Increased Adhesion Between Neutral Lipid Bilayers: Interbilayer Bridges Formed by Tannic Acid

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ABSTRACT Tannic acid (TA) is a naturally occurring polyphenolic compound that aggregates membranes and neutral phospholipid vesicles and precipitates many proteins. This study analyzes TA binding to lipid membranes and the ensuing aggregation. The optical density of dispersions of phosphatidylcholine (PC) vesicles increased upon the addition of TA and electron micrographs showed that TA caused the vesicles to aggregate and form stacks of tightly packed disks. Solution calorimetry showed that TA bound to PC bilayers with a molar binding enthalpy of −8.3 kcal/mol and ζ potential measurements revealed that TA imparted a small negative charge to PC vesicles. Monolayer studies showed that TA bound to PC with a dissociation constant of 1.5 μM and reduced the dipole potential by up to 250 mV. Both the increase in optical density and decrease in dipole potential produced by TA could be reversed by the addition of polyvinylpyrrolidone, a compound that chelates TA by providing H-bond acceptor groups. NMR, micropipette aspiration, and x-ray diffraction experiments showed that TA incorporated into liquid crystalline PC membranes, increasing the area per lipid molecule and decreasing the bilayer thickness by 2 to 4%. ^{2}H-NMR quadrupole splitting measurements also showed that TA associated with a PC molecule for times much less than 10^{-4} s. In gel phase bilayers, TA caused the hydrocarbon chains from apposing monolayers to fully interdigitate. X-ray diffraction measurements of both gel and liquid crystalline dispersions showed that TA, at a critical concentration of about 1 mM, reduced the fluid spacing between adjacent bilayers by 8–10 Å. These data place severe constraints on how TA can pack between adjacent bilayers and cause vesicles to adhere. We conclude that TA promotes vesicle aggregation by reducing the fluid spacing between bilayers by the formation of transient interbilayer bridges by inserting its digallic acid residues into the interfacial regions of adjacent bilayers and spanning the interbilayer space.

INTRODUCTION

Adhesion between plasma membranes or other membranes necessarily involves the close apposition of adjacent membrane surfaces. Because of the inherent structural complexity and heterogeneity of components of biological membranes, adhesion processes are often studied between phospholipid bilayer vesicles, where the magnitude and origin of the adhesion energy is understood at a fairly detailed level (Evans and Needham, 1988a,b). For electrically neutral phospholipid bilayers, the adhesion energy arises from a balance between a number of nonspecific repulsive (steric, hydration) and attractive forces (van der Waals, H-bonds) (Israelachvili, 1991; Rand and Parsegian, 1989).

A goal of our work is to find a molecule that can bind to neutral bilayers and increase the adhesion between apposing bilayers. In this paper, we investigate the properties of tannic acid (TA), a naturally occurring polyphenolic compound that is composed of a central sugar molecule substituted with five digallic acid moieties (Fig. 1). TA was chosen for our study with lipid bilayers for several reasons. First, Kalina and Pease (1977) showed that TA forms a "complex" with multilamellar vesicles of phosphatidylcholine (PC) and sphingomyelin that can be stabilized with OsO_4 so that the membranes survive the dehydration with ethanol that is conventionally used in electron microscopy. Kalina and Pease (1977) also found that the interaction of TA with other phospholipids, such as phosphatidylinositol and phosphatidylserine, is much weaker than it is with PC and sphingomyelin, but offered little explanation other than the specificity of TA for choline. Second, Schrijvers et al. (1989) found that the addition of TA causes unilamellar vesicles to aggregate and form large multilamellar vesicles, although the mechanism for this aggregation process was not explored. Third, TA aggregates or precipitates a variety of polymers (Nash et al., 1966) and proteins (Goldstein and Swain, 1965; Haslam, 1974; Oh et al., 1980). The physicochemical interactions involved in the aggregation of proteins and polymers by TA have been usually attributed to the formation of multiple hydrogen bonds between the hydrogen-bond donating phenolic hydroxyl groups on TA and hydrogen-bond accepting carbonyl groups or other hydrogen-bonding moieties (e.g., amine, amide) in proteins or polymers (Gustavson, 1956; Haslam, 1974; Nash et al., 1966; Oh et al., 1980). Hydrophobic interactions are also thought to be important in the interaction of TA with proteins (Oh et al., 1980). Although many investigations of the interaction of TA with biological macromolecules have been made

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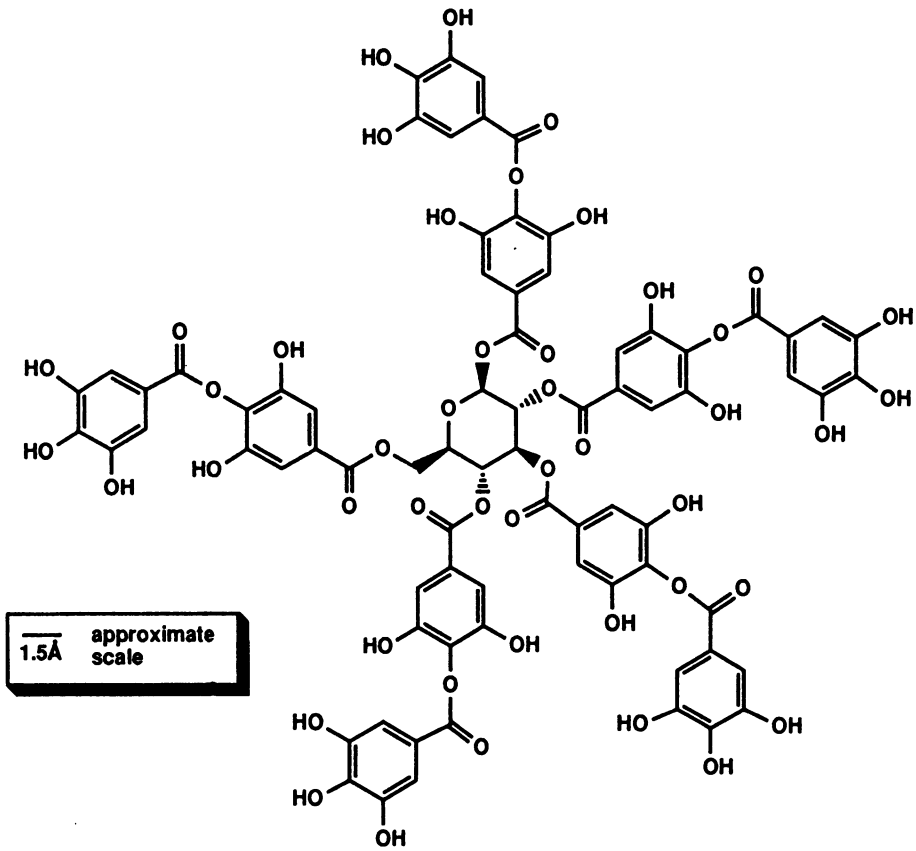

FIGURE 1 Chemical structure of tannic acid in its uncharged form.

(Hagerman, 1989; Ya et al., 1989), none of these studies has analyzed how TA modifies the underlying attractive and repulsive forces between surfaces.

Another reason to investigate the interaction of tannic acid with biological surfaces is that it may be only one example of a polyphenolic compound acting as a "molecular glue," mediating targeted attachment of derivatized molecules and surfaces. For example, it is known that polyphenolic proteins are synthesized by marine mussels to enable them to attach to surfaces (Waite, 1983) and are also effective in causing cultured cells to adhere to a variety of surfaces. (Cell-Tek, a commercial product, is produced and sold by Becton Dickinson Labware, Bedford, MA).

Our interest then is to characterize: 1) the binding of TA to lipid bilayers, 2) the location of TA in the bilayer, 3) the effect of TA on bilayer structure, and 4) the mechanism of TA-induced aggregation of lipid bilayers. In this paper we use absorbance measurements and electron microscopy to monitor TA-induced lipid aggregation, microelectrophoresis, solution calorimetry, and dipole potential measurements to assess TA-lipid binding, micropipette aspiration, NMR, and x-ray diffraction to determine changes in bilayer structure upon TA binding, and x-ray diffraction to measure the effects of TA on the fluid space between adjacent bilayers.

MATERIALS AND METHODS

Materials

Dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (EPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-stearoyl-2-oleoylphosphatidylcholine (SOPC), dimyristoylphosphatidylcholine (DMPC-d_4 which was deuterated on the α- and β-carbons in the headgroup), and DPPC-d_{62} (which had completely deuterated fatty acid chains) were purchased from Avanti Polar Lipids (Alabaster, AL). These lipids were used as purchased. Polyvinylpyrrolidone (PVP) (average molecular weight 40,000) and *N*-(2-hydroxyethyl)-piperazine-N′-(2-ethanesufonic acid (HEPES) buffer were obtained from Sigma Chemical Co. (St. Louis, MO). Salts were reagent grade. Water was passed through organic and ion exchange filters. D_2O (99.6% deuterated) obtained from Cambridge Isotope Laboratories (Woburn, MA). Unless otherwise stated, the buffer for all experiments contained either 1 mM or 5 mM HEPES adjusted to pH 7 with NaOH.

Tannic acid (pentadigalloylglucose, molecular weight 1701; Fig. 1) was purchased from Fluka Chemical Co. (Buchs, Switzerland). Its purity was checked by differential scanning calorimetry (DSC). Thermograms of TA performed at 5°C/min with a Perkin-Elmer DSC7 differential scanning calorimeter were found to have a major endothermic transition at 208°C, a temperature in good agreement with the literature value of 210°C (Weast, 1984).

Absorbance measurements

Multilamellar lipid vesicles (MLVs) were formed by dispersing lipids in buffer and then vortexing them above their phase transition temperature. Large unilamellar lipid vesicles (LUVs) were formed using the freeze-thaw high pressure extrusion method (Hope et al., 1985; Seelig and Ganz, 1991). In this freeze-thaw method, MLVs were freeze-thawed 3 to 5 times and extruded through a 0.1-μm polycarbonate filter 10–20 times.

Absorbance measurements were performed on a dual-beam Shimadzu spectrophotometer at 600 nm. Neither TA nor PC dispersions absorb appreciably at this wavelength. The temperature of the solution in the cuvette was controlled to $\pm$0.5°C by circulating heated water through the walls of the cuvette. The final lipid concentration was 0.14 mg/ml unless otherwise specified. Aliquots of stock solutions containing TA or PVP were added to the dispersion to reach the concentrations indicated in the figures.

