Interaction of the Glycoalkaloid Tomatine with DMPC and Sterol Monolayers Studied by Surface Pressure Measurements and Brewster Angle Microscopy†

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The interaction of the glycoalkaloid tomatine with monolayers of dimyristoylphosphatidylcholine (DMPC) and cholesterol, as well as other selected sterols, has been investigated using surface pressure measurements at constant area and Brewster angle microscopy (BAM). The interaction of tomatine with sterol monolayers is found to vary with the structure of the sterol. The interaction of tomatine with cholesterol-containing monolayers results in a surface pressure increase accompanied by the appearance of a mottled texture. Morphological changes are observed that suggest the formation of tomatine−cholesterol complexes that aggregate at the water−air interface. No morphology change observable by BAM is observed for monolayers containing epicholesterol, suggesting that the stereochemistry of hydrogen bonding between the sterol and the sugar units on tomatine is of particular significance. Strong interactions are observed with cholestanol- and coprostanol-containing monolayers, and BAM reveals formation of spiked aggregates upon interaction with 7:3 mole ratio DMPC/coprostanol mixed monolayers. More modest surface pressure changes are observed for cholestenone- and epicoprostanol-containing monolayers. A much smaller surface pressure increase is observed when tomatine is injected beneath a pure DMPC monolayer.

Introduction

The study of molecules that bind to membranes and disrupt their structure, either permanently or transiently, is an area of significant interest in relation to fields including drug delivery, toxicology, and therapeutics.[1−6] In addition to liposomes, monolayers at the water−air interface can serve as model systems to study the interactions of lipids and sterols with such compounds. Tomatine (Figure 1a) is a glycoalkaloid found in tomato plants and a number of other plants where it exhibits antifungal and insect and snail repellant effects, in addition to many other effects.[7] Glycolalkaloids are compounds that consist of a nitrogen-containing six-ring steroidal aglycone and an attached oligosaccharide, and are found in numerous plants, including potatoes which contain the related compounds chaconine and solanine.[8] Glycolalkaloids form 1:1 complexes with cholesterol and other 3β-hydroxy sterols and disrupt sterol-containing membranes.[9−11] Glycoalkaloids are a part of the broad class of natural products known as saponins,[12,13] many of which alter the permeability of membranes,[14,15] sometimes in a manner that does not depend on the presence of a sterol.[16] Glycoalkaloids have been considered for their potential to increase the permeability of mucosal membranes in drug delivery.[17] A range of additional biological effects have been reported for glycoalkaloids, including cholinesterase inhibition,[18,19] immunological adjuvant activity,[20,21] and antitumor activity.[22] Tomatine serves as an example of a natural product that interacts with and disrupts membranes by binding to a membrane component, the sterol. It has been established from studies using liposomes that membrane disruption only occurs when 3β-hydroxy sterols are present in the membrane, and that the glycolalkaloids form 1:1 complexes with these sterols. The structures of cholesterol and of the less commonly studied sterols epicholesterol and coprostanol are shown in Figure 1b. The strength of the effect on membrane permeability is dependent on the structure of the oligosaccharide group and on the nature of the sterol present.[10] Formation of these glycoalkaloid−sterol complexes is believed to alter the membrane permeability by perturbing the membrane structure.

The membrane-disrupting effects of glycoalkaloids have been observed both in liposome studies and in studies using animal and plant cells. The results found for model liposome systems were confirmed in studies using selected cells and mitochondria.[11] Leakage of peroxidase encapsulated inside liposomes composed of lipid and cholesterol, ergosterol, or stigmasterol upon exposure to tomatine was reported to be maximal at pH 7.2 and to decline significantly with decreasing pH.[23] Less release of peroxidase was reported from liposomes containing bovine brain sphingomyelin than from those containing egg yolk phosphatidylcholine with equivalent amounts of sterol. Tomatine induced electrolyte leakage from plant tissues, except from tomato and potato which contain low amounts of free sterol.[24] The effect of tomatine on plant organelles has been reported.[25] The effect of solanine, chaconine, and tomatine on liposomes containing egg yolk phosphatidylcholine (PC) and the sterols cholesterol, ergosterol, fucosterol, and β-sitosterol was studied.[9] Addition of glycoalkaloid resulted in the release of the encapsulated self-quenching dye carboxyfluorescein from the liposomes, with the rate and extent of release increasing with the mole fraction of cholesterol; very little release was observed from pure egg yolk PC liposomes or for sterol contents below 10 mol %. The membrane-disrupting effects were found to require the presence of the intact tetrasaccharide group, suggesting a key role for interactions between the carbohydrate groups. Removal of just one sugar unit from tomatine resulted

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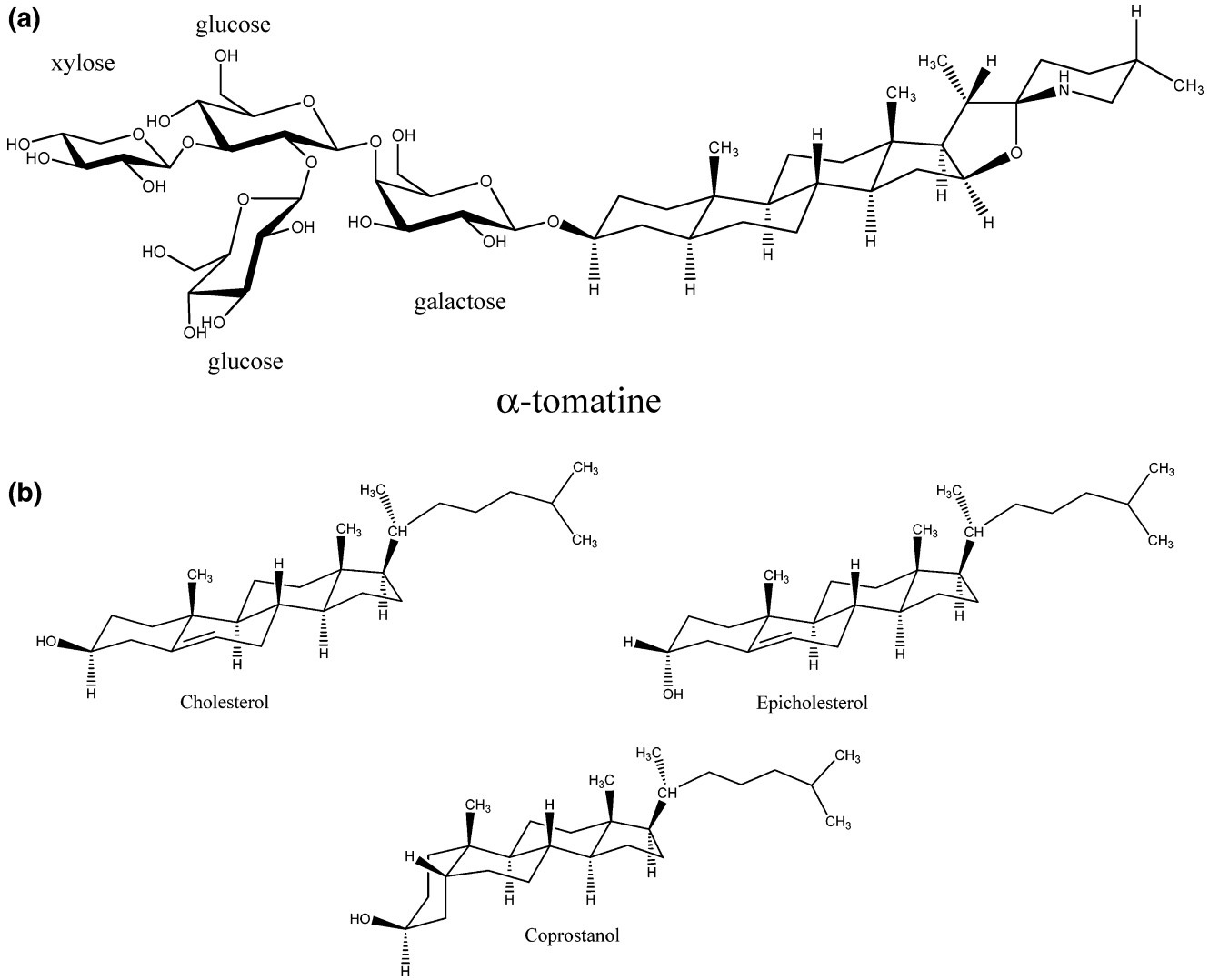

