THE PROBABLE ROLE OF PHOSPHATIDYL CHOLINES IN THE TANNIC ACID ENHANCEMENT OF CYTOMEMBRANE ELECTRON CONTRAST

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ABSTRACT

Unsaturated natural and synthetic phosphatidyl cholines (PCs), when treated with tannic acid and OsO_4, demonstrated a substantial increase in contrast as compared to PC treated only with OsO_4. This was not observed when phosphatidyl ethanolamine (PEA) was similarly exposed to tannic acid. The increased electron density observed in the lamellar organization of the PC phospholipids was limited to the hydrophilic layers corresponding to the polar regions of the phospholipid molecules. The repeating periods of lamellae were identical in PC, treated with both tannic acid and OsO_4, and when treated only with OsO_4. In each case, this approximated 45 Å. The enhancement of membrane contrast by tannic acid in the presence of OsO_4 is interpreted as being at least in part due to its multivalent capacity, binding to reactive sites on choline, as well as with OsO_4.

KEY WORDS tannic acid · cytomembrane lipids · contrast enhancement

Tannic acid originally was introduced into electron microscopy in 1971 by Mizuhira and Futaesaku (7) as a supplementary fixative. Since then a number of investigators have emphasized that it enhances the contrast of various intra- and extracellular structures, including cytomembranes (8, 9, 10, 14). Most of our information regarding the chemistry of tannic acid has derived from the interests of the leather industry which has developed a substantial literature concerning the reactions of tannic acid with proteins, particularly collagen (3, 4, 6). On that basis Futaesaku et al. (2) suggested that tannic acid might "fix" soluble proteins by hydrogen bonding or chelating via the phenolic radicals of the tannic acid, or by its electrostatic charges. These principles do not immediately suggest why cytomembranes commonly also seem to exhibit an increased contrast and sharpness after tannic acid treatment.

Simionescu and Simionescu (10, 11), as well as Wagner (15), have addressed themselves to these questions, and have come to the conclusion that tannic acid should not be regarded as a true fixative. Instead, it should be thought of as a multivalent agent, acting principally as a mordant between osmicated structures in tissue and lead stains. Thus, treatment with OsO_4 is a prerequisite for enhanced contrast. Simionescu and Simionescu (11) found that a carboxyl group and at least one hydroxyl group on the tannic acid are the minimal functional components required for the mordanting effect. However, they did recognize that tannic acid also "stabilized" some tissue components against extraction, which otherwise would have occurred during dehydration and subsequent processing for electron microscopy.

In work presented elsewhere (5) we have pro-

vided evidence that tannic acid reacts with the choline "base" of phosphatidyl choline (PC) and sphingomyelin (SPH). Although the "complex" so formed is regarded as having a stabilizing effect on model PC systems and in the secretory bodies of type II pneumocytes where fully saturated PC is the predominant phospholipid, the complex is rendered insoluble in conventional dehydrating solvents only by further reaction with OsO_4.

The present study is concerned with tannic acid interactions in model systems of purified unsaturated phospholipids from both natural and synthetic sources. Since PC demonstrates a particular reactivity, and is also a major component of most mammalian membrane systems (16, 17), the evidence presented here provides a rational basis for interpreting enhanced contrast in cytomembranes treated with tannic acid.

MATERIALS AND METHODS

The tannic acid principally used in the following experiments was AR grade, code no. 1764, obtained from Mallinckrodt Inc. (St. Louis, Mo.). This product, according to Simionescu and Simionescu (10, 11), is a relatively low molecular weight galloylglucose (≃1,000). Comparative studies were also made with a better characterized, low molecular weight tannic acid obtained as a gift from Dr. N. Simionescu (Yale University), as prepared by T. H. Beasley of the Mallinckrodt Company. Also used was tannic acid obtained from the Fisher Scientific Company (Pittsburgh, Pa.), stated to have a mol wt of ≃1,700. Parallel experiments compared the effects of the Mallinckrodt Inc. gallic acid with tannic acid.

Phospholipids studied included chromatographically pure egg and beef PC, as well as phosphatidyl ethanolamine (PEA), obtained from General Biochemical Co. (Cleveland, Ohio), and L-α-phosphatidyl choline dioleoyl (PCDO), purchased from Sigma Chemical Co. (St. Louis, Mo.).

Aqueous suspensions (5–25% wt/vol) of the various phospholipids (except PEA) were prepared by shaking for a few hours at room temperature. In the case of PEA, this was done at 100°C.