Electron microscopy

Negative staining of LUVs of EPC was accomplished by placing a drop of the dispersion (2 mg/ml) on a carbon-formvar grid (400 mesh), blotting with filter paper, adding a drop of a 2% uranyl acetate, and blotting again with filter paper. Freeze-fractured samples of EPC LUVs were prepared as described previously (Borovyagin and Sabelnikov, 1989; McDaniel et al., 1982). Dispersions were applied to copper plates and frozen at −190°C with liquid nitrogen-cooled propane. Fracturing was done at −150°C and samples were shadowed with platinum at an angle of 45° and with carbon at an angle of 90°. The replicas were then treated with acid, washed with water, and picked up on uncoated grids. All specimens were studied with a JOEL 1200 EX electron microscope.

pH titration

Titration curves of TA were obtained by measuring the changes in pH with a Corning digital pH meter of a 1% TA solution formed in 200 ml of degassed double-distilled water in which aliquots of degassed 1.0 N NaOH were added.

Microelectrophoresis

MLVs containing TA were made by two methods. In the first method (the same as that used in the absorption, microscopy, and area expansion experiments), TA in buffer was added to preformed vesicles in the same buffer (0.1 M NaCl, 1 mM HEPES, pH 7.0). In the second method dispersions of EPC or DPPC were formed in a TA solution so that the mole ratio of PC to TA was 15/1. The dispersion was vortexed, frozen in liquid nitrogen, and lyophilized to a powder. The powder was then resuspended at 10 mg/ml in 0.1 M NaCl, 1 mM HEPES. This stock solution was diluted 100X to a final concentration of 0.1 mg/ml with the same buffer containing various concentrations of TA. This approach was taken so that the bulk TA concentration was not significantly changed by the addition of the lipid to the aqueous solution. The electrophoretic mobilities (u) of EPC multilamellar vesicles were measured at 25°C with a Rank Brothers (Cambridge, UK) Mark 2 electrophoresis unit using the cylindrical cell arrangement. The ζ potential was calculated from u using the Smoluchowski equation, $\zeta = \epsilon_0\epsilon/\eta u$, where ϵ and η are the dielectric permittivity and dynamic viscosity of the suspension (assumed equal to water), respectively, and ϵ_0 is the permittivity of free space (McLaughlin and Harary, 1976). A total of 20 measurements (10 in each direction) were made per point.

Surface potential

Measurements of surface potential (V) were made on monolayers formed by spreading 10 μl of EPC hexane/ethanol (9/1) solutions (25 mg/ml) onto a subphase containing 1 mM KCl, 2 mM HEPES, pH 7.0, or 0.1 M NaCl, 1 mM HEPES, pH 7.0. Under similar conditions, MacDonald and Simon (1987) have shown that the surface monolayer is in equilibrium with liposomes in the subphase. A thoroughly cleaned glass trough with a surface area of ~30 cm^2 was used, and a small Teflon magnetic stir bar kept the subphase stirred. The surface potential was measured between a Ag/AgCl electrode in the subphase and a polonium electrode in air which was connected to a Keithly electrometer. The reported values of V represent the differences in the potential of the subphase surface in the presence and absence of the monolayer. Changes in surface potential (ΔV) due to the presence of TA and PVP were obtained by injecting into the subphase under the monolayer aliquots of concentrated solutions of these molecules that had the same salt composition and pH as the subphase.

Solution calorimetry

LUVs were formed as described above. Heats of reaction for the binding of TA to EPC large unilamellar vesicles were measured with a Microcal Omega titration calorimeter (Microcal, Northampton, MA) interfaced to a computer with software supplied by Microcal. 6.6 mM suspensions of EPC LUVs were titrated with multiple injections into the sample chamber containing 12.5 or 25 mM TA. Titrations were performed at temperatures between 22°C and 25°C. Corrections were always made for the heat of dilution of the TA into the buffer.

Nuclear magnetic resonance

^{31}P-NMR anisotropies were measured on EPC samples in the presence and absence of TA. In the absence of TA, 1 ml of 5 mM NaCl, pH 6.7, was added to 50 mg EPC. For the samples containing TA, ~1 ml of 5 mM NaCl, 6 mM TA at pH 6.7 was added to 50 mg EPC to yield a EPC/TA molar ratio of 10/1. The samples were vortexed, sonicated, frozen, and lyophilized into a powder. The lyophilized lipids were then placed in 8-mm glass tubes to which D_2O at equal weight to the lipid was added. After sealing the glass tube, the sample contents were homogenized by backward and forward centrifugation. ^{2}H spectra were obtained using deuterated lipid samples with deuterium-depleted water. All NMR experiments were performed on a Bruker MSL-300 spectrometer (Karlsruhe, Germany) using a high power probe with an 8-mm doubly tuned solenoidal sample coil. For EPC MLVs, all ^{31}P-NMR spectra were obtained at 22°C at a resonance frequency of 121.513 MHz with a phase cycled Hahn echo using a gated broadband decoupling of protons (Rance and Byrd, 1983). A delay time of 100 μs between the 90° and 180° pulse was chosen (Davis, 1983). A total of 1024 scans with a recycle delay time of 1 s were accumulated.

Quadrupole splitting measurements were performed at 31°C on deuterated-DMPC-d_4 MLVs and at 50°C for DPPC-d_{62} MLVs. At these temperatures, the lipids are in their liquid crystalline phase. The quadrupolar echo sequence {(90)x-τ-(90)y-echo} was used with τ = 100 μs and a recycle time of 0.5 s. The resulting spectra were then dePaked following the methods of Sternin et al. (1983). Peak assignments were made by integrating the dePaked spectra over all methylene resonances except for the methyl resonances. The integral was divided into 14 intensity steps. The frequencies of the intensity steps were taken as the quadrupolar splitting of the methylene groups. The quadrupolar splitting of methyl resonances were taken from their peak positions. Although the assignment of a specific resonance to a particular methylene group has its difficulties, this method gives the general shape of the order parameter profile (Davis, 1983).

^{13}C-NMR spectra of TA were obtained at a resonance frequency of 150.9 MHz in solutions containing 10 mM TA in 10% D_20 in H_2O (v/v) at pH values of 2.84 and 6.93 adjusted with NaOH.

Micropipette measurements of tannic acid adsorption

For micropipette experiments, giant (30–40 μm) unilamellar vesicles were made by gentle rehydration, with 180 mOsm sucrose, from dried lipid films deposited on a Teflon substrate (Needham and Nunn, 1990). Sucrose solution-containing lipid vesicles were resuspended in equiosmotic (180 mOsm) glucose solution in one-half of a double microchamber on the interference contrast microscope stage. The refractive index difference and density difference between these two solutions made for better visualization and ease of capture of single-walled vesicles. Micropipettes were made from 0.75 mm internal diameter capillary glass tubing, pulled to a fine point by heated pipette puller, and broken to the desired flat tip diameter (9 μm) by quick fracture using a microforge. Pipettes were filled with equiosmotic NaCl solution, connected to a water-filled manometer system, and mounted in a deFonbrune micromanipulator (Research Instruments Inc., Durham, NC) operated by a joystick (Research Instruments).

Individual single-walled lipid vesicles were selected from the glucose suspension. Each vesicle was prestressed in the glucose chamber to test for unilamellarity and to support all the excess area in the micropipette. A base suction pressure of 5000 dyn/cm^2 was then applied to the pipette so that the vesicle was held in the pipette at a low but significant membrane tension (~2 dyn/cm). The vesicle was then transferred across the air gap between the two chambers into the test TA solutions (0–0.1 mM TA in equiosmotic (180 mOsm) NaCl), by using a larger (100 μm) transfer pipette (Needham, 1992). With the measuring pipette and aspirated vesicle in focused view, the transfer pipette was pulled back, exposing the test vesicle to TA solution in

as short a time as possible (0.2 s). Once exposed to the new bathing medium, the change in projection length of the vesicle membrane in the pipette was recorded on videotape. Equiosmotic solutions were used to keep vesicle volume constant, so that any change in the projection length of the vesicle in the micropipette upon exposure to the TA solution was due to lipid bilayer area change. In this manner small changes in membrane area could be readily measured from pipette and vesicle geometry (Needham and Nunn, 1990) because the micropipette acts as a very sensitive transducer of area change. Time and micropipette suction pressure were also recorded on the videotape image by videomultiplexing.

The experiments were carried out at a temperature of 15°C, just above the dew point, so that evaporation losses were kept to a minimum. The relatively rapid area changes due to exposure to TA were therefore not influenced by the changes in volume that can occur if evaporation losses concentrate the bathing solutions. Measurements of the osmolality of the suspending and bathing solutions at the end of several transfer experiments showed that the osmolality had only changed by 5% (180 mOsm to 190 mOsm) over a typical experimental period of 3 h.

X-ray diffraction

Lipid-TA dispersions were formed above their phase transition temperature in solutions of 0.1 M NaCl, 5 mM HEPES, pH 7.0, in the presence and absence of TA. EPC dispersions were formed at concentrations of 1 mg/10 ml, whereas DPPC dispersions were formed in 70% by weight buffer. The dispersions were vortexed periodically above the phase transition temperature of the lipid and then centrifuged. The loose pellets were sealed in a quartz glass x-ray capillary tube and mounted in a point collimation x-ray camera. For all specimens, x-ray diffraction patterns were recorded at 20°C on Kodak DEF x-ray film. X-ray films were processed by standard techniques and densitometered with a Joyce-Loebl microdensitometer as described previously (McIntosh and Holloway, 1987; McIntosh and Simon, 1986b). After background subtraction, integrated intensities, $I(h)$, were obtained for each order h by measuring the area under each diffraction peak. For these unoriented patterns, the structure amplitude $F(h)$ was set equal to ${}^{2}I(h)^{1/2}$ (Blaurock and Worthington, 1966; Herbette et al., 1977). Electron density profiles, $\rho(x)$, on a relative electron density scale were calculated from

$$\rho(x) = \frac{2}{d}\sum \exp\{i\phi(h)\} \cdot F(h) \cdot \cos\left(\frac{2\pi x h}{d}\right) \quad (1)$$

where x is the distance from the center of the bilayer, d is the lamellar repeat period, $\phi(h)$ is the phase angle for order h, and the sum is over h. Phase angles were determined by the use of the sampling theorem (Shannon, 1949) as described in detail previously (McIntosh and Holloway, 1987). All electron density profiles described in this paper are at a resolution of $d/2h\text{max}$ = 6–8 Å.

