The influence of structural modifications in sterols and phospholipids on the rate of polyene antibiotic-sterol interaction was studied. For filipin and amphotericin B association with sterols in vesicles, a preferential interaction was found with sterols whose side chain length is close to that of cholesterol. Introduction of trans double bonds into the sterol side chain did not alter the rate of interaction in vesicles. The Δ⁷-bond of the sterol appears to be of critical importance in amphotericin B-sterol interaction, whereas the Δ⁵-bond is not essential. These observations are relevant to the well-known effects of amphotericin B on cell membranes containing ergosterol compared with those containing cholesterol. The dependence of the rates of sterol-polyene antibiotic interaction on the phospholipid composition of the vesicles indicates that phospholipid vesicles may be an inadequate model for reaching a comprehensive understanding of the effects exerted on biological membranes by these agents.

The binding of filipin and amphotericin B to 3β-hydroxy-sterols has generated a great deal of interest (reviewed in Refs. 1 and 2, and references cited therein). Filipin has served in rapid kinetic and cytochemical studies as a probe of sterol distribution in biological membranes (for review, see Ref. 3) and lipoproteins (4). Amphotericin B is useful clinically against a wide range of fungi (2, 5). The extent to which sterol structure influences the ability of these polyene antibiotics to form complexes has been focused mainly on comparisons of binding in ergosterol- and cholesterol-containing membranes (2, 6–11). There are indications that the binding of filipin (12–14) and the effects of amphotericin B on membranes (11, 15, 16) are sensitive to structural changes in the alphatic side chain and/or B ring of the sterol. We have undertaken kinetic studies of the binding of filipin and amphotericin B with a number of sterols to evaluate the contributions of changes in the structure of the side chain and B ring. We also report here that amphotericin B-sterol interactions are dependent on the phospholipid composition of the membrane. This finding increases the difficulty in reaching a comprehensive understanding of the membrane action of amphotericin B at the molecular level.

Materials and Methods

Chemicals—The sources of amphotericin B and filipin were cited previously (9, 17). The filipin complex was purified as described previously (17); amphotericin B was used without additional purification. Stock solutions of the polyenes (filipin in dimethylformamide; amphotericin B in dimethyl sulfoxide, under argon) were stored for several days at -20 °C. Aqueous solutions of the polyenes were prepared daily under subdued light. The concentrations were determined spectrophotometrically using the extinction coefficients cited (9, 17). The buffer used in all of the experiments was 5 mM Hepes containing 2.5% glucose, pH 7.4. For experiments with amphotericin B, the buffer was deoxygenated before use by boiling for 2 min, followed by cooling under a stream of argon. The antioxidant n-propyl gallate (Aldrich Chemical Co.) was added (20 ppm final concentration) The final concentrations of dimethylformamide and dimethyl sulfoxide in aqueous filipin and amphotericin B solutions, respectively, were 0.3 and 1%. All manipulations with filipin and amphotericin B were performed under subdued light.

Egg PC, DMPC, egg PG, bovine heart DPG (cardiolipin), cholesterol, desmosterol, 7-dehydrocholesterol, β-cholesterol (dihydrocholesterol), and 25-hydroxycholesterol were obtained from Sigma. Egg PB (Cyclo Chemical Co.) was purified by addition of cold acetone to a concentrated solution in chloroform, which precipitated egg PE. Ergosterol and β-sitosterol were obtained from both Sigma and Steraloids (Wilton, NH). Androst-5-en-3ß-ol (referred to as C19) and pregn-5-en-3ß-ol (referred to as C21) were from Steraloids. The sterols were purified by recrystallization several times from ethanol, except for desmosterol which was purified by preparative thin-layer chromatography on Silica Gel H plates (Analytich, Newark, DE) developed with diethyl ether/petroleum ether (3:1 v/v). The sterols and phospholipids migrated as single spots on Silica Gel G plates (18) when visualized by sulfuric acid spray and charring. The sources of 20-isocholesterol, the unsaturated side chain derivatives (cis- and trans-22-dehydrocholesterol and cholesta-5,22E,24-trien-3ß-ol), and the cholesterol analogs with side chains of different lengths (referred to by the total number of carbon atoms they contain—C22, C24, C26, C28, and C30) were cited previously (19).