For most experiments, suspensions (1 ml) of the several phospholipids were mixed for 30 min at room temperature with an equal volume of 2% tannic acid in 0.1 M phosphate buffer, pH 7.4. The suspensions were centrifuged, and the pellets washed with four exchanges of buffer to remove any unreacted tannic acid. The fourth wash did not contain any free tannic acid, for the supernate would no longer reduce OsO_4.

Pelletized phospholipid suspensions, either pretreated with tannic acid or sometimes untreated, were osmicated with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. In some experiments, the phospholipids were treated with tannic acid after osmication, and in some experiments, neutralized gallic acid was substituted for tannic acid.

Finally, the pelletized phospholipids were dehydrated in 70 and 100% ethanols, followed by propylene oxide, and conventionally embedded in Epon 812. Ultrathin sections were stained for 1 min only with lead citrate. The measurements of spacings were made with the aid of a Nikon "Profile Projector" (Nikon Inc., Garden City, N. Y.) at × 20 magnification of negatives initially magnified × 40,000 or × 80,000.

RESULTS

Natural PC from both egg and beef sources, and also synthetic PCDO, when treated with tannic acid as well as with OsO_4, demonstrated a substantial increase in lead-stain contrast as compared with PC treated with OsO_4 alone (compare Figs. 1, 2, and 4 with Figs. 3 and 5). The augmentation of density resulting from the pretreatment of the specimens with tannic acid before osmication was limited to the hydrophilic layers corresponding to the polar heads of the phospholipid molecules. The most commonly ordered structure was in the form of parallel lamellae exhibiting approximately a 45-Å periodicity. Electron-dense layers were 15–20 Å thick, the -lucent regions 25–30 Å thick. This corresponds to the periodicity and molecular organization previously ascribed to various phospholipid systems (1, 12, 13). The usual lamellar arrays sometimes interchanged with "tubules," arranged in hexagonal patterns when observed in transverse sections, as is seen in part of Fig. 1.

Tannic acid treatment generally resulted in the formation and preservation of relatively large precipitated masses demonstrating high degrees of order. In contrast to this, when suspended PC was treated only with OsO_4, large organized arrays were rarely to be found. The large array demonstrated in Fig. 3 was exceptional in this respect. Instead, most of the PC was arrayed in small stalks or sheets consisting of only a few lamellae.

When PEA was exposed to tannic acid before osmication, no stain augmentation could be detected when compared with the untreated controls (Fig. 6).

The effect of tannic acid in these model experiments with PC was found to be superior when it was applied before osmication, rather than in the reverse sequence. No differences were discernible in the effects of treatments with the three different samples of tannic acid employed. However, neutralized gallic acid did not demonstrate any effect. Osmication, as well as lead staining, was a prerequisite for the increase in contrast. Unstained sections exhibited no more contrast than that nor-

