Stopped-Flow Kinetic and Equilibrium Studies of Filipin III Binding to Sterols†

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ABSTRACT: Measurements were made of the rapid kinetics and the apparent dissociation constants (K_d) of the binding of the polyene antibiotic, filipin III, to sterols in aqueous dispersion with egg lecithin. Stopped-flow studies revealed that the initial rate of association of filipin III with cholesterol is faster in sonicated aqueous dispersions (vesicles) than in unsonicated aqueous dispersions (liposomes). Osmotically swollen liposomes give faster initial rates of association with filipin III than osmotically shrunken liposomes. No association reaction was observed in the stopped-flow apparatus with liposomes prepared from lecithin alone. The rate and degree of binding depend on both the absolute concentration of cholesterol and on the mol % of cholesterol in the bilayer. The initial rate of association of filipin III with aqueous suspensions of cholesterol devoid of phospholipid was lower than that with liposomes or vesicles. Changes occurring in the ultraviolet absorption and fluorescence polarization of filipin III in the presence of vesicles of varying lipid concentration were used to determine binding constants. The K_d values at 25° for the binding of filipin III to lecithin–sterol vesicles were: epicholesterol, 13 μM; ergosterol, 2.47 μM; and cholesterol, 0.80 μM. Despite the low intrinsic affinity of filipin III for lecithin (K_d = 144 μM), at high lecithin concentrations the polyene binds to vesicles prepared from lecithin alone. This is apparently a reflection of the law of mass action. Sterol accessibility, molecular packing of the lipids in the bilayer, and phospholipid/sterol molar ratio are seen to be important parameters in the binding of filipin III to sterols.

It is well established that in many cells membrane-bound sterol is required for sensitivity to polyene antibiotics. It is generally accepted that the polyene antibiotics share the same basic mechanism of action, *i.e.*, of first binding to sterols in cell membranes and thereby causing impairment of membrane function, leakage of essential metabolites, alteration in cell metabolism, and eventually cell death. Differences in potencies among the polyenes are thought to be attributable to their varying affinities for sterols. In fact, correlations of potencies and equilibrium constants for association of polyene antibiotics with aqueous suspensions of cholesterol have been noted (Bittman and Fischkoff, 1972). However, no studies have been reported concerning the quantitative estimation of binding constants of polyene antibiotics with membrane-bound sterols. Although it was noted that filipin caused rapid hemolysis of mammalian erythrocytes (Kinsky *et al.*, 1962) and that a critical factor in determining sensitivity to nystatin was absorption by the fungal cell (Lampen *et al.*, 1959), the kinetics of the binding of polyene antibiotics to sterols have not been carefully studied. The present investigation is concerned with the equilibrium and kinetic parameters of the binding of filipin III to sterols. Flipin III is the principal component of the mixture of filipins known as the filipin complex (Bergy and Eble, 1968). It is the most hemolytic of the filipins (Sessa and Weissmann, 1968) and, together with filipin II, accounts for most of the antifungal properties of the filipin complex (Bergy and Eble, 1968).

Two types of model membranes are used in this study. Unsonicated suspensions of lecithin (with or without sterols) in water spontaneously form multilamellar structures consisting of a number of concentric vesicles of lipid bilayers of various sizes (Bangham *et al.*, 1965); on prolonged ultrasonic irradiation, vesicles are formed that are bounded by a single bilayer wall enclosing a volume of aqueous solution (Huang, 1969). In this paper unsonicated dispersions are referred to as liposomes, whereas sonicated preparations are referred to as vesicles.

Experimental Section

Materials. Egg lecithin was isolated and purified as described previously (Bittman and Blau, 1972). Cholesterol was purchased from Sigma, and epicholesterol and ergosterol from Schwarz/Mann. The purification of the sterols was performed as described previously (Bittman and Blau, 1972; Bittman and Fischkoff, 1972). Dicetyl phosphate and Tris were obtained from Sigma. Filipin III was generously supplied as lot number U-25,639 by Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich. Stock solutions were prepared in dimethylformamide (DMF). Aliquots of the stock solution were transferred to buffer solutions to give the desired concentrations of filipin and DMF. Kinetic experiments were conducted in 10 mM Tris–60 mM KCl (pH 7.2) and equilibrium experiments in 1 mM Tris–10 mM NaCl (pH 7.4). The extinction coefficient of filipin reported previously (Bittman and Blau, 1972) was used for determination of the molarities of the antibiotic solutions. The final concentration of DMF was 0.5% (v/v) in kinetic experiments and 0.3% (v/v) in equilibrium experiments, except in experiments using aqueous suspensions of cholesterol. The concentrations of DMF used in aqueous suspensions of cholesterol are cited in the caption to Figure 2. The concentrations of filipin, lipids, and DMF are reported in terms of the final concentration obtained after the antibiotic solution and lipid preparation were mixed.