Preparation of Vesicles—The sonicated vesicles used in the filipin-binding studies contained DMPC, sterol, and 4 mol % dicetyl phosphoric acid (phospholipid-sterol molar ratio, 3:1). For the amphotericin-binding studies (Tables IV and V, Figs. 2 and 3), vesicles were prepared from the phospholipids indicated without any sterol or dicetyl phosphoric acid. The phospholipid dispersions were sonicated for about 30 min in a cup horn under nitrogen (Heat Systems Ultrasonics W375A). Undispersed phospholipids and multimamellar liposomes were removed by centrifugation at 12,000 rpm for 30 min at 4 °C in a SS34 rotor.

Mycoplasm gallisepticum Membranes—M. gallisepticum strain A5603 was grown with various Δ sterols and membranes were prepared by sonication of dilute cell suspensions at 0 °C as described previously (19).

Kinetic Measurements—Changes in the absorbance spectra of polyene antibiotics in the presence of vesicles reflect binding of polyene species to membrane components (reviewed in Refs. 20, 21, and 22). In some studies, correlations have been examined between

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The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid; PC, phosphatidylcholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; PG, phosphatidylglycerol; DPG, diphasphatidylglycerol; PE, phosphatidylethanolamine.
Sterol-Polyene Antibiotic Interaction

the rate of sterol-polyene antibiotic interaction and the rate of polyene-induced changes in membrane permeability and structural properties (23-25). We measured the rapid reaction of filipin with vesicle or mycoplasma membrane suspensions at 10 °C in a stopped flow apparatus. The initial rate of absorbance change of filipin per s, \( dA/dt \), was calculated from oscilloscope traces as described previously (17). Amphotericin B was added using the relation \( k_2 = dA/dt \Delta_{\text{total}}^{-1} \), where \( dA/dt \) is the initial rate of filipin-sterol association in absorbance units \( s^{-1} \) and \( \Delta_{\text{total}} \) is the total absorbance change (which is a function of filipin concentration). Since amphotericin B interacts with both sterol-free and sterol-containing vesicles (2, 9, 24), we estimated the rates of amphotericin-sterol interaction using vesicles that had been pre-equilibrated with amphotericin B. The decrease in amphotericin B absorbance accompanying the addition of an ethanolic sterol solution to the amphotericin-containing phospholipid vesicles was monitored as a function of time as follows. To a cuvette (1-cm path length) containing 1 ml of vesicles (400 \( \mu \)M total phospholipid) were added 10 \( \mu \)l of 0.2% n-propyl gallate and 10 \( \mu \)l of an amphotericin B stock solution (0.4 mM). The vesicle suspension was added to both the sample and reference cuvettes to avoid errors from light scattering of the vesicles. The absorbance of amphotericin B at 406 nm reached an equilibrium value after about 3 min, with stirring under argon at 25 °C. This suggests that partitioning between the membrane and aqueous phases and interconversions involving bound forms of amphotericin B had attained equilibrium. The kinetics of binding of amphotericin B to sterols in vesicles was initiated by adding 10 \( \mu \)l of an ethanolic sterol stock solution (1 mM). Sterol was also added to the reference cuvette. The decrease in the absorbance of amphotericin B accompanying binding was recorded at 25 °C on a Perkin-Elmer Hitachi Model 320 spectrophotometer until no further change occurred (up to ~45 min, depending on the sterol).

Analytical Procedures—Lipid phosphorus and sterol content were determined as described previously (19). The standard curve obtained with cholesterol was used for assay of the cholesterol analogs bearing side chains of different lengths or unsaturation. The wavelengths used in estimating sterol content were 542 nm for \( \beta \)-sitosterol, 555 nm for desmosterol, 590 nm for 25-hydroxycholesterol, and 550 nm for the other sterols. 25-Hydroxycholesterol was incorporated into the vesicles at about 70-75% of the input amounts.