RESULTS

Absorbance measurements

Fig. 2 shows that the addition of 0.2 mM TA to aqueous EPC dispersions containing either MLVs or LUVs increased the optical densities of the solutions. Also shown is that the TA-induced increase in absorbance of both MLVs and LUVs was partially reversed by the addition of PVP, a polymer that precipitates TA by having a H-bond acceptor for the H-bond donating phenolic groups on TA (Nash et al., 1966; Oh et al., 1980).

Electron microscopy

Electron micrographs of negatively stained LUVs of EPC formed in the absence of TA contained unilamellar vesicles with diameters of about 0.1 μm as well as small aggregates containing two and three vesicles (Fig. 3 *A*). About 10 min after the addition of 0.2 mM TA, larger aggregates containing many vesicles were commonly seen (Fig. 3, *B* and *C*). After longer times (≈30 min) in 0.2 mM TA, the unilamellar vesicles adhered and produced flattened disks of approximately uniform diameter but of variable widths, as shown by images of both negatively stained preparations (Fig. 4, *A* and *B*) and freeze-fracture replicas (Fig. 4, *C* and *D*). That is, the vesicles lost fluid from their internal compartments.

pH titration and electrophoresis

Dissolving 1% TA in water lowered its pH to near 3. The pH of a 1% TA solution as a function of added 1 N NaOH had two pKas, one at pH 3.2 and the other at 8.7. These results are in general agreement with values for the pKas of TA (Gustavson, 1956). Gustavson proposed that the strongly acidic group arises from some of the digallic acid residues that are bonded to the glucose through phenolic groups, rather than through the usual carboxyl linkages, thus leaving available titratable carboxyl groups (Gustavson, 1956). We have obtained information consistent with this interpretation by measuring the ^{13}C-NMR spectrum of TA at two different pH values. A carboxyl group was identified and its chemical shift changed from 182.3 to 179.3 ppm at pH 6.93 and 2.84,

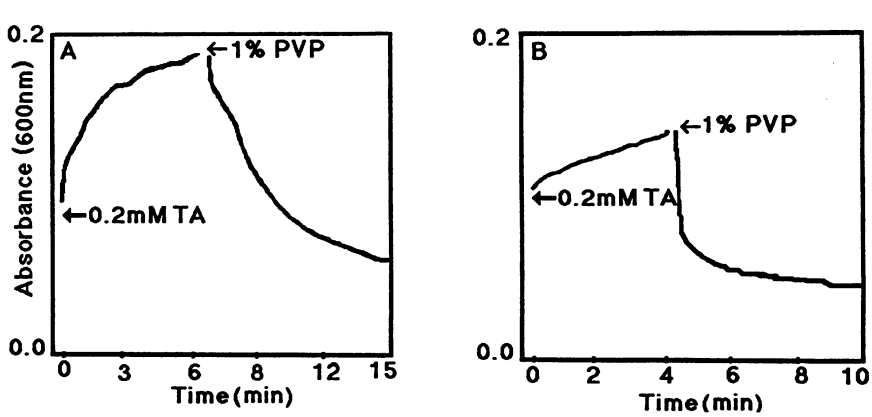

FIGURE 2 Changes in absorbance at 600 nm of MLV (*A*) and LUV (*B*) dispersions of EPC (0.14 mg/ml). At time zero min, 0.2 mM tannic acid in the same buffer (pH 7.0) was added to the cuvette. MLVs and LUVs were dispersed in buffer and buffer plus 60 mM NaCl, respectively. The increases in absorbance at early times could not be recorded because the TA was being mechanically mixed with the dispersion. After steady state was achieved, solutions of PVP were added to final concentrations of 1% (break in trace represents mixing time). The reference cuvette contained the same dispersion as the sample cuvette without TA or PVP.

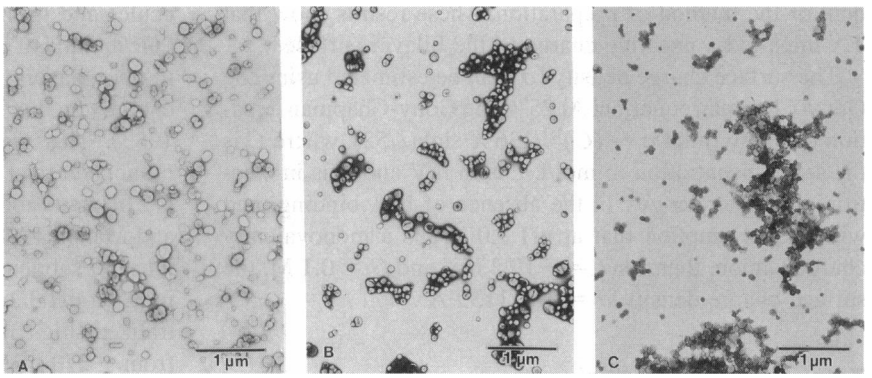

FIGURE 3 Micrographs of negatively stained large unilamellar vesicles of EPC in the absence (*A*) and in the presence (*B* and *C*) of 0.1 mM tannic acid. In the absence of TA, individual vesicles and small clusters of vesicles are observed. The addition of TA causes the formation of larger aggregates. Scale bar = 1 μm.

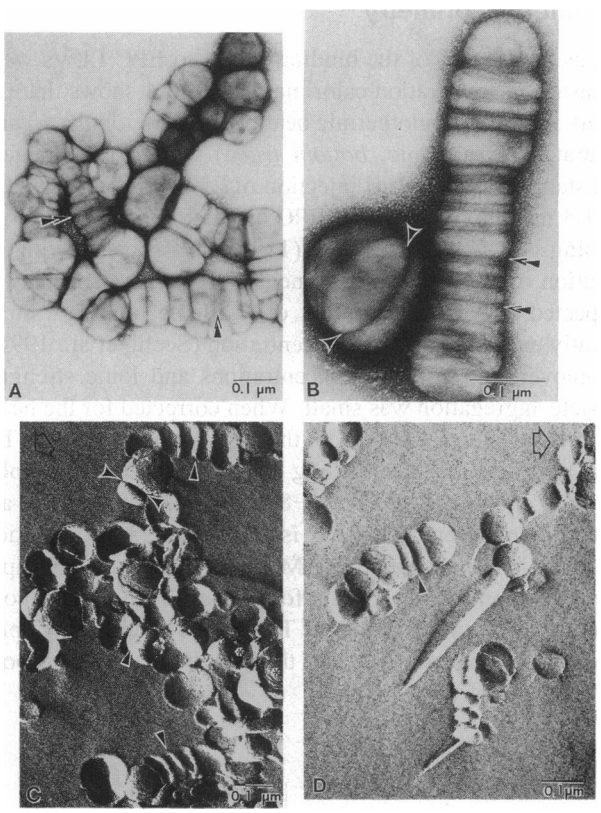

FIGURE 4 Images of negative stain (*A* and *B*) and freeze-fracture (*C* and *D*) preparations of aggregates produced by the addition of 0.1 mM tannic acid to EPC large unilamellar vesicles. The double arrowheads in *A* and *B* correspond to regions where spherical vesicles, originally ≈0.1 μm in diameter, flattened into intact disks of thickness of ≈0.01 μm. Single arrowheads correspond to regions where two intact vesicles are in contact. The large arrows in *C* and *D* refer to the direction of shadow. Scale bar = 1 μm.

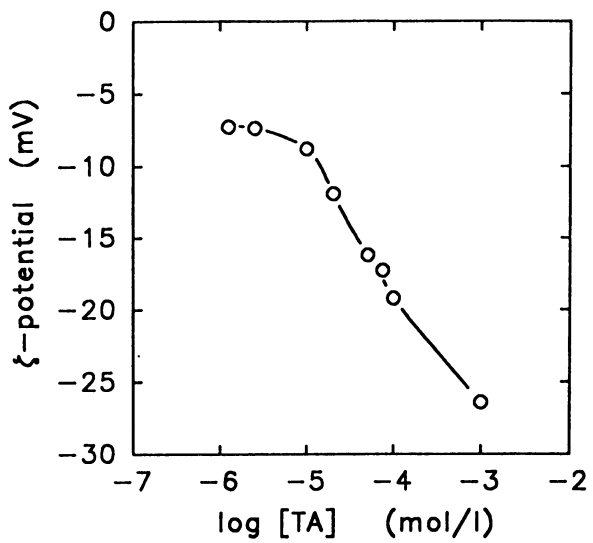

FIGURE 5 Measurements of ζ potential of MLVs of EPC as a function of TA concentration in 0.1 M NaCl, 1 mM HEPES at pH 7.0.

respectively (data not shown). The more basic moiety likely corresponds to the titration of hydroxyl groups which for polyhydroxylated compounds like 1,3,5-trihydroxybenzene have pKas in the range of 8–9 (Dean, 1987). These data demonstrate that at pH 7.0, where virtually all subsequent experiments were performed, TA is negatively charged.

ζ potentials of MLVs formed in 0.1 M NaCl at pH 7 were near 0 mV for EPC and −2 ± 2 mV for 15/1 EPC/TA. Fig. 5 shows the ζ potential (ζ) of 15/1 EPC/TA MLVs as a function of TA concentration in the buffer. For these experiments the MLVs were selected to be approximately the same size. During the addition of TA to the dispersions the vesicles aggregated to various extents depending on the TA and NaCl concentrations. When this occurred the preparation was briefly sonicated in a bath sonicator. The ζ potential increased with increasing TA concentration. Lowering the salt concentration at a fixed TA concentration also increased the ζ potential as would be expected from conventional double-layer theory (Hunter, 1986). For MLVs of EPC/TA (15/1) in 0.1 mM TA at pH 7.0, the ζ potentials were: −19.2 ± 1.4, −24.7 ± 3.4, −37.8 ± 3.5, −34.4 ± 2.1, and −37.7 ± 2.5 mV in the presence of 0.1, 0.05, 0.02, 0.01, and 0.005 M NaCl, respectively.

For experiments in which TA was added to the aqueous phase of EPC liposomes formed in 0.1 M NaCl at pH 7.0, the ζ potential was relatively constant upon the addition of TA to the the buffer, being −19.5 ± 1.4 mV at 1.4 μM TA and −22.7 ± 1.7 mV at 0.1 mM TA. Although the ζ potentials were more negative at lower TA concentrations for liposomes formed in buffer than for liposomes preformed in TA at a 15/1 mole ratio at high TA concentrations, the ζ potentials were approximately the same and hence indepen-

dent of the method of preparation. These results show that TA imparted a negative charge to the bilayer surface.