Figure 1. (a) Structure of α-tomatine. (b) Structures of cholesterol, epicholesterol, and coprostanol.

in a drastic lowering of the effectiveness of the compound at inducing the leakage of carboxyfluorescein from liposomes. A subsequent study of tomatine, chaconine, and solanine activity showed that release of carboxyfluorescein from liposomes containing 30 mol % sterol was significant only for 3β-hydroxy sterols.[10] Formation of tubular and globular structures adjacent to liposomes treated with tomatine was observed by freeze-fracture electron microscopy. Detergent-resistant complexes between cholesterol and glycoalkaloid that formed were confirmed by centrifugation to be of 1:1 stoichiometry.[10] A model was proposed in which the glycoalkaloid irreversibly binds to the sterols in the outer leaflet of the bilayer, these complexes undergo lateral aggregation, and the geometric requirements of the glycoalkaloid oligosaccharide groups result in formation of tubular or globular structures rich in the 1:1 glycoalkaloid/sterol complexes that disrupt membrane integrity and result in the leakage of encapsulated molecules. Evidence for such structures is found in electron microscopy studies of liposomes and cells treated with tomatine.[26]

Study of the interactions of tomatine as a representative glycoalkaloid with lipid + sterol monolayers was deemed to be of interest as a step toward further physical characterization of the interaction of these compounds with model membrane systems. The activity of tomatine and other saponins as adjuvants may be related to their ability to permeabilize antigen-presenting cells.[27] The antitumor activity of the glycoalkaloid solamargine has been attributed to recognition of part of its carbohydrate moiety by membrane receptors followed by endocytosis.[28] In addition, evaluating the correlation of monolayer studies with liposome studies was deemed interesting. In this paper, the effect of injecting tomatine beneath monolayers of cholesterol and other selected sterols and beneath mixed monolayers of DMPC and sterols is investigated using surface pressure measurements and Brewster angle microscopy (BAM).

Experimental Section

Experiments were conducted in a home-built Langmuir trough[29] positioned beneath a BAM-1plus Brewster angle microscope (Nanofilm Technologies). The main trough was augmented by the addition of a Teflon insert for the side of the trough where both the surface pressure transducer is located and the laser beam of the BAM is incident on the water surface. The volume of the subphase in the insert is ~14 mL with the monolayer allowed to flow into the insert from the main subphase (~100 mL) across a 4.0 mm wide, 5.0 mm long, and 3.0 mm deep canal in the Teflon wall of the insert. The exact volume of the subphase in the insert trough depends on the exact water level, which can be adjusted by eye to within 0.5 mL. The body of the insert encloses a miniature cuvette stirrer (Spinette Magnetic Cell Stirrer, Starna Cells) under the Teflon that drives a miniature stir bar used to gently stir the subphase

in the insert. The insert is placed in the trough underneath the illumination of the BAM and the surface pressure transducer (Nima ST9000) from which a piece of filter paper serves as the Wilhelmy plate. Surface pressure data are acquired from the ST9000 by a multifunction board in a PC via a GW-Basic program, which also controls the barrier motion. With this configuration, experiments in which the monolayer is compressed to an initial surface pressure prior to injection of the tomatine solution are carried out. To isolate the monolayer in the insert prior to injection of the tomatine, a T-shaped Teflon plug is placed sideways in the canal to prevent flow of the monolayer back into the main trough. This results in the experiment being carried out as a measurement of surface pressure change at constant area after the tomatine is injected. Separate measurements of surface pressure (Π) versus molecular area (A) compression isotherms were carried out using the same trough with the insert removed, which results in a traditional rectangular Langmuir trough (water surface = 22.0 cm × 7.0 cm). Compression isotherms were measured at a rate of 2.0 Å^2 molecule^{-1} min^{-1} for pure sterols and at a rate of 4.0 Å^2 molecule^{-1} min^{-1} for DMPC and DMPC + sterol mixed monolayers.

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL) and used as received. Cholesterol, epicholesterol, cholestanol, cholestenone, coprostanol, and epicoprostanol were obtained from Steraloids (Newport, RI) and used as received. The subphase was buffered to pH 7.0 using a 0.05 M phosphate buffer prepared in Millipore water, which was also 0.10 M in NaCl. Potassium dihydrogen phosphate (99.99%), potassium hydrogen phthalate (99.5%−100.5%), and sodium hydroxide (99.99%) were obtained from Sigma-Aldrich (St. Louis, MO); sodium chloride (metals-basis grade, 99.99%) was obtained from Alfa-Aesar (Ward Hill, MA). Tomatine (minimum 90%) was obtained from Sigma (St. Louis, MO) and used as received. Commercial tomatine extracted from plants is a ∼9:1 mixture of α-tomatine (α refers to the presence of the intact tetrasaccharide) and α-dehydrotomatine, which has a double bond present between C-5 and C-6.[30] Solutions of tomatine of concentration 280−300 μM were prepared in a 0.010 M pH 5.0 phthalate buffer that was also 0.60 M in NaCl. The addition of NaCl to the phthalate buffer was done so that the injected solution would have a greater density than that of the subphase in the trough, to prevent the injected drops from rising as they exit the syringe needle and thus preventing artificially rapid increases in surface pressure after injection. Observation of the injection of 80 μL of a dilute aqueous dye solution in this buffer with stirring at ∼20 rpm indicated that the dye was evenly dispersed 10 min after injection. Tomatine solutions were prepared fresh for each day of experiment. The solubility of tomatine increases with decreasing pH, and has been reported as 6 mM at pH 5, 1 mM at pH 6, 0.040 mM at pH 7, and 0.030 mM at pH 8.[31] The tomatine solution was injected through a needle hole in the side of the insert using a 100 μL microsyringe into the subphase beneath the monolayer. Injection volumes near 80 μL resulted in a tomatine concentration of ∼1.3 μM in the subphase, well below the reported solubility limit of 40 μM at pH 7. Higher concentrations near 3 μM were not found to produce larger effects with cholesterol monolayers, and injection to a subphase concentration of ∼1.3 μM was chosen for the majority of the experiments. The injection of ∼80 μL of buffer had a very small effect on the water level and the immersion depth of the Wilhelmy plate, resulting in an apparent shift in surface tension of ∼0.10 mN m^{-1}, which is well within the error in these measurements.

