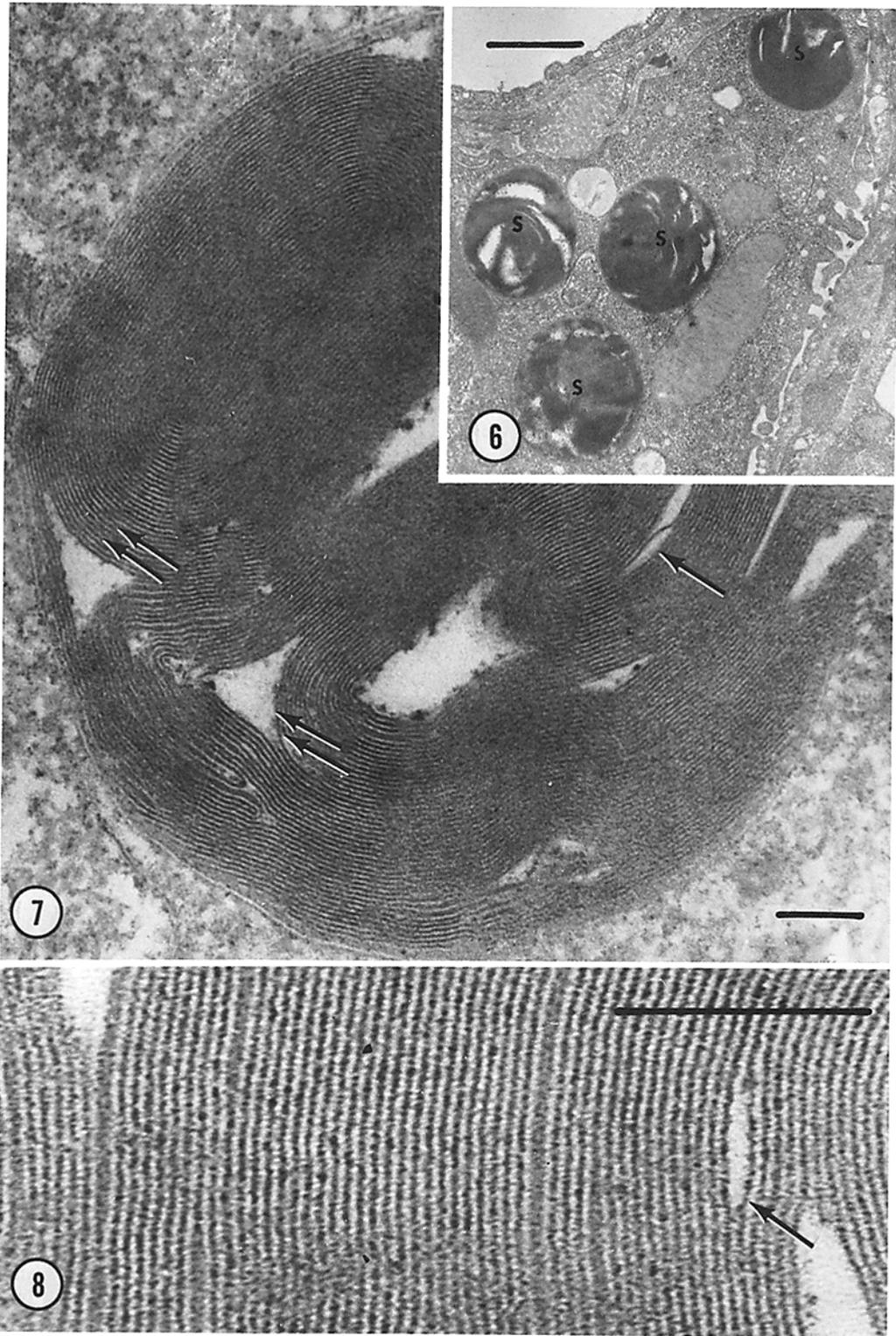
Most of the information regarding the chemistry of tannins, and their possible interaction with tissue components, comes from the leather industry (reviewed in references 20 and 23). Therefore, it is mainly applicable to effects on proteins (17, 18, 20, 23). It is generally assumed then that tannins react with peptide bonds, and probably also with the amine and amide residues present in polar amino acid side chains, which hydrogen bond with the phenolic groups of tannin (17, 18). More specifically, Futaesaku et al. (13), who first introduced tannic acid into electron microscopy, proposed that its fixation effects on tissue-soluble proteins might be either by hydrogen or chelating bonds through the phenolic radicals, or by electrostatic bonding due to charges on tannic acid in solution. Simionescu and Simionescu (33) have taken a cautious position in stating that tissue active groups that may be reactive with tannic acid really remain unknown, although they quote evidence that suggests that reaction does not depend upon the presence of such active groups as ketones, Schiff bases, nitriles, disulfides, acid chlorides, or inorganic ions.