The surface charge density (σ) can be estimated using the Gouy-Chapman equation. At 25°C the Gouy-Chapman equation can be written $\sigma \approx (C)^{1/2}/136\, X\cdot\sinh(\zeta/52)$, where C is the salt concentration in mol/l, ζ is in mV and σ is in electronic charges per Å^2. In the absence of Na^+ binding, and with the assumption that at pH 7.0 TA is a monovalently charged anion, then for $\zeta = -19.2$ mV and $C = 0.1$ M, the surface charge density $\sigma = 1e^-/1139$ Å^2.

Total surface potential

Fig. 6 shows a measurement of the total surface potential (V) of an EPC monolayer before and after the injection of 0.1 mM TA into the subphase. The injection of TA reduced V by over 200 mV. Moreover, the reduction in surface potential was partially reversed by injection into the subphase of PVP.

As shown in Fig. 7, injections of various concentrations of TA into subphases containing 5 mM KCl, 10 mM HEPES at pH 7.0 reduced the surface potential of both EPC (liquid crystalline) and DPPC (gel) monolayers. The magnitude and concentration dependence of the responses of fluid and gel phase monolayers to TA were similar in shape. For all TA concentrations, the change in V was greater for EPC than for DPPC. For EPC, ΔV increased smoothly with TA concentration so that the data could be fit to a Langmuir type isotherm: $-\Delta V = -\Delta V_{max}\, TA/Ka_{1/2} + TA$, where $Ka_{1/2} = 1.5$ μM and $\Delta V_{max} = -270$ mV. For DPPC monolayers, the dipole potential data could not be fit in a straightforward way to a Langmuir isotherm, but it is nevertheless clear that the TA concentration where TA has half of its maximal value (≈0.1 mM) is about 100 times higher than $Ka_{1/2}$ for the liquid crystalline EPC monolayer.

The total surface potential arises from contributions of both oriented dipoles of the lipid, solvent, and solute molecules and from fixed or absorbed charges (Hunter, 1986). One method of determining the contribution of fixed charges in monolayers containing TA is to neutralize the fixed charges on TA by lowering the pH (Lakhdar-Ghazal et al., 1983). This was partially achieved by forming the EPC monolayer over a bulk phase of 5 mM KCl at pH 3.2, which is 2 pH units above the pKa of the PC phosphate group (Cevc and Marsh, 1988) and injecting TA in 5 mM KCl at pH 3.2 into the subphase to achieve a final TA concentration of 1 mM. At pH 3.2, much of the negative charge on TA is neutralized (Fig. 2). This operation increased ΔV only 20 mV from −270 mV at pH 7.4 to −250 mV at pH 3.2.

Solution calorimetry

Heats of reaction of the binding of TA to EPC LUVs were measured with titration calorimetry. Fig. 8 *A* shows that the heats adsorbed (endothermic heat of dilution, *top trace*) and liberated (exothermic, *bottom trace*) were approximately constant for each 0.2 μl injection of 25 mM TA (≈3.9 μM in 1.4 ml) into the 6.6 mM EPC dispersion in a buffer containing 1 mM NaCl at pH 7.0. (The heats associated with the dilution of the vesicles were negligible.) This constancy is expected if the concentration of available binding "sites" greatly exceeded the TA concentration (Seelig et al., 1991). Moreover, at these TA concentrations and ionic strength, vesicle aggregation was small. When corrected for the heats of dilution (Fig. 8 *B*) and with the assumption that all the TA was bound to the lipid (Seelig and Ganz, 1991), the molar binding enthalpy (ΔH) was −8.3 ± 0.4 kcal/mol. The assumption of complete binding is a reasonable approximation given that $Ka_{1/2}$ was ≈1.5 μM (Fig. 7) and that the lipid concentration was a thousandfold greater than the TA concentration. Even though all the TA may be bound to the lipid, it does not necessarily mean that TA binds lipids in both monolayers of the bilayer.

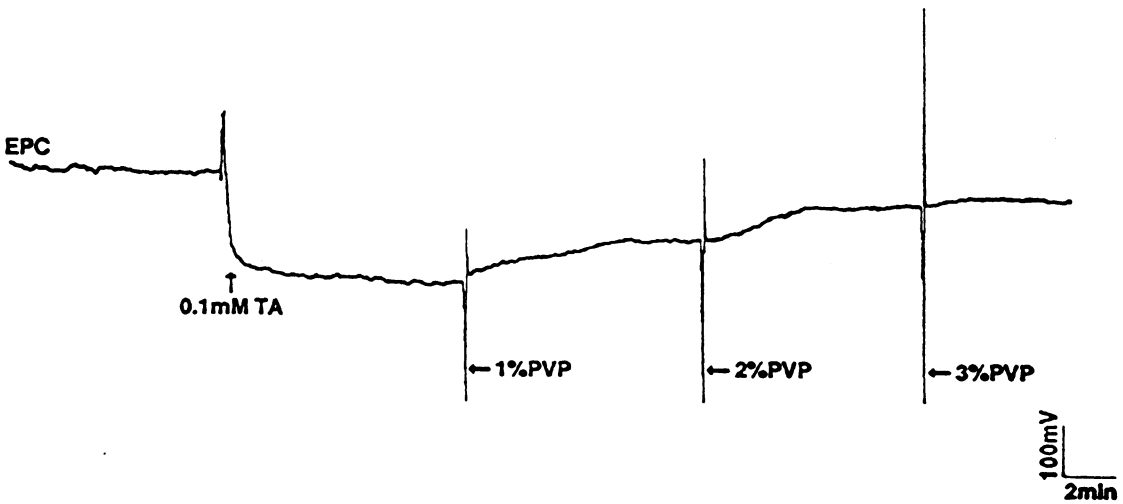

FIGURE 6 Decrease in surface potential (V) of an EPC monolayer produced by injection of 0.1 mM TA into a stirred subphase. Injections of PVP into the subphase partially reversed the decrease in surface potential.

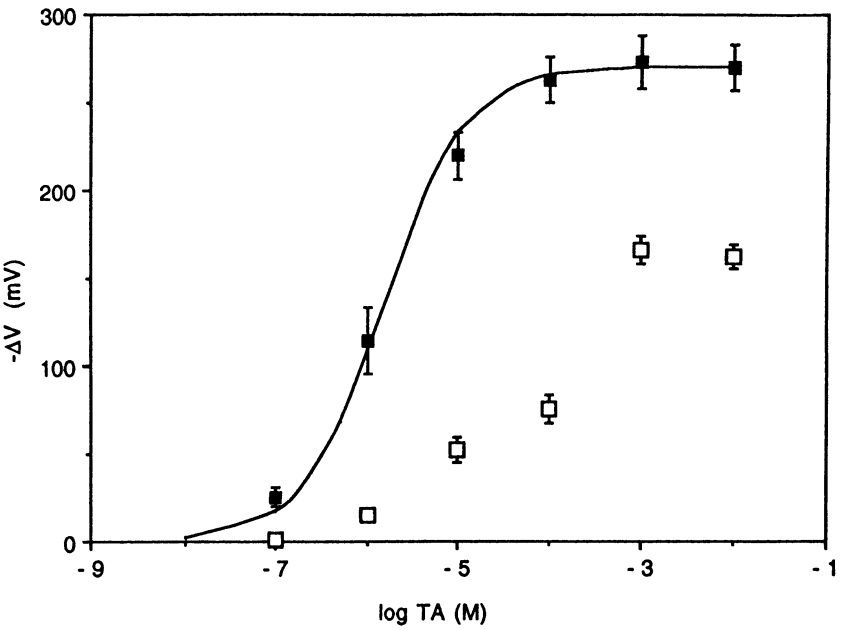

FIGURE 7 Change in surface potential ($-\Delta V$) of monolayers of EPC and DPPC induced by injection of tannic acid into the subphase. The solid line for the EPC data is a fit to a Langmuir type isotherm with $Ka_{1/2} = 1.5\ \mu M$ and $-\Delta V_{max} = 270$ mV. These data are the means of at least five experiments.

Nuclear magnetic resonance

^{31}P-NMR

The ^{31}P chemical shielding anisotropy of fully hydrated EPC MLVs in 50 wt % H_2O remained unchanged at -46 ± 1 ppm upon the addition of TA up to mole ratios of EPC/TA of 10/1 (data not shown).

^{2}H-NMR

Fig. 9 *A* shows one-half of spectra of DPPC-d_{62} (*a*), 120/1 DPPC-d_{62}/TA (*b*), 60/1 DPPC-d_{62}/TA (*c*), 30/1 DPPC-d_{62}/TA (*d*), and 15/1 DPPC-d_{62}/TA (*e*). The appearance of these spectra are the result of quadrupolar splitting ($\delta\nu$) of the deuterated methylene and methyl groups where $\delta\nu = 3e^2Q/4h\ |S_{CD}|$, $e^2Q/h = 167$ kHz, and $|S_{CD}|$ defines the order parameter of the C-D bond vector (Davis, 1983). The overall square shape of the profile provides evidence of a well defined order parameter plateau (Davis, 1983). The large peak at 2.5 kHz reflects the fluid terminal methyl groups. The multiple small peaks between 5 and 13 kHz reflect the deuterated methylenes of the acyl chains, with the peaks with the higher frequencies representing the methylenes closest to the hydrocarbon water interface. The addition of TA markedly reduced the width of the spectrum, indicating that TA disordered the bilayer and decreased the average acyl chain conformational order parameter (Seelig and Seelig, 1980; Sternin et al., 1983). To obtain additional information regarding the interaction of TA with the acyl chains, spectra were dePaked (Fig. 9 *B*) and individual order parameters calculated. Fig. 10 *A* shows the order parameter profile for DPPC at 50°C by itself (*open circles*) and with TA at a mole ratio of 15/1 (*solid circles*). TA decreased the quadrupolar splitting for all carbon positions. In both cases the splitting remained relatively constant until carbon 8, whereupon they rapidly decreased toward the terminal methyl groups. Plots of the ratio of order parameters S_{TA}/S_{DPPC} for DPPC/TA at 60/1 and 15/1 are shown in Fig. 10 *B*. TA produced two major changes. First, TA reduced all order parameters in a concentration-dependent manner. Second, after TA addition, the changes in order parameters were relatively constant up to carbon 8, whereupon they rapidly increased toward the terminal group.