RESULTS

Sterol-Filipin Interaction in Vesicles—Filipin binds to sterols that have a planar ring system, a free 3\( \beta \)-hydroxyl group, and an aliphatic side chain (12, 13). We studied the initial rates of filipin binding to sterols with modified side chain structures. Fig. 1 presents the dependence of the initial rates on the concentrations of sterols in DMPC vesicles containing 25 mol % sterol. The linear increase of \( dA/dt \) with sterol concentration is indicative of a binding process. The rates of association are slightly higher with desmosterol, ergosterol, and \( \beta \)-sitosterol than that with cholesterol. These sterols have side chains of approximately the same length, but differ in that desmosterol contains a double bond at C-24, ergosterol a double bond at C-22 and a methyl group at C-24, and \( \beta \)-sitosterol an ethyl group at C-24. The substitution of a polar group into the side chain, as in 25-hydroxycholesterol, results in a marked decrease in the initial rate of filipin binding (Fig. 1A). Fig. 1B shows that reversal of the configuration at C-20 enhances the initial rate of filipin binding relative to that shown in Fig. 1A for cholesterol; however, the total absorbance change was also increased (to be considered in Table I). Introduction of trans double bond(s) into the side chain does not alter the rate of interaction with filipin, as shown with trans-22-dehydrocholesterol and cholesta-5,22E,24-trien-3\( \beta \)-ol. The latter sterols have greatly extended side chains. cis-22-Dehydrocholesterol, which has a side chain with a kinked conformation, interacts with filipin at a markedly lower rate. The influence of side chain length on the kinetics of filipin-sterol interaction was examined using sterols in the \( 20(R) \)-n-alkylpregn-5-en-3\( \beta \)-ol series. The terminal methyl branch at C-25 of cholesterol is absent in these analogs. Fig. 1C shows that sterol C26, whose side chain is almost the same length as that of cholesterol, has a much faster rate of binding to filipin than the other cholesterol analogs in this series. It is interesting that C28 and C24, whose side chains are similar in length to that of cholesterol, have lower rates than C26 at each sterol concentration we studied. Cholesterol analogs with very short side chains (C21 and C22) and analogs with no side chain (C19) and a long aliphatic chain (C30) have very low rates of interaction with filipin. The slopes of the \( dA/dt \) versus [sterol] plots and the values of the total absorbance changes measured in the binding process are listed in Table I. The relative second order rate constants for binding to filipin (\( k_2 \) values of the various sterols relative to that found with cholesterol) fall into the following three categories: 1) desmosterol, cholesterol, \( \beta \)-sitosterol, and 20-isocholesterol > 2) trans-22-dehydrocholesterol, cholesta-5,22E,24-trien-3\( \beta \)-ol > 3) cis-22-dehydrocholesterol, 25-hydroxycholesterol. A similar analysis of the kinetic data for sterols of differing side chain lengths is given in Table II. The relative second order rate constants are in the order C26 > C24, C28 > C22, C30, C21, C19. Thus, the rate of the binding process is sensitive to the length of the sterol side chain. The slope of the \( dA/dt \) versus [sterol] plot and the \( \Delta_{\text{total}} \) side chain lengths is given in Table II. The relative second order rate constants for \( \beta \)-sitosterol-filipin interaction are also much lower than that for cholesterol-filipin interaction. Thus, side chain bulk and conformational rigidity reduce the rate of interaction in the membranes but not in the vesicles. However, alteration of side chain length, without an increase in bulk or rigidity, does not result in a change in second order rate constant in M. gallisepticum membranes. The reasons for the differences between vesicles and mycoplasma membranes with respect to the influence of sterol structure on \( k_2 \) are not clear, but differences in protein and phospholipid composition may be considered.