Monolayers were spread from solutions (∼0.5 mg ml^{-1}) in chloroform (Fisher Scientific, Optima grade). After spreading and allowing 15 min for solvent evaporation, monolayers were compressed to the target surface pressure at a rate of 2.0 Å^2 molecule^{-1} min^{-1}. All measurements were conducted at room temperature, 23 ± 1 °C. Upon reaching the target surface pressure, the Teflon plug was inserted into the canal to isolate the monolayer in the insert trough. After allowing 3−5 min, stirring of the subphase beneath the monolayer was initiated at ∼20 rpm and another 3−5 min allowed for surface pressure relaxation. Tomatine dissolved in pH 5.0 phthalate buffer in 0.50 M NaCl was then injected into the subphase. Prior to the injection, the monolayer surface pressure would typically relax by 0−2.5 mN m^{-1}, with the greatest relaxations observed for some of the pure sterol monolayers and much less or no relaxation observed for mixtures with DMPC. There can be a small disturbance in surface pressure when the plug is inserted, which was done as gently as possible. Subsequently, the surface pressure versus time was followed for a period of ∼4000−5000 s while the subphase was stirred. All experiments were repeated to verify reproducibility of the observed effects. The data are shown as change in surface pressure $\Delta\Pi$, defined as surface pressure minus the value at the time of injection, versus time. The monolayer was observed by BAM throughout the experiment and after the experiment when the stirring was stopped. Stirring resulted in a slow motion of the monolayer and some flickering in the recorded BAM images but was necessary to achieve uniform mixing within a reasonable period of time. The BAM images shown are from the same experiments for which the surface pressure data are reported.

Results

A series of sterols was selected to represent different perturbations of the structure of cholesterol. Epicholesterol was chosen to investigate the effect of inverting the stereochemistry of the hydroxyl group at the 3-position. In Cholestanol (dihydrocholesterol), the double bond between C-5 and C-6 present in cholesterol is saturated. Cholestenone has a ketone functionality on the 3-position instead of a hydroxyl group, and is no longer a possible hydrogen-bond donor. Coprostanol, in which the steroid *A* ring is cis instead of trans relative the *B* ring of the steroid ring system, was chosen to examine the effect of deforming the ring system. Epicoprostanol includes both the *A*/*B* cis ring conformation and inversion of the stereochemistry of the hydroxyl group at the 3-position. The compression isotherms for the sterols included in this study are shown in Figure 2. The observed collapse pressures (Π_c) are for cholesterol, 41.0 mN m^{-1}; for cholestanol, 38.5 mN m^{-1}; for epicholesterol, 33.4 mN m^{-1}; for coprostanol, 36.7 mN m^{-1}; for epicoprostanol, 30.7 mN m^{-1}; and for cholestenone, 25.9 mN m^{-1}. The sterols all exhibit a single condensed-phase region upon compression. At molecular areas where $\Pi \sim 0$ for the sterols, BAM shows coexistence of the condensed phase and the gas phase. In Figure 3, compression isotherms are shown for pure DMPC, and for mixtures of DMPC with the various sterols, all of 7/3 DMPC:sterol mole ratio. The Π−A isotherms are all featureless, and the collapse pressures are all near 40 mN m^{-1} or above. The condensing effect of sterols on phospholipid monolayers (negative deviation from the molecular area expected on the basis of ideal mixing) is observed with cholesterol exhibiting the greatest condensing effect. Calculations of the condensing effect as a function of composition and surface pressure for phospholipid + sterol systems have been reported in detail by others.[32−36] DMPC monolayers compressed

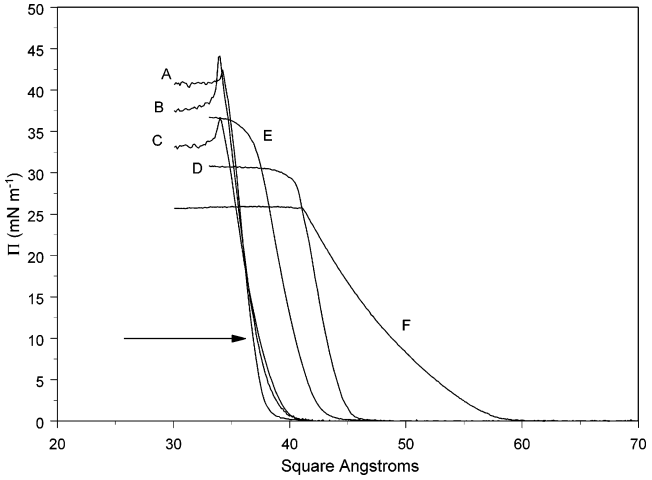

Figure 2. Surface pressure (Π) versus molecular area (*A*) isotherms for sterols (0.05 M phosphate buffer, 0.10 M NaCl): (A) cholesterol, (B) cholestanol, (C) epicholesterol, (D) epicoprostanol, (E) coprostanol, and (F) cholestenone. An arrow shows the surface pressure of 10 mN m^{-1} to which the monolayers were compressed prior to the tomatine injection experiments.

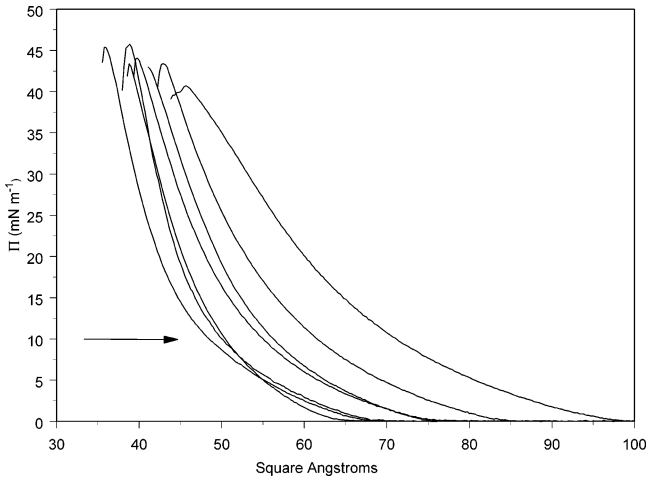

Figure 3. Surface pressure (Π) versus molecular area (*A*) isotherms for DMPC + sterol mixtures of mole ratio 7/3 DMPC/sterol (0.05 M phosphate buffer, 0.10 M NaCl); from right to left at 10 mN m^{-1}: DMPC (pure), DMPC/cholestenone, DMPC/epicoprostanol, DMPC/epicholesterol, DMPC/coprostanol, DMPC/cholestanol, and DMPC/cholesterol. An arrow shows the surface pressure of 10 mN m^{-1} to which the monolayers were compressed prior to the tomatine injection experiments. The graphs have been truncated just after collapse to avoid clutter near the top of the figure.

below ∼100 $Å^2$ $molecule^{-1}$ are in a liquid-expanded state at room temperature[37] and appear featureless under BAM observation. For these studies of tomatine−monolayer interactions, DMPC was chosen due to its simple phase behavior both alone and in mixtures with sterols.