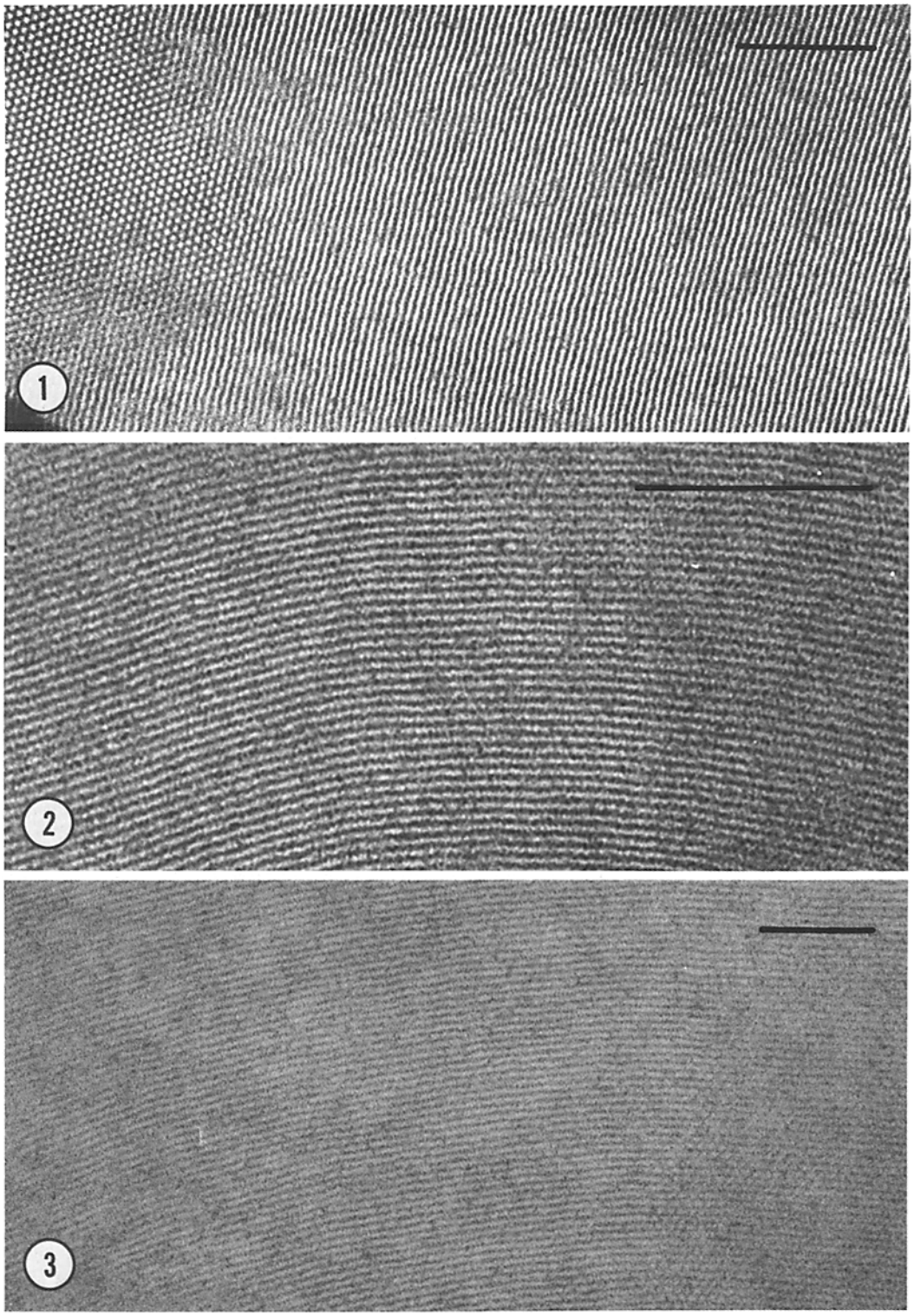

FIGURE 1 Egg PC (lecithin), suspended in water, and then treated first with tannic acid, followed by OsO_4. A sharp alternation of electron-dense hydrophilic bands with electron-lucent hydrophobic ones generally was to be seen. More complicated organizational arrangements occasionally produced highly ordered tubular arrays as may be seen at the left of this figure. Bar, 0.1 μm. × 210,000.

FIGURE 2 PC from beef, treated as the material of Fig. 1. The hydrophilic polar zones exhibit intense staining. Bar, 0.1 μm. × 360,000.

FIGURE 3 Egg PC treated only with OsO_4 before staining with lead citrate. In the absence of tannic acid mordanting, the lamellar arrays exhibit only a low contrast (compare with Figs. 1 and 2). Bar, 0.1 μm. × 180,000.