Our chemical data provide some information concerning the nature of the complex formed by the interaction of tannic acid and choline. The complex demonstrates a significantly different mobility than choline alone. The pH dependency of the complex suggests an ionic interaction. Thus, it seems likely that reaction may be between the

FIGURE 3 PCDP suspended in water at 50°C, but otherwise treated with tannic acid and OsO_4 as in Figs. 1 and 2. The elevated preparative temperature tended to produce small assemblages of lamellae rather than the large arrays found after room temperature suspension. Bar, 0.1 μm . $\times 190,000$.

FIGURE 4 PCDS, suspended at 80°C, was treated first with tannic acid and then OsO_4 . The lamellar organization of this fully saturated phospholipid is similar to that shown for PCDP. Bar, 0.1 μm . $\times 240,000$.

FIGURE 5 SPH, suspended at room temperature, was treated first with tannic acid and then with OsO_4 . A lamellar organization of this phospholipid was also preserved by this treatment. Bar, 0.1 μm . $\times 225,000$.



quaternary ammonium radical of the choline, which is a base, and carboxylic groups available in tannic acid. Free carboxylic groups might be contributed by gallic or digallic acids originating from tannic acid, either as a result of solution in water, or by direct interaction with the phospholipids. Additionally, the instability of the PC-tannic acid complex under hydrophobic conditions suggests that hydrogen bonding involving the multiple hydroxyl groups of the tannic acid might be a contributing factor in its formation and maintenance in an aqueous environment. The fact that there was no detectable reaction with monomeric gallic acid suggests that molecular (polymeric) size may be a limiting factor. Free carboxyl groups, as well as hydroxyl groups, have also been implicated as essential for the mordanting effect of tannic acid (34).

Simionescu and Simionescu (33) and Wagner (41) have suggested that the mordanting effect of tannic acid is most pronounced after tissue osmication. Our own data on PC indicate that treatment with tannic acid before osmication is a requisite for the optimal preservation of ordered structure in PC. However, tannic acid treatment after osmication was indeed found to be most effective in favoring high-density lead staining of the contents of the secretory bodies of type II pneumocytes, even though very little ordered structure then was observed. A possible interpretation of these results is that substantial OsO_4 can be dissolved in unreduced form in saturated phospholipids (21). This, then, would be available, so that secondary treatment of the tissue with tannic acid could reduce the OsO_4 present, while concomitantly achieving PC insolubility. However, OsO_4 acting on PC before tannic acid largely blocks the "stabilization" effect of the latter. The electron micrographs cannot assure us that there is a complete

quantitative preservation of PC in either case, a question presently being investigated.

Whatever the mechanism of osmication may be, it seems certain that the final disposition of the tannic acid- OsO_4 -lead stain lies in the polar heads of the PC molecules. Support for this thesis derives from our own measurements of alternating electron-lucent and -dense lamellae in both saturated PC model experiments, and in the secretory bodies of type II pneumocytes. Of the ≈ 45 Å-repeating periodicity, the electron-dense zone was only 15–20 Å thick, while the electron-lucent layer measured 25–30 Å. These dimensions are basically in agreement with measurements made by others, on various unsaturated phospholipids (37, 38), as well as on saturated ethanolamines (3).

The chemistry involved in permitting OsO_4 to act as a true fixative for saturated PC in the presence of tannic acid remains speculative for the present. However, it seems likely that the reaction may be similar to that described for the formation of diesters of OsO_4 which cross-link molecules of olefins (4, 5, 38, 42). Thus, diesters of OsO_4 may cross-link phenolic groups of gallic acid moieties. Since the tannic acid would also be complexed with PC and SPH, it would then have the effect of fixing the already established phospholipid lamellar organization.