Figure 4 shows the change in surface pressure versus time after the injection of tomatine to a concentration of ∼1.3 μM beneath a monolayer of cholesterol compared to that for injection beneath a monolayer of DMPC. The monolayers were compressed to a surface pressure of 10 mN m^{-1} prior to the injection of tomatine. This surface pressure was chosen as being well below the collapse pressures for these monolayers and as a convenient initial pressure that could allow for a significant surface pressure rise before approaching surface pressures where collapse could become a complicating issue. As is evident from the data in Figure 4, tomatine interacts strongly with the more tightly packed cholesterol monolayer but has a very small effect

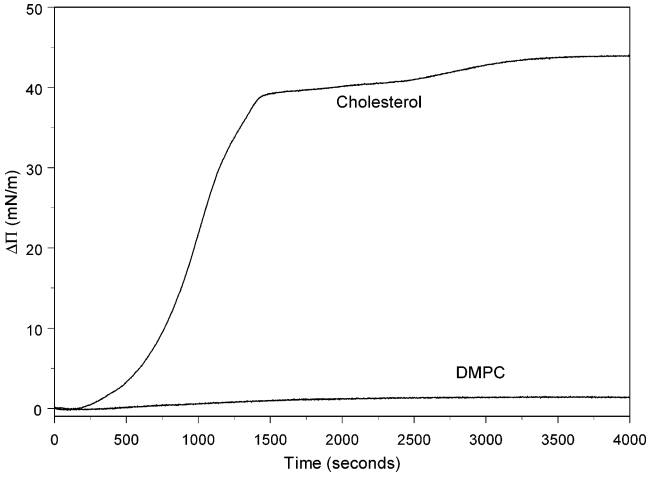

Figure 4. Surface pressure change (ΔΠ) versus time after the injection of tomatine to a concentration of 1.3 μM beneath monolayers of dimyristoylphosphatidylcholine (DMPC) and of cholesterol, both initially compressed to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl).

on the DMPC monolayer despite the ease with which molecular insertion into this fluidlike monolayer could occur. Thus, a specific association of tomatine with cholesterol is responsible for the surface pressure increase. The interaction with DMPC results in a total surface pressure increase near 1.5 mN m^{-1} suggesting a distinct, but much smaller and most likely nonspecific interaction with the DMPC monolayer. BAM reveals no change in the featureless appearance of the DMPC monolayer during and after the interaction with tomatine. Given the presence of the sugars on one end and a nitrogen-containing ring on the opposite end of the molecule, some surface activity may be expected for tomatine, but at the concentrations used we found it to be minimal. Injection of tomatine to a concentration of ∼1.3 μM into the stirred buffered subphase with no monolayer present resulted in a surface pressure of ∼0.2 mN m^{-1} after 80 min with no noteworthy structures observed by BAM. The larger effect observed with DMPC suggests some modest interaction between the two compounds, perhaps related to the sterol-like structure of tomatine which may promote some insertion into the lipid monolayer.

In Figure 5, the surface pressure versus time variations for the interaction of tomatine with the various pure sterols is shown. The largest surface pressure increases are observed for cholesterol and cholestanol, which can be expected since cholesterol is a natural binding partner for tomatine and the two molecules share similar steroid ring systems. Cholestanol, also known as dihydrocholesterol, has been recommended as a substitute for cholesterol in monolayer experiments to remove concerns about the possible photooxidation of cholesterol, and the two were found to exhibit very similar phase behavior in mixtures with phospholipids.[38,39] The overshoot observed in the time dependence of the surface pressure for cholestanol was observed in each run. A significant increase is observed for coprostanol despite the *A*/*B* cis ring conformation. The surface pressure increase observed for epicholesterol is less than half that observed for cholesterol and is similar to that observed for cholestenone. The smallest surface pressure increase is observed for epicoprostanol and is still significantly greater than that observed for DMPC. The quantitative form of the time dependence of these surface pressure responses is likely to depend on a range of factors including diffusion of tomatine to the monolayer, the rate at which it inserts into the monolayer and binds to the sterol, the strength of sterol/tomatine binding,

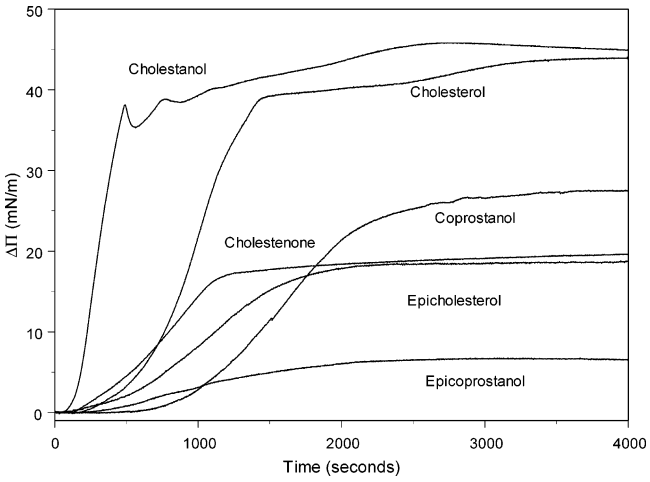

Figure 5. Surface pressure change ($\Delta\Pi$) versus time after injection of tomatine to a concentration of 1.3 μM beneath monolayers of sterols initially compressed to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl): cholesterol, cholestanol, epicholesterol, cholestenone, coprostanol, and epicoprostanol.

and the slope of the isotherm of the individual sterol, which reflects the sterol monolayer compressibility. In addition, the ultimate surface pressures achievable are likely influenced by the instrinsic stability of the sterol monolayer through its collapse pressure and the stability of the mixed monolayer of tomatine and sterol that is formed. A detailed treatment of these factors requires further study.

Cholesterol monolayers, as well as the other sterol monolayers, appear featureless at 10 mN m^{-1} by BAM prior to the injection of tomatine. Figure 6A shows the cholesterol monolayer just prior to injection, a featureless view also seen for the other sterols. Figure 6B shows the cholesterol monolayer 15 min after tomatine injection, and a mottled texture of brighter and darker regions is now evident. Cholesterol monolayers interacting with tomatine, as observed by BAM, begin to show formation of visible aggregates in the form of many small brighter spots as soon as the total surface pressure increases to about $\sim$13$-$14 mN m^{-1}. The motion of the monolayer due to the stirring of the subphase makes it difficult to capture a good still frame image very soon after injection. During the rest of the experiment, this texture becomes somewhat finer and then stops changing. As the pressure rises, the monolayer stops undergoing any motion induced by the stirrer about 30 min into the experiment. Figure 6C shows a BAM image of the cholesterol monolayer 73 min after tomatine injection at the end of the experiment. Monolayers of cholestanol resemble those of cholesterol visually, perhaps with somewhat finer features. When epicholesterol is spread as a monolayer and subjected to tomatine injection, BAM shows no change in the appearance of the monolayer during the course of the experiment and it remains featureless despite the increase in surface pressure. No signs of the formation of any collapse-induced features are seen.

Monolayers of cholestenone are noteworthy in that their interaction with injected tomatine results in somewhat larger domain formation, as seen in Figure 7A. These islands grow larger (Figure 7B) but do not completely merge (Figure 7C). No further change in appearance is observed beyond that shown in Figure 7C. In the case of coprostanol, aggregates form (Figure 8A) which cluster into large islands (Figure 8B), and then result again in a rather uniform field of view (Figure 8C). Epicoprostanol monolayers show very little if any visual change during the course of their interaction with tomatine, with the possible exception of some faint streaks in the image.