The decrease in spectral width reflects a decrease in bilayer thickness that can be calculated by determining the average order parameters, $\langle S_{CD} \rangle$, of the spectra. The hydrophobic thickness (d_h) can be expressed as $d_h = d_{max}\ (\alpha \langle S_{CD} \rangle + \beta)$, where d_{max} is the maximum hydrophobic thickness (19.7 Å for DPPC) and $0.5\ \alpha + \beta = 1$ (Ipsen et al., 1990). [See Nagle (1993) for detailed discussion of this method.] For DPPC, d_h was 25.8 Å and was 25.7 Å, 25.5 Å, 25.3 Å, and 25.2 Å for DPPC/TA mole ratios of 120/1, 60/1, 30/1, and 15/1, respectively. That is, at the highest concentration tested, TA reduced the hydrophobic thickness about 0.6 Å or by 2.3%.

The ^{2}H-NMR spectra of DMPC-d_4 taken at 31°C in the absence of TA and at a DPPC/TA ratio of 15:1 revealed that TA caused an increase in the splitting of the deuteron on the α-carbon from 6.3 kHz to 6.5 kHz and a decrease in the splitting of the deuteron on the β-carbon from 5.8 to 5.1 kHz (data not shown).

In all NMR experiments, changes of the order parameters were proportional to the TA concentration, meaning that there was rapid exchange between lipids associated with TA and free lipids. A lower bound of the TA-PC complex can be estimated from the difference (Δ) in the magnitude of the quadrupolar splitting of PC in the presence and absence of

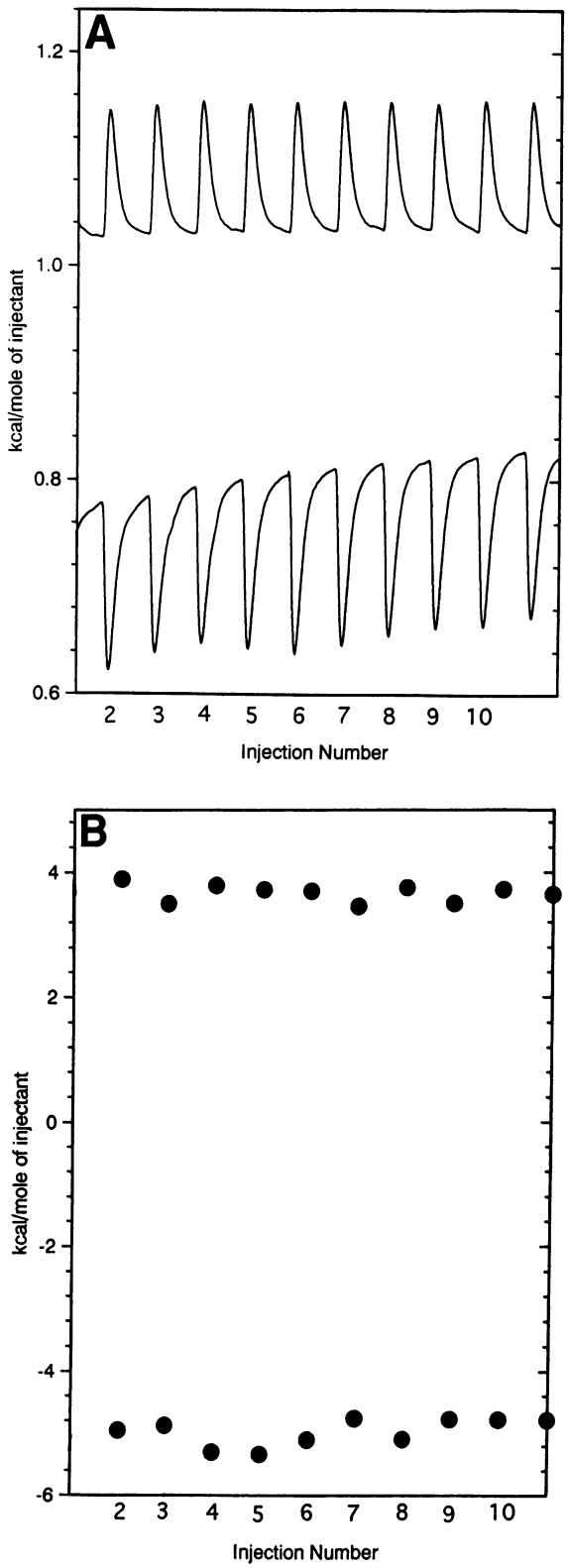

FIGURE 8 (*A*) Heats of solution and binding of tannic acid to EPC large unilamellar vesicles. Injections of 0.2 μl of 25 mM tannic acid in 1 mM NaCl, 5 mM HEPES, pH 7.0 into 1 mM NaCl, 5 mM HEPES, adjusted to pH 7.0 yield endothermic transitions (*top trace*). Injections of 0.2 μl of 25 mM tannic acid (in 1 mM NaCl, 5 mM HEPES, pH 7.0) into 6.6 mM EPC in 1 mM NaCl, 5 mM HEPES, pH 7.0 yield exothermic transitions (*bottom trace*). (*B*) Heats of solution (*top*) and binding (*bottom*) of TA calculated from the data presented in *A*.

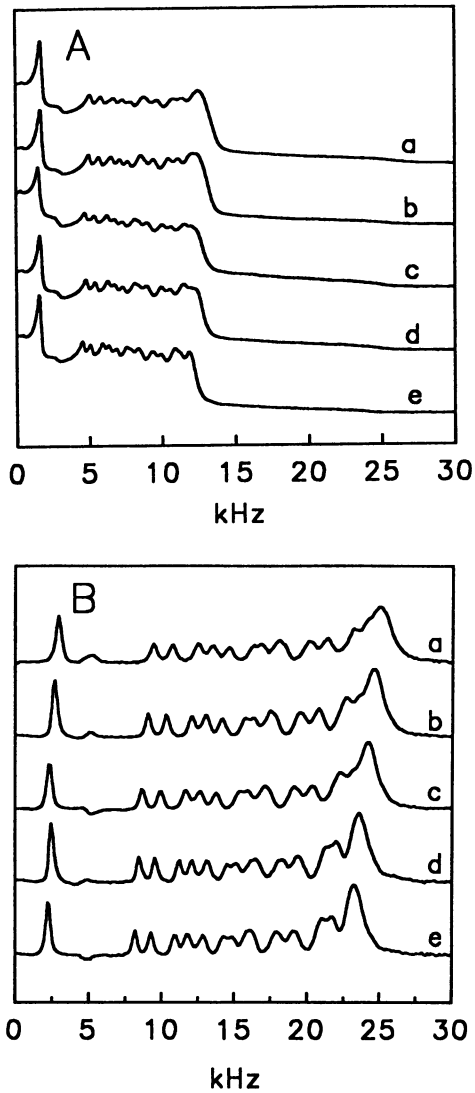

FIGURE 9 (*A*) Right-hand side of ^{2}H-NMR powder spectra of DPPC-d_{62} at 50°C as a function of TA concentration. (*a*) DPPC; (*b*) 120/1 DPPC/TA; (*c*) 60/1 DPPC/TA; (*d*) 30/1 DPPC/TA; (*e*) 15/1 DPPC/TA. (*B*) dePaked spectra of the data shown in *A*.

TA ($\Delta \approx 1$ kHz; Figs. 9 and 10). Two sets of quadrupolar splitting would be observed if the lifetime of the complex (τ) $\frac{1}{2}\pi\Delta \approx 10^{-4}$ s. For $\tau \approx 10^{-4}$ s, spectra would be broadened by medium speed exchange and for $\tau \ll 10^{-4}$ s, well resolved spectra with average quadrupolar splitting would be observed. Thus, in the case of TA-PC complexes, the lifetime of the complex was much less than 10^{-4} s.

Micropipette measurements of adsorption of tannic acid to PC vesicles

The relative change in vesicle area versus time after exposure of vesicles in 180 mOsm NaCl to various concentrations of TA (0, 1.5 μM, 10 μM, 50 μM, and 1 mM, all in 180 mOsm NaCl) is presented in Fig. 11. In the absence of TA, there was no perceivable change in projection length over a period of 10 min, as expected from the constant osmolarity condition.

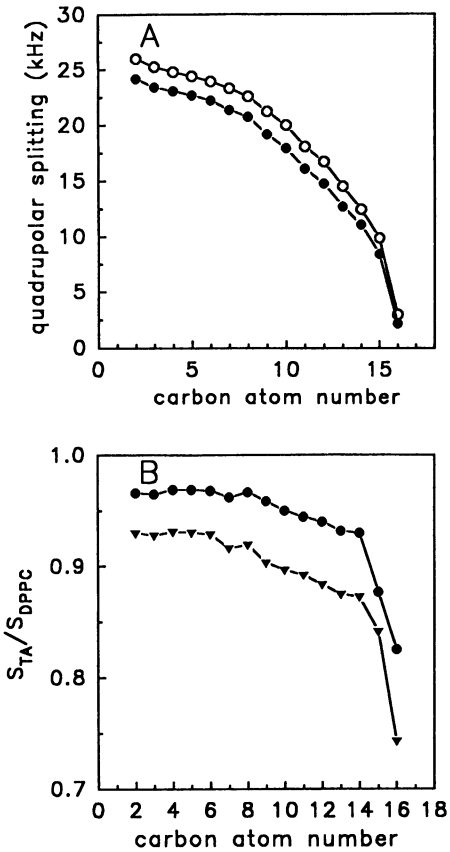

FIGURE 10 (*A*) Quadrupolar splitting of methylene groups along the DPPC-d_{62} acyl chains as derived from the dePaked spectra. ○, DPPC; ●, 15/1 DPPC/TA. (*B*) Order parameter ratios, S_{TA}/S_{DPPC}, at 60/1 DPPC/TA (●) and 15/1 DPPC/TA (▲).

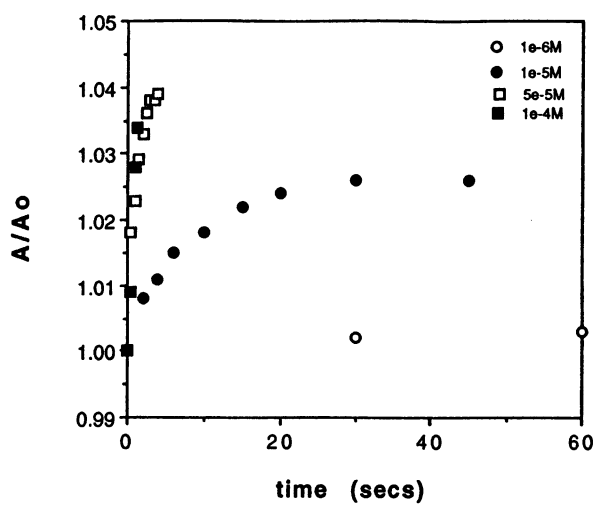

FIGURE 11 Relative change in vesicle area upon (A/A_0) transfer into equiosmolar TA solutions.