The difficulties inherent in preserving intact the phospholipid content of the secretory bodies of type II pneumocytes are so generally appreciated that an extended review of the scientific literature seems unwarranted. It should be noted, however, that Stratton (39) initiated an investigation of tannic acid as a fixative for these structures, although the primary thrust of his publication concerned nonconventional dehydration procedures. No attempt was made to correlate tannic acid

FIGURE 6 Part of a type II pneumocyte from a rat perfused with glutaraldehyde and tannic acid, subsequently treated with OsO_4 . The secretory bodies (S) were relatively well preserved with much content. Bar, 1.0 μm . $\times 14,500$.

FIGURE 7 Higher magnification of a secretory body, prepared as those of Fig. 6. A lamellar organization is apparent with a spacing similar to that observed in saturated PC. Divergent groups of lamellae sometimes created lens-shaped cavities (arrow), or more complicated angular cavities (double arrows). Bar, 0.1 μm . $\times 130,000$.

FIGURE 8 A high-resolution micrograph of lamellae in a secretory body; tissue prepared as that of Figs. 6 and 7. Diverging lamellae are seen (arrow). The dense layers, corresponding to polar heads of the phospholipid molecules, can be seen to consist of discrete units, as in the PC model system illustrated in Fig. 2. Bar, 0.1 μm . $\times 390,000$.