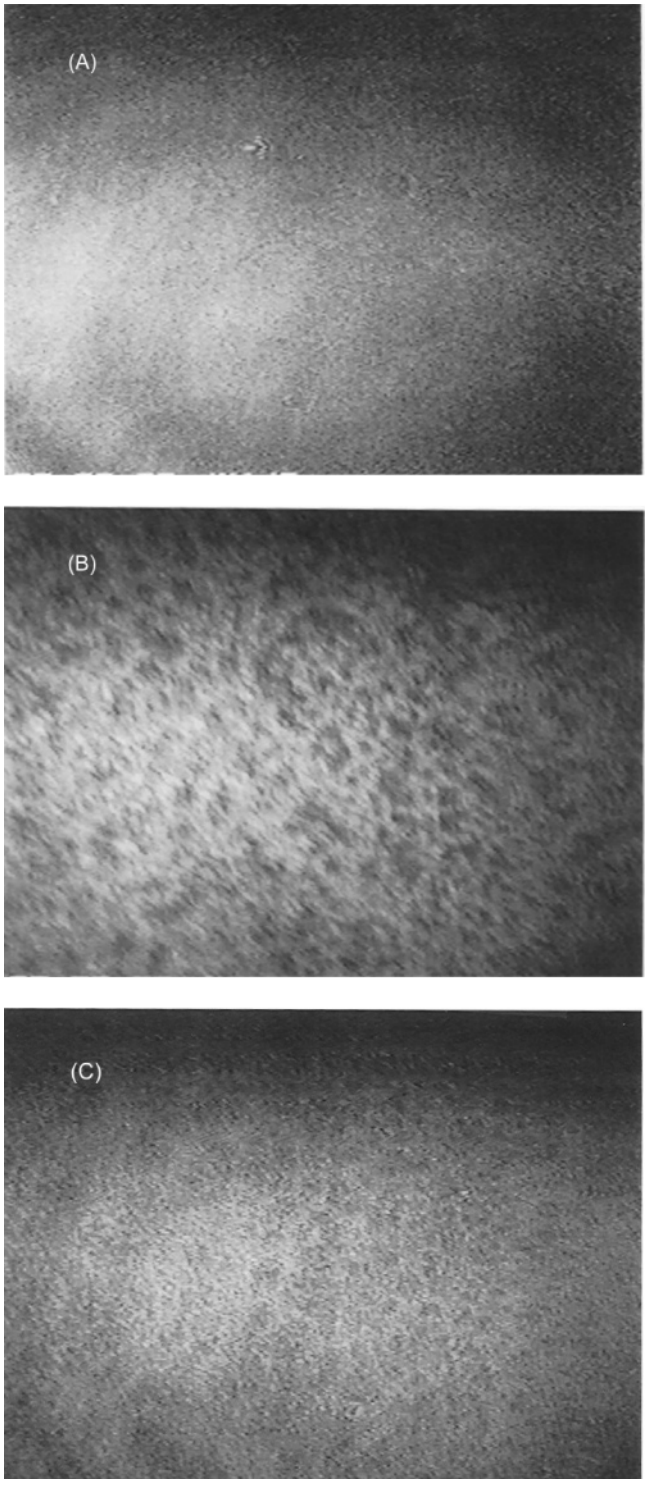

Figure 6. BAM micrographs of a cholesterol monolayer initially compressed to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl) and then interacting with 1.3 μM tomatine: (A) before injection, (B) 15 min after injection, and (C) 73 min after injection. The field of view is 900 μm $\times$ 700 μm.

The interaction of tomatine with 7/3 mixtures of DMPC and cholesterol, cholestanol, epicholesterol, cholestenone, coprostanol, and epicoprostanol was examined. The DMPC/cholesterol system was chosen since it provides an initially uniform monolayer prior to any interaction with tomatine. The reports of McConnell[39] and Keller[40] indicate that, for DMPC + dihydrocholesterol (cholestanol) monolayers, the mixtures are in a one phase state near 10 mN/m and above, under which conditions the monolayer is above a critical point between two

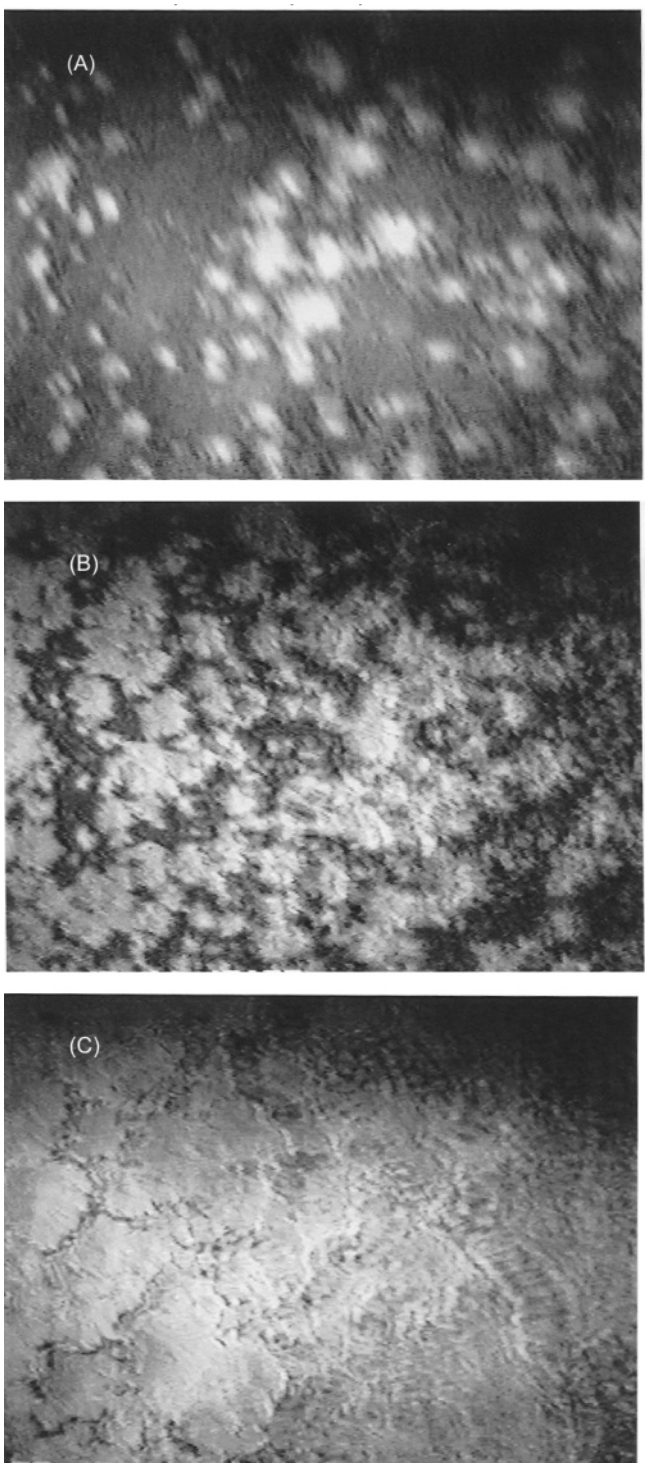

Figure 7. BAM micrographs of a cholestenone monolayer initially compressed to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl) and then interacting with 1.3 μM tomatine: (A) 8 min after injection, (B) 16 min after injection, and (C) 31 min after injection. The field of view is 900 μm $\times$ 700 μm.