Transfer into 1 μM TA gave a very slow rate of change that did not come to a perceived equilibrium when monitored over a time period of 5 min. In 10 μM TA, equilibrium adsorption was observed as vesicle area expansion reached saturation in about 20 s. The equilibrium area increase in 1 μM TA was 2.6 ± 0.6% (mean ± SD). At the higher concentrations of 50 μM and 0.1 mM TA vesicles showed a very rapid expansion and broke at average critical area changes of 3.2 ± 0.8% and 2.5 ± 0.3%, respectively. The rate of adsorption, taken to be $\partial(A/A_0)/\partial t$, increased linearly with TA concentration, showing that the absorption was diffusion-limited.

X-ray diffraction

For each specimen of either DPPC or EPC in the presence or absence of TA, the x-ray diffraction pattern consisted of a series of low-angle reflections, which indexed as orders of a lamellar repeat period, and one or two wide-angle bands. For DPPC suspensions in the absence of TA, the lamellar repeat period was 63.6 Å and the wide-angle pattern contained a sharp reflection at 4.2 Å and a broad band at 4.08 Å. Such patterns are typical of lipids in the gel (Lβ') phase (Tardieu et al., 1973). The addition of 10 mM TA decreased the lamellar repeat period 44.0 Å and changed the wide-angle pattern to a single sharp reflection at 4.09 Å. Such patterns are typical of gel phase bilayers with interdigitated hydrocarbon chains (Ranck et al., 1977; Simon and McIntosh, 1984).

For EPC suspensions in the presence or absence of TA, the wide-angle band was quite broad and centered at 4.5 Å, typical of bilayers in the liquid crystalline phase (Tardieu et al., 1973). The lamellar repeat period of the EPC suspensions plotted against bulk TA concentration is given in Fig. 12. The repeat period was 63.2 Å in absence of TA and decreased slightly to about 61 Å at 10^{-4} M TA. Increasing the TA concentration to 6×10^{-4} M produced a pattern with two repeat periods, at 61 Å and 51.4 Å. Higher TA concen-

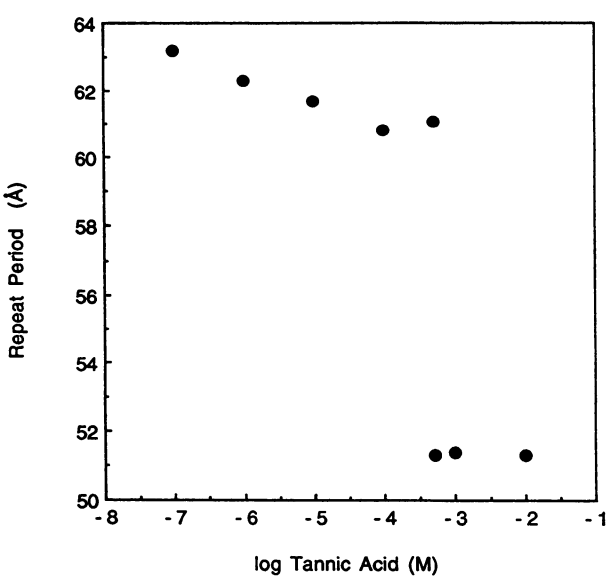

FIGURE 12 Repeat period of egg phosphatidylcholine liposomes plotted versus TA concentration.

trations gave patterns with a single repeat period at 51.4 Å. No intermediate repeat periods were observed between 61 Å and 51.4 Å.

Electron density profiles for DPPC and EPC bilayers in the presence and absence of TA are shown in Figs. 13 and 14, respectively. For each profile the geometric center of the bilayer is placed at the origin so that the low density region in the center of the profile corresponds to the lipid hydrocarbon chains and the high density peaks on either side correspond to the lipid headgroups. For DPPC in the Lβ' phase in the absence of TA (Fig. 13, *top*) the distance between headgroup peaks across the bilayer was 42 Å, as shown previously (McIntosh and Simon, 1986b). The middle electron density profile in Fig. 14 shows a DPPC in the presence of 10 mM TA. This profile differed from the top profile in several significant aspects. First, the distance between headgroup peaks across the bilayer was reduced from 42 Å to 30 Å, indicating that the width of the bilayer was markedly reduced by the incorporation of TA. Second, the shape of the hydrocarbon region in the center of the bilayer was modified by the addition of TA. For DPPC in the absence of TA (Fig. 13, *top*) there was a sharp trough in the geometric center of the bilayer, corresponding to the localization of the terminal methyl groups in the center of the bilayer. For DPPC in 10 mM TA, there was no terminal methyl trough. The shape of this profile is consistent with that previously found for bilayers, where the hydrocarbon chains from apposing monolayer interpenetrate or interdigitate (Simon and McIntosh, 1984). That is, TA, like several other amphipathic molecules (McDaniel et al., 1983; Simon and McIntosh, 1984; Simon et al., 1988), caused the hydrocarbon chains from apposing bilayers to interdigitate. For comparison, the bottom profile shows the interdigitated phase of DPPC in the presence of 1,2,3-heptanetriol. Although the bilayer portions of the profiles were similar for DPPC with TA or 1,2,3-heptanetriol, the fluid spaces between adjacent bilayers were significantly smaller for the DPPC bilayers in the presence of TA than in buffer (*top profile*) or in the presence of 1,2,3-heptanetriol (*bottom profile*). This implies that the reduction of the fluid space caused by TA is not due to the interdigitation of the lipid hydrocarbon chains in the bilayer.

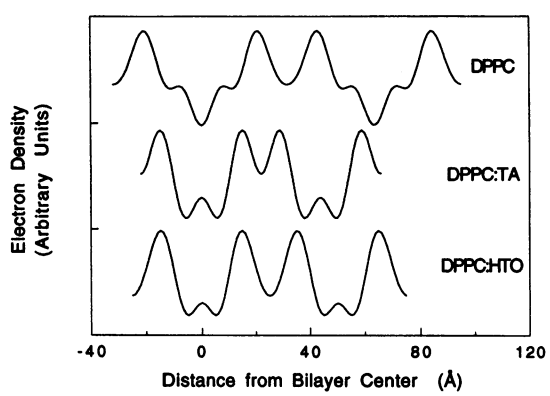

FIGURE 13 Electron density profiles for DPPC in water (*top profile*), DPPC in 10 mM TA (*middle profile*), and DPPC with 1,2,3-heptanetriol (*bottom profile*).

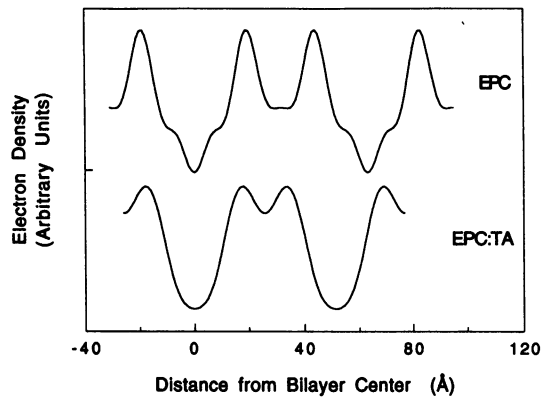

FIGURE 14 Electron density profiles for EPC in water (*top profile*) and EPC in 0.1 mM mM TA (*bottom profile*).

Fig. 14 shows that TA had four major effects on the electron density profile of EPC: 1) the distance between headgroup peaks across the bilayer was reduced by about 2 Å from 38 Å to 36 Å; 2) the terminal methyl trough in the center of each bilayer was broadened; 3) the distance between headgroups across the fluid space between adjacent bilayers was reduced; and 4) the electron density in the interbilayer fluid space was increased.

These profiles can be used to estimate the fluid spacing between adjacent bilayers (McIntosh et al., 1986b). For profiles of DPPC and EPC bilayers at this resolution ($d/2h_{max} \approx$ 6–8 Å), the high electron density headgroup peaks are located between the phosphate moiety and the glycerol backbone (Hitchcock et al., 1974; Lesslauer et al., 1972). As noted previously (Hitchcock et al., 1974; Lesslauer et al., 1972; McIntosh et al., 1989a, 1987; McIntosh and Simon, 1986a), the definition of the lipid/water interface is somewhat arbitrary, because the bilayer surface is not smooth, the lipid headgroups are mobile (Hauser et al., 1981), and water penetrates into the headgroup region of the bilayer (Simon et al., 1982; Worcester and Franks, 1976). We operationally define the bilayer width as the total thickness of the bilayer assuming that the headgroup conformation is the same as it is in single crystals of dimyristoylphosphatidylcholine (Pearson and Pascher, 1979). That is, we assume that the phosphocholine group is, on average, oriented approximately parallel to the bilayer plane, so that the edge of the bilayer lies about 5 Å outward from the center of the high density peaks in the electron density profiles (McIntosh et al., 1987, 1986b). We estimate the total bilayer thickness (d_b) to be the distance between headgroup peaks (d_{pp}) plus 10 Å. Thus, assuming the orientation does not markedly change from the co-planar orientation in the presence of TA, we estimate $d_f \approx d - d_b = d - (\text{dpp} + 10\ \text{Å})$.

Using the above definition for fluid spacing, we estimate that $d_f \approx 12$ Å for DPPC in water and $d_f \approx 4$ Å for DPPC in 10 mM TA. For DPPC containing 1,2,3-heptanetriol in water, $d_f \approx 10$ Å. For EPC, the presence of 0.01 M TA

reduced d_f from about 15 Å to about 5 Å. Because the electron density profiles for EPC in TA were at somewhat lower resolution (8 Å) than they were for EPC in water (6 Å), estimates of d_f were more precise for DPPC than for EPC in TA.

DISCUSSION

The data presented in this paper provide information on the binding of TA to bilayers and the effects of TA on both bilayer structure and width of the interbilayer fluid space.