Sterol-Amphotericin B Interaction in Vesicles—When ethanolic sterol solutions are added to amphotericin-containing vesicles, the absorbance of amphotericin B decreased at 385 and 408 nm. However, the absorbance remained the same at shorter wavelengths (330, 346, and 364 nm). This suggests that an increase in the state of aggregation of amphotericin B does not take place under these conditions, since self-association involves an increase in the absorbance at the shorter wavelengths. Fig. 2 shows the time course of the absorbance change accompanying the addition of sterols to vesicle suspensions containing bound amphotericin B. The lag period of about 3 min may reflect the partitioning of the sterols into the bilayer. It has been shown that when thyrocholsterol is added as an ethanolic solution to egg PC vesicles containing trypared 5,5'-dithiobis-(2-nitrobenzoic acid), sterol movement from the external to internal surfaces of the bilayer
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is rapid at 20 °C (t₀ ~ 1 min) (26). Several other studies have also shown that cholesterol undergoes very rapid uptake from buffer to membranes, as well as very rapid transbilayer movement (27, 28). Although no data are available for the rates at which aggregates of other sterols partition from aqueous alcohol suspension into unilamellar vesicles, sterols that are similar in structure and polarity to cholesterol and thiocholesterol are also expected to undergo rapid transbilayer movement. However, we cannot exclude the possibility that the lag period may indicate that an initially formed amphotericin-sterol complex undergoes conversion to another form of the complex.

The rate and extent of absorbance change of amphotericin B on addition of sterol to egg PC-egg PE vesicles follows the order of 7-dehydrocholesterol > cholesterol > β-sitosterol (Fig. 2). The inset to Fig. 2 shows the influence of the PE content of the bilayer on the initial rates of amphotericin B interaction with each of these sterols. (The initial rates were estimated from the slopes of the tangent to the absorbance decrease after the lag period is completed.) The initial rate of 7-dehydrocholesterol-amphotericin B binding is particularly sensitive to the PE content. Table IV lists the results of a series of experiments in which we examined the influence of phospholipid composition on the rate of interaction of 10 μM sterol with 4 μM amphotericin B. Experiments 1-5 show that when the egg PE content in PC-PE vesicles is increased from 0 to 67.5 mol %, the rate of 7-dehydrocholesterol-amphotericin B binding increases by a factor of 2. We also compared results obtained using vesicles from PC, PE, and PG with the same fatty acyl chain content. A comparison of the results of experiments 6 and 7 indicates that the rate of binding with each sterol decreases on substitution of PG for PE in vesicles containing 50 mol % PC and 25 mol % DPG. However, a comparison of experiments 1 and 8 suggests that the rates of binding of these sterols to amphotericin B are similar in vesicles prepared from egg PC and egg PG alone. Finally, experiments 8-10 show that the rates with each sterol decrease on increasing the DPG content of bilayers prepared from mixtures of egg PG and DPG.

The rates of interaction of amphotericin B in DMPC vesicles with different sterols are presented in Fig. 3 and Table V. The fast rates are observed with the Δ⁶,⁷-dienes ergosterol and 7-dehydrocholesterol (as noted earlier in Table IV and Fig. 2 using vesicles from other phospholipids). The rates with cholesterol, β-sitosterol, and cis- and trans-22-dehydrocholesterol are significantly slower. The finding that the rate of interaction with cholesta-4,8-dien-3β-ol exceeds that with cholesterol indicates that the Δ⁶,⁷-diene structure is not re-
Comparison of filipin binding kinetics to sterols in DMPC vesicles: effects of changes in side chain alkylation, unsaturation, configuration, and hydrophobicity

Initial rates were measured at 10 °C. The final concentrations of filipin and total lipid were 8 μM and 2 mM, respectively. DMPC:sterol molar ratio, 3:1. The number of separate vesicle preparations used is shown in parentheses.