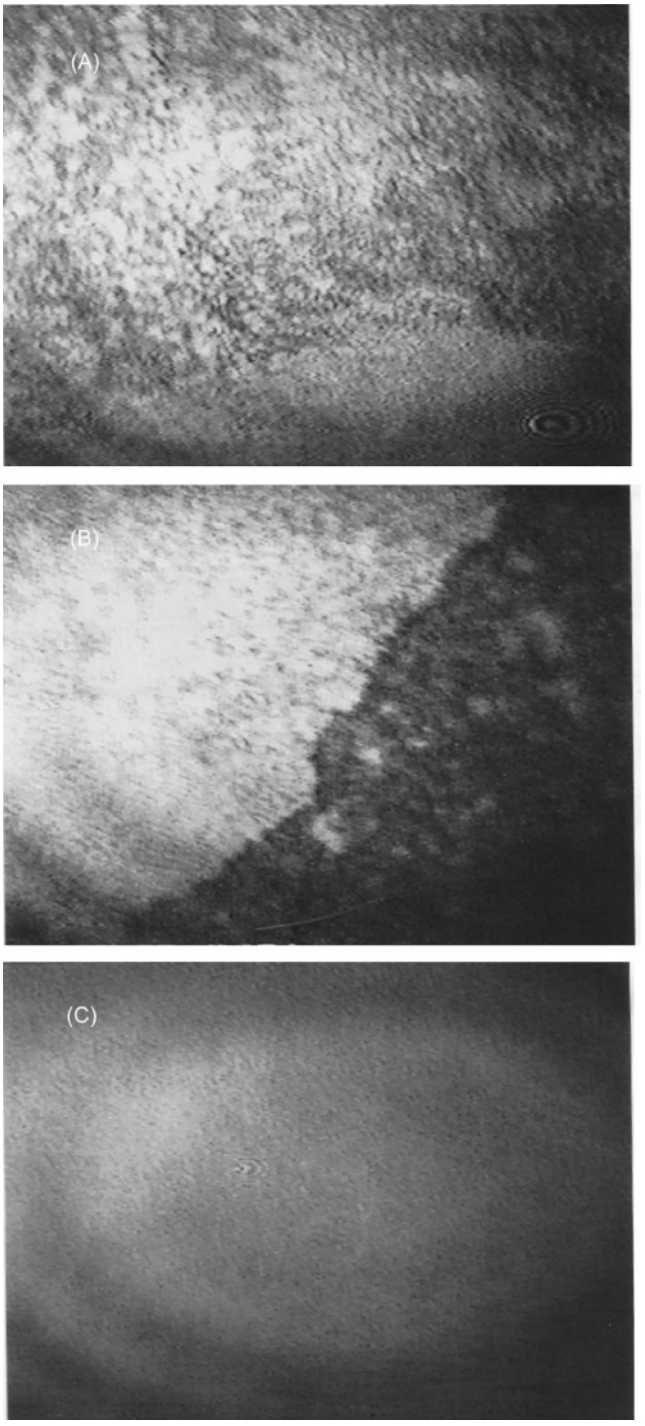

Figure 8. BAM micrographs of a coprostanol monolayer initially compressed to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl) and then interacting with 1.3 μM tomatine: (A) 10 min after injection, (B) 15 min after injection, and (C) 30 min after injection. The field of view is 900 μm $\times$ 700 μm.

coexisting liquidlike phases. At 10 mN m^{-1}, all of the DMPC + sterol mixed monolayers studied here appear featureless under BAM. Coexisting regions of the two liquidlike phases were visible at earlier points in the compression. The gas phase is also observed when $\Pi \sim 0$. For monolayers of the 7/3 DMPC/cholesterol mixture, reducing the final subphase concentration of tomatine reduced the observed surface pressure response, as can be seen in Figure 9. The surface response of mixed monolayers of DMPC with various sterols in a 7/3 mole ratio of DMPC:sterol to $\sim$1.3 μM tomatine is shown in Figure 10. The response of mixed monolayers containing cholesterol and cholestanol is the greatest, and the response is also substantial for 7/3 DMPC + coprostanol. The surface pressure response of the mixed monolayers of DMPC of 7/3 mole ratio with cholestenone, epicholesterol, and epicoprostanol is much less.

Figure 11 shows images of DMPC/sterol monolayers of 7/3 mole ratio interacting with tomatine. The DMPC/sterol monolayers all initially appear featureless. Figure 11A shows the DMPC/cholesterol monolayer, and the appearance of a sort of woven texture is seen which becomes of finer texture by the

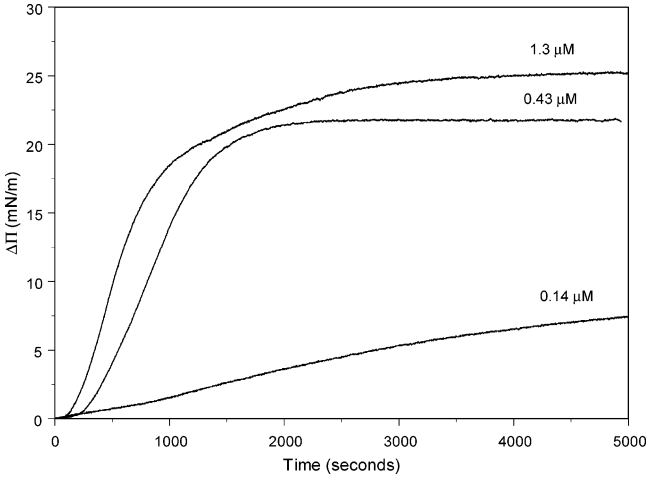

Figure 9. Surface pressure change ($\Delta\Pi$) versus time after injection of tomatine beneath mixed monolayers of DMPC and cholesterol (7/3 mole ratio) compressed to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl). The subphase tomatine concentrations shown are 1.3, 0.43, and 0.14 μM.

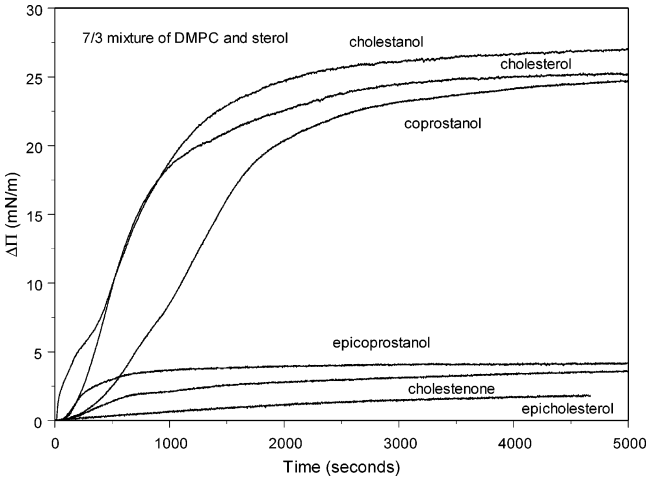

Figure 10. Surface pressure change ($\Delta\Pi$) versus time after injection of tomatine to a subphase concentration of 1.3 μM beneath mixed monolayers of 7/3 mole ratio of DMPC and different sterols, each monolayer initially compressed to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl). The sterols are cholesterol, cholestanol, epicholesterol, cholestenone, coprostanol, and epicoprostanol.

end of the experiment. A similar result occurs for DMPC/cholestanol monolayers. Monolayers of DMPC/epicholesterol appear entirely featureless throughout the interaction with tomatine (not shown). Monolayers of DMPC/cholestenone show the formation of a relatively small number of brighter spots, as shown in Figure 11B, which do not seem to increase much in size or numbers during the experiment. The DMPC/coprostanol system is the most interesting visually, as seen in Figure 11C. In the DMPC/coprostanol monolayers, small starfish-like domains form upon interaction with tomatine, many of which appear to possess a counterclockwise sense of curvature in their arms. These domains become somewhat greater in number by the end of the experiment, but do not change their shape. Such chiral domains have been observed in monolayers of a number of amino acid surfactants, in which condensed phase domain arms curved in one sense can be observed for one enantiomer and arms curved in the opposite sense can be observed for the opposite enantiomer.[41−43] Domains observed in *N*-docosyl-D-leucine[42] are quite similar to those seen in Figure 11C. Similar domains have also been observed in monolayers of a racemic monoglycerol ester, with both directions of arm curvature observed.[44] Monolayers of DMPC/epicoprostanol show the formation of a range of indistinct bright spots during the interaction with tomatine, as seen in Figure 11D.