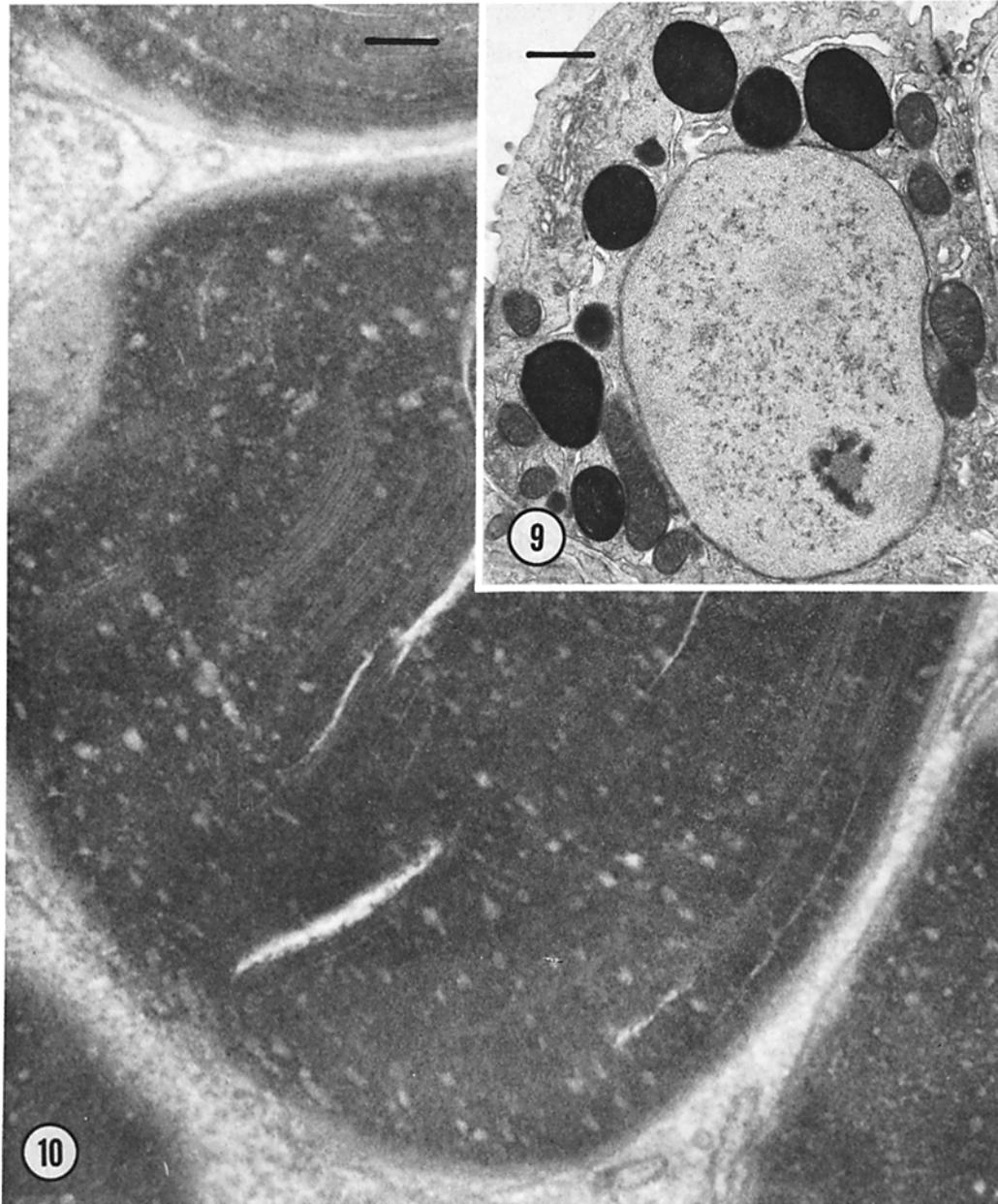


FIGURE 9 Part of the type II pneumocyte from a rat perfused with glutaraldehyde, then postosmicated, and finally treated with tannic acid. There was little evidence of phospholipid extraction, and lead citrate staining resulted in high electron density. Bar, $1.0 \mu\text{m}$. $\times 7900$.