Lipid vesicle aggregation

The increases in absorbance produced by the addition of TA to EPC (Fig. 2) are likely the result of aggregation of the vesicles to form larger sized particles. This conclusion is consistent with the observation that the increase in absorbance was reversed by the TA chelator PVP (Fig. 2), and also with the electron microscopic studies, which showed that TA indeed induced PC vesicles to aggregate while remaining intact (Figs. 3 and 4). The addition of higher concentrations of TA leads to vesicle breakage ((Schrijvers et al., 1989) and Fig. 11). The addition of TA to EPC to LUVs initially produced small clusters of mostly spherical vesicles (Fig. 3). Subsequently, these spherical vesicles lost water and formed stacks of flattened disks of variable widths with some as small as 100 Å, or about twice the width of the bilayer (Fig. 4). The freeze-fracture images (Fig. 4, *C* and *D*) demonstrated that vesicle flattening was not an artifact of the addition of the electron dense salts in the negatively stained preparation. In this stacked disk arrangement, the vesicles could maximize their interaction or adhesion energy, since vesicles in a stack have about 80% of their surfaces in contact. Opposing the formation of these stacked disks is the mechanical work to deform a sphere into a disk and the osmotic pressure that develops as the vesicle volume decreases and the trapped solutes become concentrated (Evans and Parsegian, 1983). These data showed that the adhesion energy gained by the interaction of TA with these surfaces was sufficient to compensate for the energy of deformation and osmotic pressure developed during shrinking. Thus, TA increases the adhesion between PC bilayers. Stacks of bilayer disks were previously observed when small unilamellar vesicles of beef brain phosphatidylserine were incubated for 60 min in 1.0 M NaCl (Day et al., 1980). In this case the adhesion energy was decreased by a reduction in the electrostatic repulsive pressure. For TA-induced adhesion of electrically neutral bilayers, the mechanism is more complex, as described below.

Binding of tannic acid to bilayers

Evidence that TA binds to lipids is provided by six independent measurements based on different physical principles: microelectrophoresis (Fig. 5), surface potential (Figs. 6 and 7), solution calorimetry (Fig. 8), NMR (Figs. 9 and 10), micropipette manipulation (Fig. 11), and x-ray diffraction (Figs. 12 and 13). Based on titration studies, ^{13}C-NMR chemical shifts, and ζ potential measurements (Fig. 5), TA at pH 7.0 is negatively charged. This may explain some of the specificity TA has for different types of lipids. Kalina and Pease (1977) found that MLVs of both PC and sphingomyelin were stabilized against dehydration with ethanol (in the presence of OsO_4) after interaction with TA, whereas MLVs of the negatively charged lipids phosphatidylserine and phosphatidylinositol were not. In addition, Schrijvers et al. (1989) found that TA caused PC, but not PC/phosphatidylserine vesicles, to aggregate. The most likely reason that TA does not associate strongly with negatively charged lipids, or cause negatively charged vesicles to aggregate, is because TA itself is negatively charged and, consequently, would be repelled by highly negatively charged bilayer surfaces.

For either monolayers or bilayers, the total surface potential (V) can be written as $V = \psi(0) + V_d$, where $\psi(0)$ is the potential that arises from fixed or absorbed charges and V_d is the dipole potential that arises from the dipoles (and multipoles) of the lipid and water (Cevc and Marsh, 1988; Tocanne and Teissie, 1990). For PC membranes, it has been shown that carbonyl groups significantly contribute to V_d, as do all dipoles, including dipoles from water molecules close to the water/hydrocarbon interface (Simon and McIntosh, 1989; Smaby and Bockman, 1990; Smejtek and Wang, 1990; Zhang and Vanderkooi, 1992). For EPC monolayers in the presence of 1 mM TA, we argue that V_d must be much larger than $\psi(0)$ since the magnitudes of the ζ potential (−25 mV, Fig. 5) is much smaller than the magnitude of ΔV (−270 mV, Fig. 6) and since ΔV was reduced only about 20 mV when the charge of TA was partially neutralized by lowering the buffer pH to 3.2. We note that for conditions of low ionic strength $\zeta \approx \psi(0)$. Large lipophilic anions such as tetraphenylboron, dipicrylamine, and phloretin show similar behavior to TA, because with all of these molecules, changes in $\psi(0)$ are small compared with changes in V_d (Reyes et al., 1983; Smejtek and Wang, 1990). Moreover, all of these large lipophilic anions decrease V_d in PC membranes on the order of 200 mV at maximum concentrations (Andersen et al., 1976; Reyes et al., 1983; Smejtek and Wang, 1990), which is the same magnitude of decrease in V_d seen with maximum concentrations of TA.

The greater decrease in V for liquid crystalline EPC than gel phase DPPC (Fig. 6) cannot be explained by a model simply involving TA binding to the choline, phosphate, or carboxyl groups of the lipid, since the surface density of all these groups is larger for gel phase DPPC, where the area/molecule (A) is 48 Å^2, than for liquid crystalline EPC, where A = 64 Å^2 (McIntosh et al., 1986b). Rather, these data (and our structural data, see below) are consistent with a model in which TA binds to bilayers by penetrating deep into its interfacial region. It would cost much less energy (i.e., energy of melting) for TA to partition into the liquid crystalline phase than into gel phase of PC. Therefore, if TA partitions into the interfacial region to a depth where the dielectric

constant changes rapidly (such as the deeper carbonyl group (Simon and McIntosh, 1985), and if changes in dipole rearrangement of this region are largely responsible for changes in the dipole potential (V) (Flewelling and Hubbell, 1986; Franklin and Cafiso, 1993; Smejtek and Wang, 1990), then larger changes in V should be observed for liquid crystalline phase lipids than for gel phase lipids, as indeed is observed (Fig. 7). This interpretation is consistent with measurements of binding of lipophilic anions (such as tetraphenylboron and dipicrylamine) to PC MLVs that show that the free energy of adsorption is more negative in the liquid crystalline than gel phases (Smejek and Wang, 1990). In addition, ζ potential measurements also show that lipophilic anions bind better to liquid crystalline than gel phase PC MLVs (Tatulain, 1983; Tatulian et al., 1991). Therefore, in general, large lipophilic anions bind better to fluid than gel phase membranes, and TA is no exception to this rule.

It is not evident with TA, or even for symmetric lipophilic anions like tetraphenylboron (Andersen et al., 1978; Ellena et al., 1987), what properties of the lipid, interfacial water, or adsorbed molecule are most responsible for the reduction of the dipole potential. Many of these compounds reduce the dipole potential in a variety of lipid bilayers, including bilayers formed from glycerol monooleate (Haydon and Hladky, 1972), which shows that the reduction in V_d is not very sensitive to the details of the polar headgroup. A common feature of bilayers of all of these lipids is the presence of both carbonyl groups and interfacial water at the hydrocarbon-water interface, and both the carbonyl groups and the interfacial water have been shown to contribute to the dipole potential (Gawrisch et al., 1992; Simon and McIntosh, 1989). A possible clue to TA interaction with PC monolayers and bilayers comes from the fact that its action is at least partially reversible by compounds, such as PVP (Figs. 2 and 6), which have H-bond donating groups. TA has been shown to interact with surfaces having H-bond accepting groups (Haslam, 1974; Nash et al., 1966). For PC bilayers, these groups would include the carbonyl and phosphate oxygens (Sundaralingam, 1972). Neutralization of the carbonyl dipole by TA is an attractive possibility for the reduction of the dipole potential by TA. However, given that 1) TA has numerous H-bond donating phenolic groups, 2) TA binds in the interfacial region rearranging interfacial water dipoles, and 3) only a small percentage of either the water or phenolic groups need to be rearranged to account for the change in dipole potential (Flewelling and Hubbell, 1986; Haydon and Hladky, 1972), it is impossible to attribute the reduction in V_d to a particular moiety on TA.

There is a large exothermic heat of binding ($\Delta H = -8.3$ kcal/mol) of TA to EPC LUVs, as there is with most other amphipathic compounds that bind to the interfacial region of PC bilayers. Such amphipathic compounds include proteins, peptides, positively charged anesthetics, and lipophilic anions such as tetraphenylboron (Beschiachvili and Seelig, 1992; Seelig and Ganz, 1991). Although there are several possible sources for this negative enthalpy of binding (Beschiachvili and Seelig, 1992; Seelig and Ganz, 1991; Wimley and White, 1993), from this single measurement the various contributions to ΔH cannot be separated.

Structural changes and localization of TA in PC bilayers

Three different methods, micropipette manipulation, x-ray diffraction, and NMR, gave complementary information on the changes in bilayer structure induced by the addition of TA.

In the micropipette experiments where TA was added to the outside of preformed vesicles, changes in area per vesicle of 3–4% were measured upon TA adsorption (Fig. 11). For TA concentrations greater than 50 μM PC, the bilayers become unstable and broke (Fig. 11). Previous measurements of bilayer stability (Needham and Nunn, 1990) showed that, under tension, PC bilayers fail at critical area expansions on the order of 3–4%. While the outer monolayer is expanded due to TA adsorption, the inner monolayer cannot expand more than a critical area of about 3–4%. Therefore, if neither lipid nor TA can "flip-flop" rapidly enough, the integrity of the whole bilayer is compromised and the bilayer will break.

The micropipette and electrophoresis measurements can be combined to provide a picture of TA incorporation into the bilayer. At 0.1 mM TA, the micropipette experiments indicate that TA increases the membrane area about 3–4% and the electrophoresis experiments indicate that there is one charge (and thus one TA molecule) per 1139 Å^2 of surface area. Assuming that 1139 Å^2 represents a 4% increase in surface area upon TA binding, we estimate that the area before binding one TA molecule was about 1095 Å^2 and that this area was increased about 44 Å^2 by binding one TA molecule. Given that the area per lipid molecule in an EPC bilayer is 64 Å^2 (McIntosh et al., 1989a), this means that one TA binds to an area containing about 17 lipid molecules. The gallic acid arms of TA are likely to partition into the headgroup region of the bilayer in an endwise or edgewise configuration, rather than in a flat configuration, since this would create smaller bilayer deformations and would also lead to less bond stress on the TA molecule (Fig. 1). To obtain values for the dimensions of gallic acid, we compare it to a structural analogue, 1,2,3-benzenetriol, whose edgewise and endwise dimensions are 26.9 Å^2 and 24.8 Å^2, respectively, and whose flat area is 57.7 Å^2 (Soriaga and Hubbard, 1982). Thus, on average, one TA molecule binds to an area occupied by about 17 lipid molecules and increases this area by an amount (44 Å^2) equivalent to the area of about two gallic acids in an endwise conformation. This estimate of 1 TA per 17 lipids is also consistent with the magnitude of the quadrupolar splitting of the deuterons at the α- and β-carbons at a DMPC/TA mole ratio of 15/1 as calibrated from the splitting of DMPC/DMPG (20/1) bilayers (Marassi and Macdonald, 1992).