Discussion

These results demonstrate a strong interaction of tomatine with sterol-containing monolayers that is very sensitive to the molecular structure of the sterol. In the case of DMPC/sterol mixed monolayers, the interaction occurs in the presence of the lipid matrix and it is possible for the complexes to phase separate away from the lipid. The BAM observations indicate that, beyond insertion and complex formation, the complexes can aggregate and form either a textured microstructure or domains. The complexes formed are highly likely to be the 1:1 sterol:tomatine complexes described in liposome studies, and their aggregation is most likely driven by hydrogen bonding between oligosaccharide units and sterol hydroxyl groups in addition to steroid ring stacking. The aggregates formed in some cases may be analogous to the detergent-insoluble pellets obtained on centrifugation of sterol-containing liposomes after their interaction with tomatine or other glycoalkaloids.[10] There are a number of possibilities for the case of tomatine interaction with a pure sterol monolayer: tomatine:sterol complexes could remain uniformly dispersed or could aggregate. It is also possible that the surface pressure increase could result in collapse or squeeze-out of those portions of the monolayer that contain uncomplexed sterols, those regions containing tomatine:sterol complexes, or some combination of both. In the case of DMPC/sterol monolayers, the surface pressures observed after the interaction are all below 40 mN m^{-1} and thus collapse is unlikely. In the case of the DMPC/sterol monolayers, phase separation of the tomatine:sterol complexes away from the lipid matrix is a more likely outcome.

The surface pressure increases observed for the pure sterol monolayers should be related to the strength of the interaction of tomatine with the sterol, the nature of any aggregates formed, and the stability of the resulting mixed tomatine−sterol monolayer. A mixed monolayer of complexes and sterol need not have the same collapse pressure as the pure sterol monolayer; however, it seems likely that the surface pressure increases attainable should be limited or influenced by the stability of the sterol monolayer as given by its collapse pressure. If the insertion and complex formation pushes the surface pressure above the sterol collapse pressure, then uncomplexed portions of the sterol monolayer should begin to collapse. It is not presently clear how to spread and compress mixed films of tomatine and sterol since tomatine is not soluble in chloroform. It is likely that the films observed after the interaction with tomatine have three-dimensional topographic features. It is possible that a combination of tomatine:sterol complex aggregates and collapsed sterol or complex regions are present for some of the sterol monolayers studied. The collapse pressures (Π_c) for the sterols studied vary with sterol structure as seen in Figure 2, and have also been reported by some other workers. Demel and co-workers[32] reported $\Pi_c = 37.2$ mN m^{-1} for cholesterol, $\Pi_c = 34.7$ mN m^{-1} for cholestanol, $\Pi_c = 26.5$ mN m^{-1} for epicholesterol, $\Pi_c = 27.4$ mN m^{-1} for cholestenone, and $\Pi_c = 33.6$ mN m^{-1} for coprostanol, all measured on pure water. The significantly higher lift-off area observed for cholestenone and the higher lift-off area observed for coprostanol were also reported by Demel. An isotherm similar to that shown in Figure 2 for cholestenone was reported by Slotte.[45] Subsequently, $\Pi_c \sim 43.5$ mN m^{-1} has been reported for cholesterol

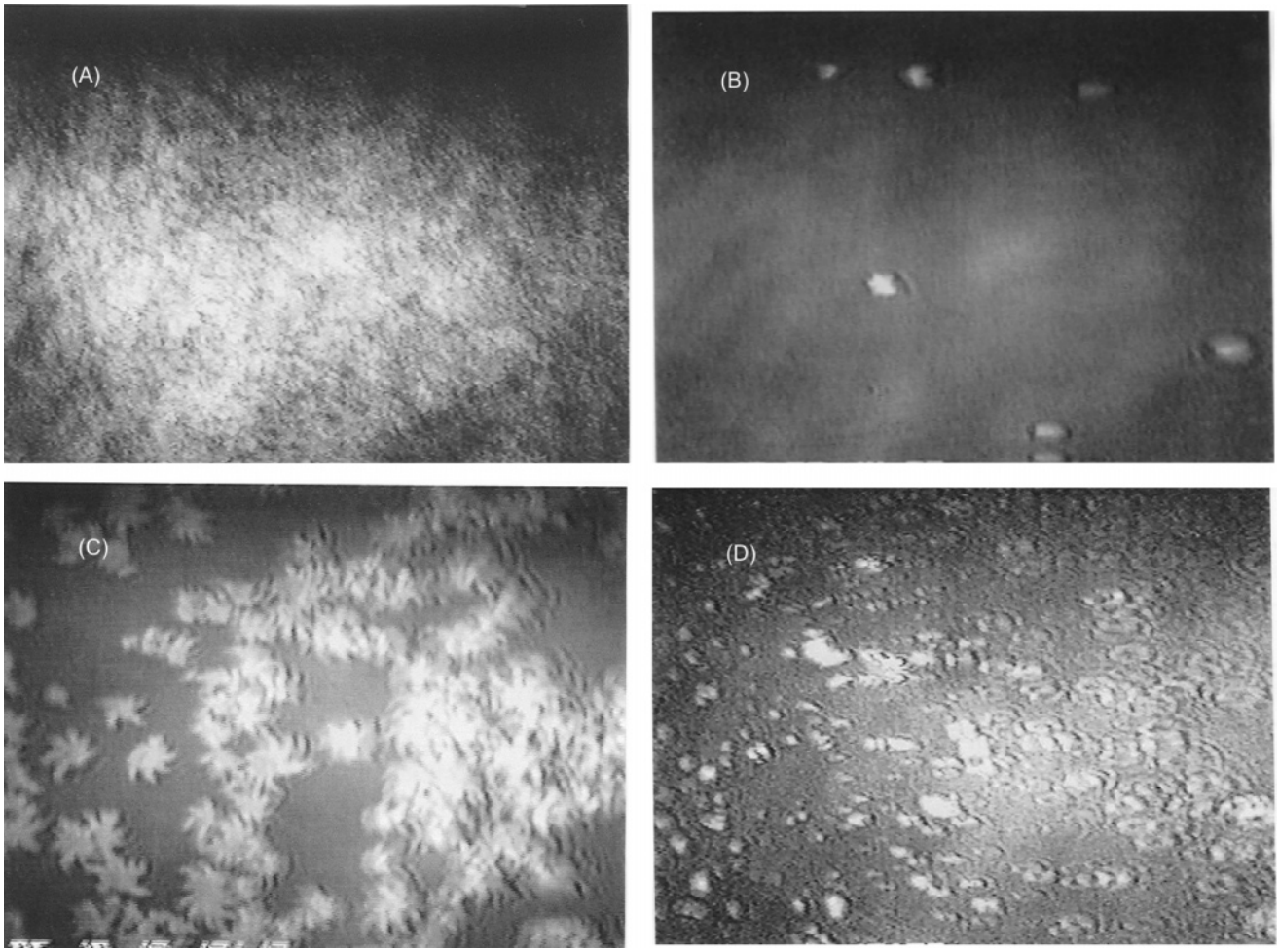

Figure 11. BAM micrographs of mixed monolayers of DMPC/sterol of 7/3 mole ratio interacting with 1.3 μM tomatine after compression to 10 mN m^{-1} on a pH 7 subphase (0.05 M phosphate buffer, 0.10 M NaCl). (A) DMPC/cholesterol 41 min after injection, (B) DMPC/cholestenone 9 min after injection, (C) DMPC/coprostanol 15 min after injection, and (D) DMPC/epicoprostanol 38 min after injection. The field of view is 900 μm $\times$ 700 μm.