FIGURE 10 A higher magnification of secretory bodies from a type II pneumocyte, prepared as in Fig. 9. Although much material obviously was preserved, only a few localized regions demonstrated arrays of lamellae, seen without good contrast. Bar, $0.1 \mu\text{m}$. $\times 100,000$.

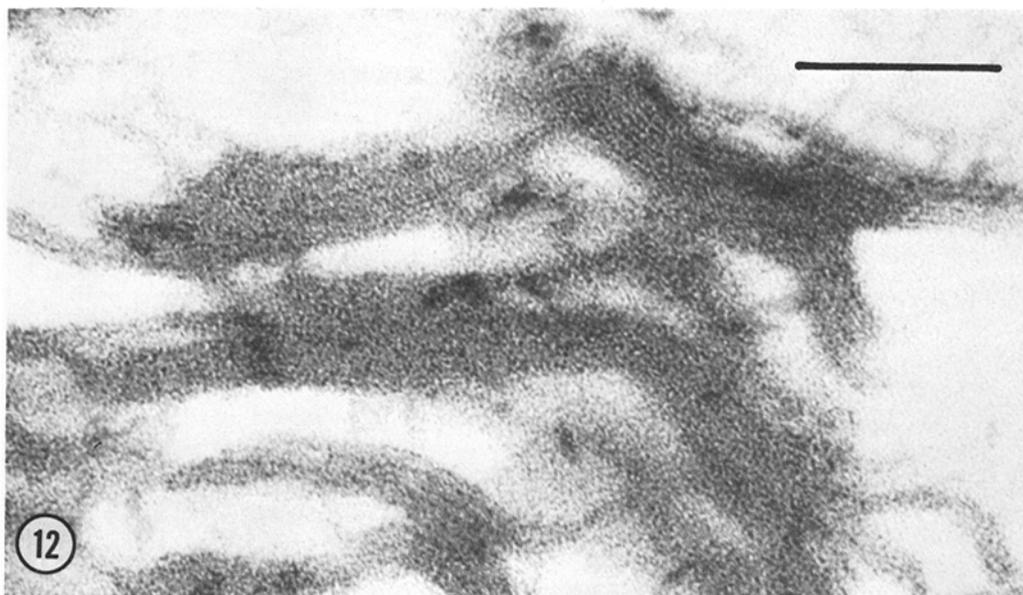
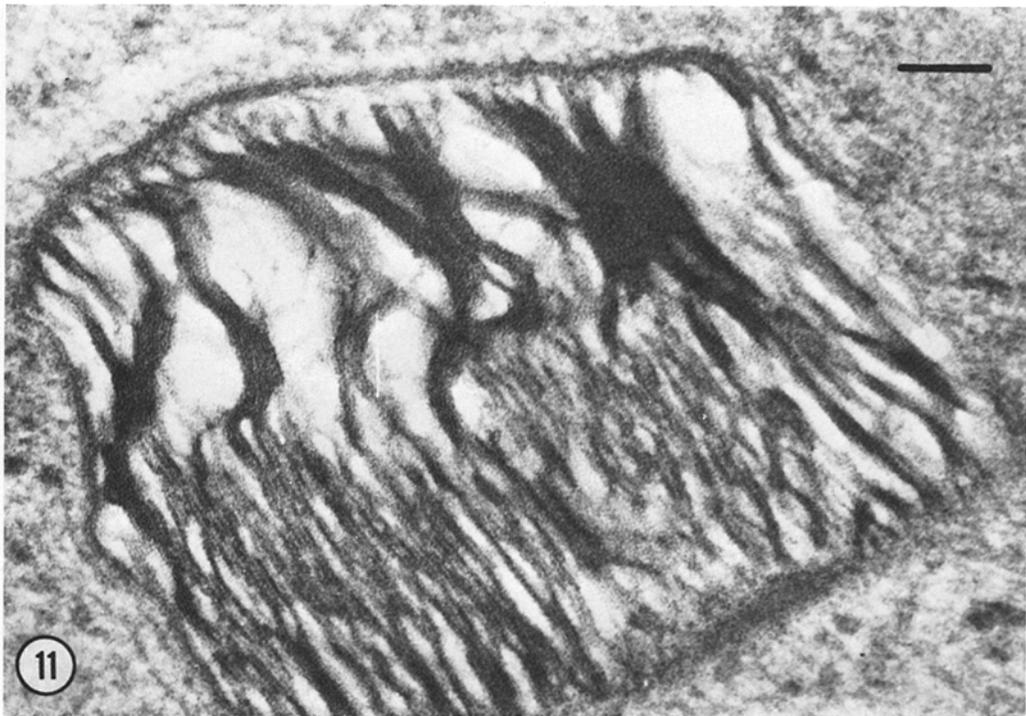