A molecule increasing the area per molecule and partitioning part of the way into the acyl chain region would be expected to decrease the bilayer thickness. If the density of the acyl chains remains constant, and if TA partitions only into the interfacial region, then one may write $\Delta A/A_0 \approx$

$-\Delta L/L_o(1 + \Delta A/A_o)$ where A_o and L_o are the area/molecule and hydrocarbon thickness in the absence of TA, respectively. If for EPC bilayers, $\Delta A/A_o \approx 0.04$ and $L_o \approx 30$ Å (McIntosh et al., 1980), then $\Delta L \approx 1.2$ Å. Thickness decreases of this magnitude were also observed in x-ray diffraction ($\Delta L \approx 2$ Å for EPC, Fig. 13) and NMR quadrupolar splitting measurements on DPPC-d_{62} bilayers at 50°C ($\Delta L \approx$ 0.6 Å). Thus, all three methods indicate that TA decreases the thickness between 0.6 Å and 2 Å. Considering the experimental uncertainty and that experiments were done with different PC lipids, with different TA concentrations, at different temperatures and ionic conditions, the agreement between the three methods is quite good. Other amphiphilic compounds such as tetracaine (Boulanger et al., 1981; Kelusky and Smith, 1984), benzyl alcohol (Ebihara et al., 1979; Turner and Oldfield, 1979), phloretin (Reyes et al., 1983), and nimodipine (Young et al., 1991) also decrease the bilayer thickness. The uniqueness of TA in causing aggregation thus does not lie with the relatively small changes in bilayer structure that ensue upon its binding, since many molecules will produce similar changes.

The ^{31}P-NMR study showed that TA did not alter the chemical shift anisotropy for phosphorus and consequently TA did not markedly influence the motions available to the phosphate group. Although these data cannot be used as definitive evidence that TA does not form strong bonds with the phosphate oxygens, they nevertheless provide supporting evidence. Since TA is a H-bond donor, it is therefore likely to associate with the H-bond accepting carbonyl groups, which then, as discussed above, would lead to a change in the dipole potential (Franklin et al., 1993). We have, however, obtained good evidence that TA influences the headgroup conformation. That is, the quadrupole splitting of the deuterons on the α- and β-carbons change in the presence of TA consistent with the $—N^+(CH)_3$ group rotating toward the plane of the membrane (Roux et al., 1989; Seelig et al., 1987). For TA, this direction of the conformational change could arise from adsorbed negative charges (Marassi and Macdonald, 1992; Roux et al., 1989; Seelig et al., 1987), from a reduction in dipole potential (Macdonald and Seelig, 1988), or from a reduction in interbilayer water (Bechinger et al., 1988). We have not performed experiments to distinguish among these alternatives. If, however, the conformational change were generated simply by the induction of a surface charge (the simplest hypothesis), then it unlikely that the large TA-induced change in dipole potential arises from this small headgroup rotation, since other charged compounds which cause similar rotations (such as fatty acids), do not markedly reduce the dipole potential.

The increase in molecular area caused by the binding of TA has an influence on the acyl chain conformation. The ^{2}H-NMR quadrupole splitting measurements show that TA decreases the order parameters of all C-D bonds in a concentration-dependent manner (Figs. 9 and 10). In addition, at a given TA concentration, the order parameter ratio is almost constant for the first half of the acyl chains and decreases toward the bilayer center (Fig. 10). To accommodate the area increase produced by TA binding, the acyl chains may either tilt cooperatively and/or fill the available space by increasing the number of gauche conformations. To account for the constant order parameter ratios for the first half of the acyl chain, we argue that the first 8 to 9 carbons cooperatively tilt. To account for the rapid decrease in the order parameter ratios in the second half of the bilayer, we propose that there is an increase in the formation of gauche conformers toward the end of the chain (see Fig. 7 in Ellena et al., 1987, for example). The changes in order parameter profile observed with TA are the same as those observed with benzyl alcohol (Turner and Oldfield, 1979). However, benzyl alcohol does not decrease the fluid spacing between fluid PC bilayers like TA (Ebihara et al., 1979), again showing that the unique effects of TA in reducing the fluid spacing and aggregating bilayers is not a consequence of how it interacts with a single bilayer.

Reduction in fluid space caused by tannic acid

The electron density profiles (Figs. 12 and 13) show that TA decreases the fluid space between adjacent DPPC bilayers from about 12 Å to 4 Å. Thus, the interdigitated lipid surfaces are separated by about the width of the glucose molecule in TA (Fig. 1), implying that there can be at most one "layer" of TA molecules between adjacent bilayers. We have previously shown that when $d_f \approx 5$ Å, there is steric repulsion between mobile phosphocholine groups (McIntosh and Simon, 1993) that may prevent further collapse of the fluid space upon TA binding. Other exogenous compounds that induce interdigitation of the hydrocarbon chains do not reduce the fluid space to the extent of TA. For example, the fluid spaces between interdigitated phase DPPC bilayers in the presence of benzyl alcohol (Ebihara et al., 1979), ethanol (Simon and McIntosh, 1984), and 1,2,3-heptanetriol (Fig. 13) (Simon et al., 1988) are 10 Å, 8 Å, and 10 Å, respectively. Thus, amphipathic compounds that contain up to three hydroxyl groups do not collapse the fluid spacing (even at higher concentrations) to the extent of TA. The one obvious difference between TA and these compounds is that TA has five arms of digallic acid (Fig. 1), each of which can penetrate into a bilayer.

For EPC there is an abrupt decrease of about 12 Å in lamellar repeat period at a TA concentration of about 5×10^{-4} M. Electron density profiles (Fig. 13) indicate that this change in repeat period is primarily due to an abrupt TA concentration-dependent decrease in fluid separation. That is, the TA-induced reduction in bilayer thickness of about 1–2 Å is much smaller than the total decrease in lamellar repeat period.

Mechanisms for tannic acid-induced collapse of the interbilayer fluid space

As noted in the Introduction, the fluid space between adjacent PC bilayers in water is determined by a balance between the attractive van der Waals pressure and several nonspecific

repulsive pressures, including hydration and steric pressures (Evans and Rawicz, 1990; Helfrich, 1975; Marra and Israelachvili, 1985; Israelachvili and Wennerström, 1990; McIntosh and Simon, 1993, 1986b; Parsegian et al., 1979). There are several possible mechanisms by which TA could reduce the fluid space between adjacent bilayers: 1) by applying osmotic pressure to the lipid multilayers; 2) by reducing the repulsive undulation steric pressure; 3) by increasing the attractive van der Waals pressure; 4) by reducing the repulsive hydration pressure (P_h); or 5) by forming molecular bridges between adjacent surfaces. We now consider these possibilities in turn. The first possibility can be eliminated since TA reduces the fluid spacing of EPC bilayers at a concentration of only 0.1 mM. An electrolyte concentration of 0.1 mM *completely* on the outside of the multilamellar vesicles would produce an osmotic pressure of only 4.8×10^3 dyne/cm^2, which would produce less than a 1 Å change in fluid spacing (McIntosh and Simon, 1986b). The second possibility can be eliminated in the case of gel phase DPPC bilayers, where the undulation force is negligible (McIntosh et al., 1987). The third possibility cannot explain the data, since the addition of TA should have a negligible effect on the Hamaker constant of the van der Waals pressure, and therefore TA binding should have little direct effect on the van der Waals pressure. (We note, however, that if the fluid space were reduced by another mechanism, then the van der Waals pressure would increase). In terms of the hydration pressure, several studies have shown that the magnitude of the repulsive hydration pressure (P_o) is proportional to V^2, where V is the surface potential (McIntosh et al., 1989a,b; Simon et al., 1988, 1991; Simon and McIntosh, 1989). Since TA has been shown to markedly reduce V for both DPPC and EPC (Fig. 7), it would be expected that hydration repulsion would be reduced by the addition of TA. For EPC in water, $P_o = 4 \times 10^8$ dyne/cm^2 and $V = 415$ mV (Simon and McIntosh, 1989). At 1 mM TA, the concentration where the x-ray measurements were performed, V was reduced by 250 mV (Fig. 7) to 165 mV. Thus, assuming that $P_o \alpha V^2$, it would be expected that the addition of 1 mM TA would reduce P_o by 84%. The shape of the $(\Delta V)^2$ vs. TA plot (obtained from Fig. 7) would predict an abrupt decrease in fluid spacing with increasing TA. That an abrupt decrease in fluid spacing is observed (Fig. 12) suggests that decreasing the dipole potential of the membrane may be part of the mechanism by which TA decreases the fluid spacing between bilayers.

The final possibility to rationalize the collapse of the fluid space caused by TA is the formation of TA mobile bridges between apposing bilayers. The possibility that TA forms bridges between adjacent surfaces is supported by two pieces of evidence. First, an abrupt decrease in repeat period (Fig. 12) would be expected at the concentration where a critical number of bridges form (1 TA/17 lipids, see above) such that the adhesion energy becomes greater than the thermal energy (Evans and Parsegian, 1983). Second, and most important, in the presence of TA the fluid spacing is decreased to 5 Å and consequently, given its size (Fig. 1), *TA must be in physical contact with monolayers from apposing bilayers*. That is, the length of each digallic acid residue is about 12 Å (Fig. 1), the central glucose moiety of TA is about 5 Å thick, and the width of each lipid headgroup is about 10 Å (McIntosh and Simon, 1993, 1986b). Therefore, the dimensions of the TA molecule, the bilayer interfacial regions, and the interbilayer space are such that one TA molecule with extended digallic acid arms could easily span the distance between bilayer interfacial regions.

The ability of TA to induce aggregation cannot simply be attributed to its having multiple hydroxyl groups capable of hydrogen bonding, as several molecules (e.g., 1,2,3-heptanetriol; Fig. 13) possess this characteristic and do not collapse the fluid space. Apparently the key feature of TA is that it has multiple digallic acid arms that are sufficiently lipophilic to partition into the interfacial regions of apposing bilayers and also large and flexible enough to span the interbilayer space.

Thus we argue that the TA-induced reduction in fluid space between PC bilayers is primarily due to the formation of bridges between adjacent bilayers, with a reduction in repulsive hydration pressure possibly being a contributing factor.

CONCLUSIONS

TA binds strongly to fluid PC membranes, increasing the bilayer area, decreasing the bilayer thickness, and decreasing the membrane dipole potential. In biological membranes, TA has been shown to decrease ion and nonelectrolyte in a variety of systems (Hunter, 1960; Schiffman et al., 1992; Shrager, 1969; Shuchter et al., 1973; Simon et al., 1992). In principle, changes in bilayer area, thickness, or dipole potential could affect the activity of transport proteins (Haydon and Hladky, 1972). Moreover, TA has the unusual property of forming bridges between apposing neutral membranes, causing them to tightly adhere. In this regard, TA may be an analogue of annexins or polyphenolic adhesion proteins.

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