by other workers.[46−48] $\Pi_c \sim 41$ mN m^{-1} was reported for epicoprostanol[33] on pure water at a compression rate of 20 Å^2 molecule^{-1} min^{-1}. A $\Pi-A$ isotherm for epicoprostanol that begins rising just above 45 Å^2 molecule^{-1} and with a collapse pressure near 28 mN m^{-1} at 298 K was reported[49] and is similar to that shown in Figure 2. The structures observed by BAM during the interaction of the sterols with tomatine appear well below the collapse pressures. For those sterols (epicholesterol and epicoprostanol) for which a surface pressure increase is observed with no changes observed by BAM, it is possible that some complexation occurs but the interactions, such as optimal hydrogen bonding between the complexes, needed to drive aggregation are missing or weakened by the inversion of the hydroxyl group stereochemistry.

At present, little is known about the detailed molecular structure of tomatine:sterol complexes, such as could be determined if crystals of these complexes could be grown for diffraction studies. The 1:1 stoichiometry for tomatine association with sterols, and the structural match between the steroid ring system of cholesterol and the central steroid-like portion of tomatine, suggests that at the molecular level the complexes involve the side-by-side stacking of one tomatine molecule next to one sterol molecule. Hydrogen bonding between the hydroxyl group on the sterol and some hydroxyl of the oligosaccharide on tomatine, possibly on the galactose residue, should also contribute to the complex stability. Since it is known that removal of even one sugar unit from the oligosaccharide portion essentially removes the ability of glycoalkaloids to disrupt liposomes,[10] the interactions between the intact oligosaccharides are required to drive aggregation of 1:1 complexes. Molecular mechanics calculations of the interaction between cholesterol and the solanidine aglycone portion shared by the potato glycoalkaloids chaconine and solanine, which is similar to the tomatine aglycone tomatidine in structure, favored an orientation in which the methyl groups on the steroid rings of cholesterol and those on the aglycone were oriented away from each other.[10] A complex with the methyl groups oriented toward each other was found to be of higher energy. This view of complex structure would require that tomatine insert into the monolayer next to the sterol with the oligosaccharide immersed and the first four rings stacked next to those of the sterol but most likely in an opposing manner so that the methyl groups projecting from the steroid ring system do not interfere with each other. Methyl group interdigitation between neighboring parallel complexes was postulated as an additional possible driving force for complex aggregation. Tomatine insertion into the monolayer and reorientation would deplete those tomatine molecules adsorbed near the surface beneath the monolayer and then promote further adsorption, insertion, and complex formation.

The structures of the other sterols perturb different aspects of the interactions required to form the molecular complexes or to promote their aggregation. The saturation of the C-5−C-6 double bond of cholesterol in cholestanol has a modest effect. It has been reported that 3α-hydroxy sterols such as

epicholesterol are less effective at condensing phospholipids and that this decrease in the condensing effect may be due to a tilted orientation of this sterol relative to the water−air interface required in order to immerse the −OH group.[33,34] The change in orientation of the −OH group could potentially affect the interaction with tomatine in two ways: interference with the hydrogen bonding between the sterol and tomatine oligosaccharide at the level of complex formation, and a tilting of the complexes due to the tilt of epicholesterol that may alter the interactions between neighboring tomatine oligosaccharides. The cis *A*/*B* ring configuration of coprostanol likely requires stacking of this sterol next to tomatine so that the methyl groups on the steroid rings face either in the same direction or toward each other, but not away from each other, to avoid steric interference from the *A* ring. The large difference in the response of coprostanol versus that of epicoprostanol in interacting with tomatine is noteworthy. The orientation of the hydroxyl group on coprostanol versus that on epicoprostanol suggests that epicoprostanol is likely more tilted at the water−air interface than coprostanol and could alter complex orientation as suggested above for epicholesterol. In addition, the hydroxyl group of coprostanol appears to be oriented in a manner that would be more favorable for hydrogen bonding to a sugar on the oligosaccharide while the hydroxyl group on epicoprostanol appears much less favorably oriented. The interaction of tomatine with cholestenone is perturbed by the loss of hydrogen-bond-donating capacity on replacing the hydroxyl group with the ketone, and by the orientation of cholestenone at the interface, which from the expansion of the cholestenone isotherm is likely significantly tilted. Molecular modeling or spectroscopic data are required to definitively elucidate the structure of these complexes.

In the case of mixed monolayers with DMPC, for which a 7/3 mole ratio was chosen here, the interaction of tomatine with the sterols is also strong and selective and follows a pattern similar to that observed for the pure sterol monolayers. BAM results suggest formation of tomatine:sterol complexes that can phase separate away from the DMPC matrix. Tomatine has the strongest interaction with cholesterol and the similar molecule cholestanol. We observe collapse pressures for 7/3 DMPC/sterol monolayers all near or above 40 mN m^{-1}; thus the surface pressures reached in these tomatine interaction experiments are below collapse so the structures seen by BAM are likely to be true monolayer domains. A collapse pressure near 50 mN m^{-1} has been reported for 7/3 DPPC/cholesterol monolayers.[46] Aggregate formation resulting in a textured appearance is clearly observed for mixed monolayers of DMPC and cholesterol. The observation of no aggregate formation by BAM in the mixtures with epicholesterol is consistent with observations for the pure epicholesterol monolayer. The observation of a weak interaction with epicholesterol is consistent with the reported liposome studies and emphasizes the key role of the hydroxyl group. The observation of very little aggregate formation for the mixed monolayer with cholestenone is in contrast to the observation of domains during the interaction with the pure cholestenone monolayer. Since cholestenone can only accept a hydrogen bond and not donate one and likely has a strongly tilted orientation, it appears that there is an insufficient driving force for aggregate formation in the lipid matrix. The domains that appear in the interaction of tomatine with DMPC/coprostanol are structured and appear to be chiral, suggesting the phase separation of an asymmetric and organized phase of tomatine:coprostanol complexes away from the lipid matrix. The monolayer results for coprostanol showing significant interaction are in contrast to the results reported for liposomes;[10] this is possibly related to the difference between interactions occurring on a curved liposome surface versus those occurring in a flat monolayer. The much lower response for DMPC/epicoprostanol monolayers as compared to DMPC/coprostanol monolayers is likely related to a change in orientation due to the inverted stereochemistry of the hydroxyl group.

Given their medicinal and agricultural significance, study of the interaction of glycoalkaloids and related saponins with model membrane systems using physical methods appears of interest and likely to yield further interesting observations. It is hoped that these studies will ultimately provide insight into possible further application of these compounds in drug delivery and immunological formulations. The saponins and glycoalkaloids have recently begun to attract the attention of synthetic carbohydrate chemists, and the unique interactions between the oligosaccharide portions of these molecules may soon be able to be exploited in synthetic bioconjugates.[50,51]

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