FIGURE 11 A control secretory body of a type II pneumocyte from a rat perfused first with glutaraldehyde and then postfixed with OsO_4 , but without treatment with tannic acid. In such conventional preparations, there was always evidence of much extraction (compare with Figs. 7 and 10). Ordered lamellar arrays were observed in only a few regions. Bar, $0.1 \mu\text{m}$. $\times 120,000$.

FIGURE 12 A high magnification portion of a secretory body of a type II pneumocyte from a rat lung infused only with OsO_4 . There was always evidence of much extraction and disorder. Only small residual regions demonstrated some evidence of lamellar organization. Bar, $0.1 \mu\text{m}$. $\times 270,000$.

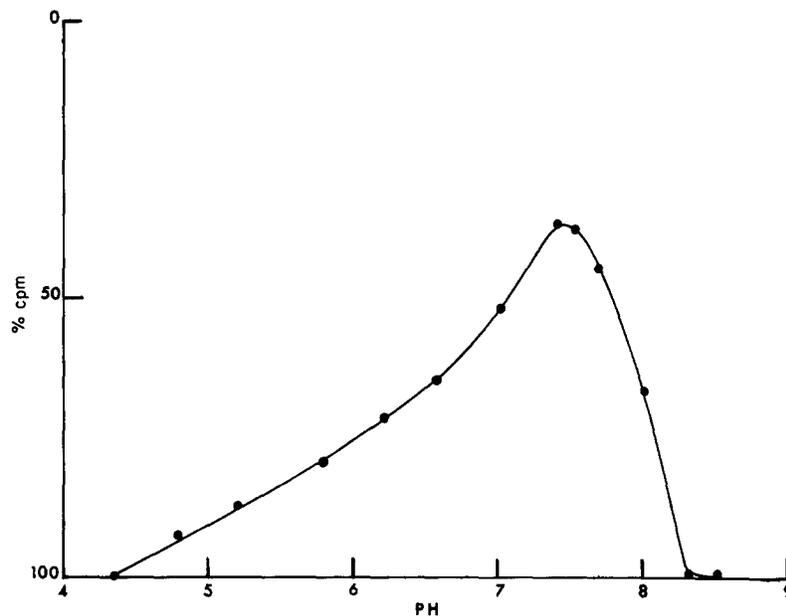


FIGURE 13 The curve demonstrates the pH dependency of choline precipitation by tannic acid. It expresses the percentage of residual radioactivity remaining in the supernate after the addition of 1 ml of 5% tannic acid to 1 ml of 0.075 M choline chloride, containing $0.1 \mu\text{l}$ of choline chloride- ^3H (1 mC/ml). The precipitate was removed after 30 min, and 0.15-ml aliquots of the supernate were analyzed in a scintillation counter.

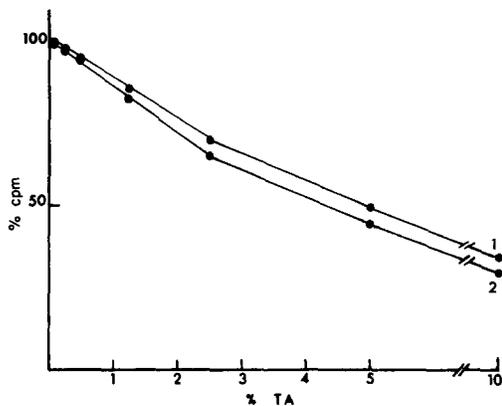


FIGURE 14 The effect of various concentrations of tannic acid (TA) on choline precipitation was determined by measuring the residual radiocholine left in the supernate after adding 1-ml samples of different concentrations of tannic acid to 1 ml of 0.075 M choline, containing $0.1 \mu\text{l}$ of choline chloride- ^3H (1 mC/ml), the same conditions as for the test described in Fig. 13. Curve 1 obtained using low molecular weight tannic acid (Mallinckrodt Inc.); curve 2 employed high molecular weight tannic acid (Fisher Scientific Co.).

treatment specifically with the preservation of PC. Other investigators have also attempted to minimize preparative extraction of the secretory bodies

by using polar dehydrating agents such as Durcupan and dioxane (30), and glycol methacrylate (9), with only moderate success. Dermer, (6, 7) tried to apply a "tricomplex" fixation procedure, depending upon phospholipid interaction with selected multivalent ions, as originally introduced by Elbers et al. (11). In spite of Dermer's extended efforts, however, this technique clearly did not solve the problem, as Gil has indicated (15). Freeze-substitution, incorporating OsO_4 fixation, by Kuhn (22), retained some secretory body contents, but without exhibiting more than a few isolated groups of lamellae. Freeze-fracturing has resulted in the observation of some of the most perfectly ordered secretory bodies of type II pneumocytes by Belton et al. (2) and by Smith et al. (35). Both groups employed long periods of pre-glycerination with relatively strong solutions of glycerol before exposing the tissue to deep cold. Belton et al. have particularly stressed the interpretative problems and hazards of the general technique.

Model experiments, concerned with the preservation of phospholipids, particularly such as those initiated by Stoeckenius (36, 37) and Dreher et al. (10), generally have dealt with mixed systems, and/or unsaturated systems that could be stabi-