| Sterol                                    | 10^6 × [dA/dt]/[sterol]^a | ΔA_{final}b | Relative k_2 |
|-------------------------------------------|-----------------------------|-------------|--------------|
|                                           | absorbance                  | absorbance  |              |
|                                           | units s^-1                   | units       |              |
| Cholesterol (4)                           | 1.6                         | 0.102       | 1.00         |
| Desmosterol (3)                           | 1.8                         | 0.105       | 1.10         |
| Ergosterol (2)                            | 1.6                         | 0.105       | 0.97         |
| β-Sitosterol (3)                          | 1.6                         | 0.108       | 0.94         |
| trans-22-Dehydrocholesterol (2)           | 1.5                         | 0.125       | 0.76         |
| cis-22-Dehydrocholesterol (2)             | 0.93                        | 0.120       | 0.49         |
| Cholesta-5,22E,24-trien-3β-ol (3)         | 1.5                         | 0.124       | 0.77         |
| 20-Isolcholesterol (3)                    | 2.5                         | 0.177       | 0.89         |
| 25-Hydroxycholesterol (4)                 | 0.53                        | 0.088       | 0.35         |

* The parameter 10^6 × [dA/dt]/[sterol] was calculated by least squares analysis of the slopes of the straight lines in plots of dA/dt versus [sterol] (see Figs. 1 and 2). At least four different sterol concentrations were used in measurements of dA/dt, with the filipin concentration maintained constant.

a ΔA_{final} is the difference between the initial and final absorbances at 390 nm, which were measured in the stopped flow apparatus.

Effect of sterol side chain length on the kinetics of filipin-sterol association in DMPC vesicles

The temperature and concentrations are the same as in Table I. Values are the means from two (C30, C28, and C22) or three (C26, C24, C21, and C19) separate vesicle preparations.

| Sterol                                    | 10^6 × [dA/dt]/[sterol]^a | ΔA_{final} | Relative k_2 |
|-------------------------------------------|-----------------------------|------------|--------------|
|                                           | absorbance                  | absorbance |              |
|                                           | units s^-1                   | units      |              |
| C30                                       | 0.45                        | 0.140      | 0.21         |
| C28                                       | 1.2                         | 0.184      | 0.42         |
| C26                                       | 3.8                         | 0.250      | 0.97         |
| C24                                       | 1.3                         | 0.186      | 0.45         |
| C22                                       | 0.50                        | 0.138      | 0.23         |
| C21                                       | 0.22                        | 0.079      | 0.18         |
| C17                                       | 0.14                        | 0.075      | 0.12         |

* The parameter 10^6 × [dA/dt]/[sterol] was calculated as in Table I. The second order rate constants are relative to the k_2 obtained with cholesterol (see Table I).

DISCUSSION

Although the knowledge of the effects of polyene antibiotics on sterol-containing membranes has reached a detailed level of understanding, the influence of structural modifications in the sterols and phospholipids is only beginning to be investi-
was reported previously that the requirements with respect to sterol structure are the same for the interaction with filipin and for the exertion of maximal effects on the properties of bilayers (12). The low values of the second order rate constants found with cis-22-dehydrocholesterol and 25-hydroxycholesterol (Table I) support this hypothesis. The kinked conformation of cis-22-dehydrocholesterol and the presence of a polar substituent in the side chain of 25-hydroxycholesterol have been proposed to reduce the ability of these sterols to interdigitate between the fatty acyl chains of phospholipids (32, 33). Nakamura et al. (16) also reported that amphotericin B requires a sterol side chain with at least five carbon atoms for maximal interaction in liposomes. Our kinetic data also show that sterols with very short (C19, C22) or very long (C32) side chains do not give measurable rates of interaction with amphotericin B under the conditions used (Table V).