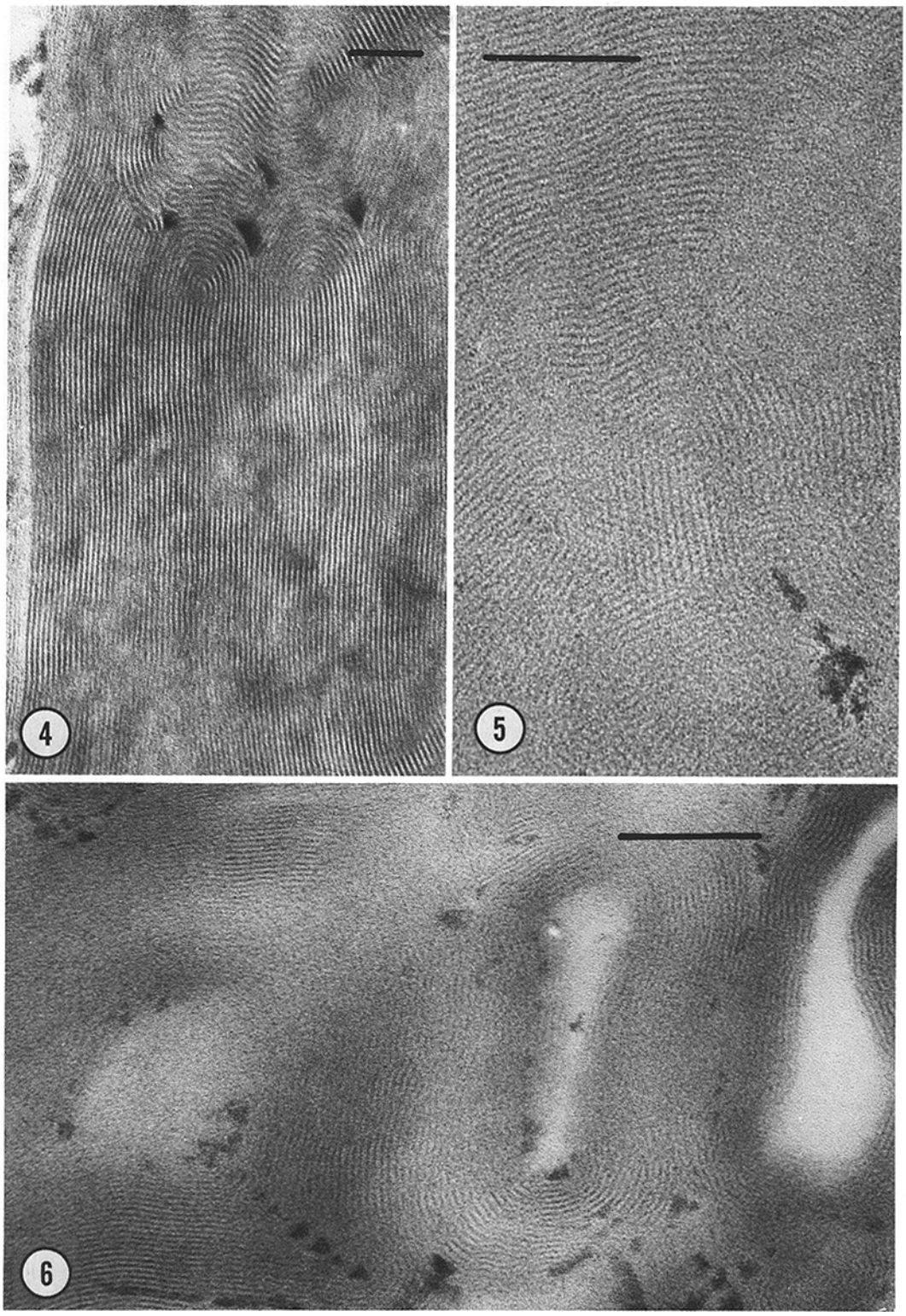

FIGURE 4 PCDO treated with tannic acid and OsO_4. Highly ordered lamellae are evident, exhibiting intense staining of the polar regions. Bar, 0.1 μm. × 120,000.

FIGURE 5 PCDO treated only with OsO_4 before lead citrate staining. The electron density and visual contrast of the lamellae are much reduced in comparison to that demonstrated in Fig. 4. Bar, 0.1 μm. × 240,000.

FIGURE 6 PEA, treated first with tannic acid, and then with OsO_4. The lamellar organization appears similar to that exhibited by the various PCs. However, the tannic acid had no effect in increasing the electron density and the contrast of the polar zones after lead citrate staining. Bar, 0.1 μm. × 220,000.

mally attributable to osmication. Uranyl acetate seemed not to have any effect.

DISCUSSION

PCs represent a major component of most mammalian membranes; often over 50% of the total phospholipid present (16, 17). In a separate study (5), using thin-layer chromatography, as well as radiolabeled choline, we have demonstrated that tannic acid is capable of interacting with the choline base of PC and SPH to form a complex. (Comparable reactions were not observed with PEA, serine, or inositol.) Evidence was presented that although the PC-tannic acid complex had a stabilizing effect on the ordered structure of PC systems, the tannic acid primarily acted as a multivalent agent capable of binding with OsO_4, which then imparted insolubility to the system as it is conventionally processed for electron microscopy. Simionescu and Simionescu (10, 11), as well as Wagner (15), have suggested specifically that tannic acid in essence serves as a mordant between osmicated structures and lead stains. Thus, the demonstrated capacity of tannic acid also to interact with choline could readily explain at least part of the increased contrast observed in osmicated and lead-stained cytomembranes after its use.

Our knowledge concerning the reactions of tannic acid with active tissue groups is limited (10, 11), and mostly it concerns substantial interaction with proteins as the major component involved in the tanning process of the leather industry (3, 4). This does suggest the possibility that the active groups of membrane proteins might also be involved in stabilizing and/or mordanting interactions with tannic acid. Thus, the membrane proteins are by no means ruled out as a contributing factor in the ultimate appearance of membranes treated with tannic acid in early preparative stages. However, the present results clearly indicate that PC contains a reactive site which also must be expected to contribute to the total effect.

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