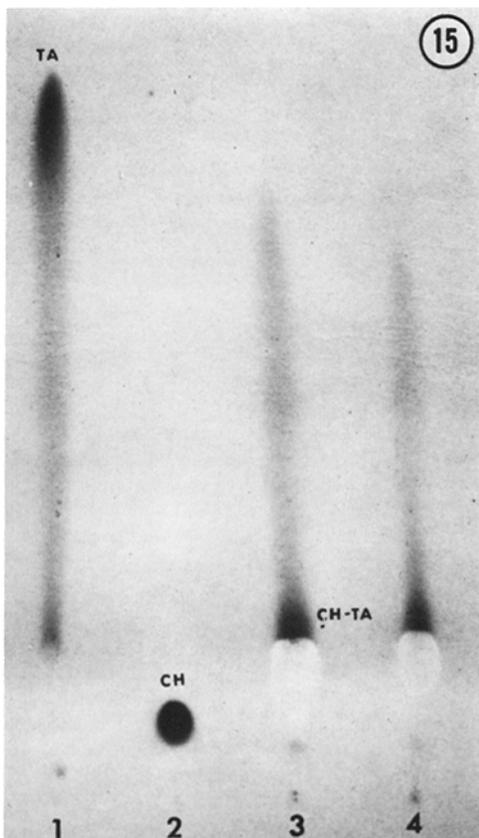


FIGURE 15 TLC of: 1, tannic acid (*TA*) (Mallinckrodt Inc.); 2, choline chloride (*CH*); 3 and 4, choline-tannic acid complexes (*CH-TA*), respectively, using Mallinckrodt and Fisher tannic acid. The solvent system was *N*-butanol-ethanol-acetic acid- H_2O (8:2:1:3). Spots were revealed by charring with dichromate-sulphuric acid. Trails probably are of tannic acid breakdown products.

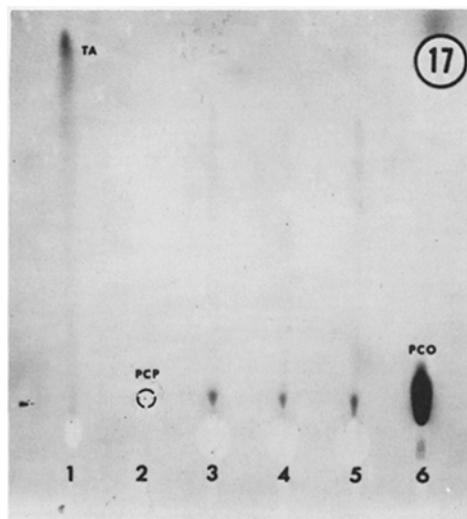
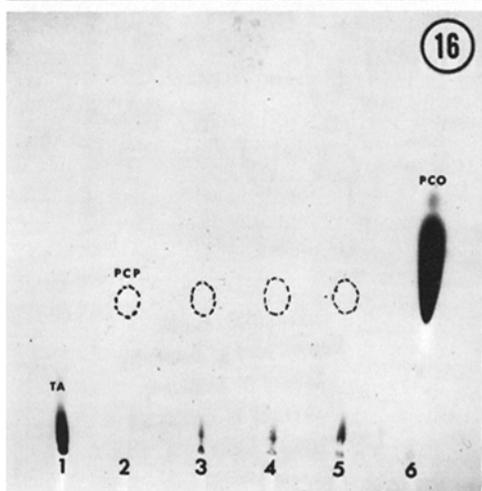


FIGURE 16 TLC of: 1, tannic acid (*TA*) (Mallinckrodt Inc.); 2, phosphatidyl choline dipalmitoyl (*PCP*); 6, phosphatidyl choline dioleoyl (*PCO*). Numbers 3, 4, and 5 are of *PCP*-tannic acid complexes, prepared, respectively, with tannic acid from Mallinckrodt Inc., Fisher Scientific Co., and the especially defined tannic acid preparation described in the text. The solvent system was chloroform-methanol- H_2O (65:25:4). Spots were revealed by exposure of the chromatograph plates to OsO_4 vapor at $60^\circ C$ for 2 h. *PCP* spots which did not react with OsO_4 were revealed by iodine vapor treatment (dotted circles). A complete separation of the phospholipid from the tannic acid occurred in this solvent system.

FIGURE 17 TLC as in Fig. 16, but in the solvent system *N*-butanol-acetic acid- H_2O (14:1:5). Spots revealing the position of tannic acid were revealed by exposure to OsO_4 vapor, the position of *PCP* by iodine vapor, as in Fig. 16. Complexes of *PCP*-tannic acid moved as *PC* alone. Thus, the complexes did not dissociate greatly in this solvent system as compared to Fig. 16, although faint trails, probably representing some free tannic acid, are visible in 3, 4, and 5.

lized and fixed simply with OsO₄. The focus of their interest usually has been in relation to cytomembranes generally, and not specifically to the surfactant system of the lung. Surfactant is so dominated by saturated lecithin that there has not so far been much rational success achieved in relation to this particular problem. We conclude that tannic acid, in its reactions with phospholipids, must be regarded as a multivalent agent, capable of simultaneous interaction with choline bases, OsO₄, and lead "stains."

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