The change in the absorbance of amphotericin B is larger per unit time with 7-dehydrocholesterol than with cholesterol (Table IV, Fig. 2). Since 7-dehydrocholesterol is as effective as cholesterol in increasing the degree of lipid order of phospholipids in the liquid-crystalline phase (34), this result does not appear to reflect a difference in membrane fluidity in the sterol-containing bilayers. Two other sterols that have additional unsaturation in the ring system compared with cholesterol, i.e., ergosterol and cholesta-4,6-dien-3α-ol, gave a result similar to that obtained with 7-dehydrocholesterol. It should also be noted that the effects of ergosterol and cholesterol on the lipid order parameter are very similar in vesicles (35). Thus, the lack of a differential perturbation of bilayer properties by these sterols suggests that the preferential binding of amphotericin B to ergosterol may explain why ergosterol-containing cells are more sensitive to this antibiotic than cholesterol-containing cells. Ergosterol differs structurally from cholesterol with respect to both the B ring and side chain, and cholesta-4,6-dien-3α-ol contains double bonds in the A and B rings. Since the ΔA/Δt values are not changed significantly compared with cholesterol when unsaturation (cis- and trans-22-dehydrocholesterol) or an alkyl group (β-sitosterol) is introduced into the side chain, we conclude that the increase in the rate of interaction with ergosterol arises from the additional double bond in the B ring.

There has been speculation that ergosterol-containing cell
membranes are more sensitive to amphotericin B than cholesterol-containing cell membranes because of differences in the structure of the amphotericin-stereol complexes (see references in Ref. 2). The differences in circular dichroism spectra (10) and in the numbers of binding sites per sterol molecule and apparent association constants (9) of amphotericin complexes with ergosterol or 7-dehydrocholesterol compared with cholesterol support the conclusion that the B ring (especially the $\Delta^1$-bond) is one of the specific sites involved in the interaction with the polypeptide. A plot of the rate of amphotericin B binding to sterols in the egg PC vesicles versus sterol concentration shows a break at $\sim 3.6$ mol % for ergosterol and 7-dehydrocholesterol, and at $\sim 4.8$ mol % for cholesterol and 5-sitosterol (Fig. 3). Above this point, the rate becomes zero order in sterol. Since different conformations of amphotericin B have been detected in sterol-containing vesicles (10) and since amphotericin may be localized in environments of different rigidity in the bilayer (10), the break in Fig. 3 may represent the transition between different types of amphotericin-sterol complexes.

Phospholipid structure influences the interaction of amphotericin B with sterol-containing vesicles. The effects of varying the degree of lipid order (by changing the length or unsaturated content of the hydrocarbon chains) (17, 36) and the bilayer thickness (37) have been considered previously. The rate of interaction of 7-dehydrocholesterol with amphotericin B in PC-PE vesicles increases significantly with increasing egg PE content. Since these phospholipids have identical hydrocarbon chains, the rate enhancement must arise from the change in head-group structure (which may consequently cause changes in the extent of hydration and intermolecular hydrogen bonding). We previously interpreted stopped flow measurements of amphotericin-sterol association in vesicles in terms of a competition between amphotericin-lipid and lipid-lipid interactions (24). The rate enhancement found on PE incorporation may be explained by the increased intermolecular hydrogen bonding of PE, which may increase the accessibility of cholesterol for interaction with amphotericin B (38). It should be noted also that calorimetry studies with saturated PE and PC are not in agreement the data in Table IV also show that increasing the DPG content of the bilayer causes the rate of amphotericin B binding to 7-dehydrocholesterol, cholesterol, and 5-sitosterol to decrease.

The rate of filipin association with cholesterol in vesicles also varies with phospholipid composition. For example, $k_2$ values increase when phosphatidylserine (17) or lysoPC (41) is substituted for PC or when the extent of unsaturation in the fatty acyl chains of PC increases (41). It should be noted, however, that the initial rate was not affected on incorporation of ribonucleic acid, albumin, and cytochrome $c$ into vesicles (41). Since phospholipid structure influences the rates of filipin and amphotericin B association with sterols, phospholipid vesicles may be an inadequate model system for reaching conclusions about the molecular specificity of the interaction between these polypeptide antibiotics and sterols.

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