Preparation of Liposomes and Vesicles. Liposomes were prepared from egg lecithin and from mixtures of egg lecithin and sterols as described previously (Bittman and Blau, 1972). Vesicles were prepared by ultrasonic irradiation under nitrogen at 4° with a 20-kHz Branson sonifier (Model S-110)

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fitted with a solid tap horn at power level 4. All liposomes and vesicles contained 4 mol % of dicetyl phosphate. Lipid dispersions subjected to differing periods of ultrasonic irradiation were examined by Dr. O. R. Anderson using negative-staining electron microscopy as described in the accompanying paper (Bittman *et al.*, 1974). In each preparation the proportion of single-shelled vesicles present was estimated by counting a minimum of 100 lipid particles in four different areas of the grid. Unsonicated liposome preparations consisted of large multiconcentric particles; few single bilayer vesicles were present. The percentage of single bilayer vesicles present increased with sonication time as follows: 15 sec, 29%; 30 sec, 68%; 2.5 min, 73%; 20 min, 88%.

Kinetic and Spectral Measurements. Rapid mixing of filipin solutions with liposomes, vesicles, or aqueous suspensions of cholesterol was performed in a stopped-flow apparatus (Durrum Instrument Co., Palo Alto, Calif.). The initial change in transmittance at 360 nm was monitored on a Tektronix storage oscilloscope equipped with a Polaroid camera. A slit width of 1 mm was used. The amplitude of the initial reaction that was analyzed represents about 10–15% of the total amplitude of the reaction. The kinetic results reported represent the analysis of at least five separate photographs of the reaction under study. Initial rate values are reported as the average of these determinations, followed by the average error of the mean. Absorption measurements were carried out on a Cary 14 spectrophotometer using cells having path lengths of 1, 5, and 10 cm. Fluorescence measurements were performed on a Hitachi-Perkin-Elmer spectrofluorometer, Model MPF-2A, equipped with a polarizer accessory, using excitation and emission slit widths reported previously (Bittman and Fischkoff, 1972). Spectra were corrected for light scattering of the vesicles. Fluorescence polarization measurements were made at 18°. Kinetic and absorption measurements were made at 25°.

Results

Initial Rate of Filipin–Cholesterol Association. Figure 1 shows that the dependence of the initial rate of association of filipin with liposome- and vesicle-bound cholesterol is characterized by a lag period at very low filipin concentrations, followed by a region of rapidly increasing rate with increasing filipin concentration, and finally by a region where the rate approaches saturation at high filipin concentrations. Reactions occurring after the initial association reaction were also observed, but their reproducibility was poor. Therefore, kinetic results reported in this paper deal only with the rapid initial association step. At a given concentration of filipin, the initial rate of association of the polyene with vesicle-bound cholesterol is greater than that with liposome-bound cholesterol, despite the use of lower concentration of cholesterol in the vesicles. Sonication reduces the average size of the liposomes and increases the surface area. The observations that higher initial rates are found for association with vesicle-bound cholesterol and that the rate at saturation is greater in vesicles than in liposomes suggest that the rate of association depends on the accessibility of cholesterol for filipin. Table I summarizes data concerning the effect of sonication time on the initial rate of association of filipin with vesicles. As the proportion of single-walled vesicles increases, the initial rate of association increases. Further support for the dependence of the initial rate of association on liposome or vesicle structure is reported in Table II. The degree of molecular packing of lipids in liposomes was modified by subjecting liposomes prepared in KCl or sucrose to osmotic pulses with the same electrolyte or nonelectrolyte. The initial rate of interaction of filipin with cholesterol in liposomes that had been subjected to osmotic shrinking conditions (tighter packing of lipid molecules) was decreased compared to that with cholesterol in liposomes that had not been exposed to osmotic shock; conversely, the initial rate with liposomes that had been subjected to osmotic swelling conditions (looser packing of lipid molecules) was increased compared to that with liposomes that had been diluted under isotonic conditions (Table II).

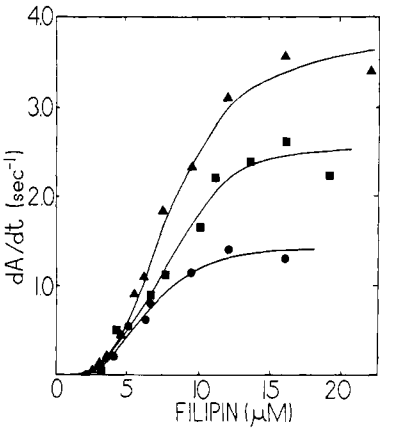

FIGURE 1: Effect of filipin concentration on the initial rates of association of filipin with lecithin–cholesterol liposomes and vesicles. Liposomes and vesicles were prepared from equimolar concentrations of lecithin and cholesterol. The cholesterol and lecithin concentrations were: (▲) 60 μM; (●) 240 μM; and (■) 480 μM. The vesicles (▲) were sonicated for 2.5 min.

Figure 2 illustrates that the initial rates of interaction of filipin with cholesterol in aqueous suspension are low, but increase markedly when cholesterol is incorporated into liposomes. More dramatic rate enhancements are observed with cholesterol-containing vesicles. The slopes of log–log plots of these data give the order of the reaction with respect to cholesterol. For aqueous suspensions of cholesterol having DMF concentrations of 2, 5 (not shown), and 10% (v/v), the order with respect to cholesterol in aqueous suspension is approximately 1; however, a more complex behavior applies in liposomes and vesicles, where the order is 0.6. (The data shown in Figure 1 for the binding of filipin to cholesterol in liposomes and vesicles also lead to noninteger values for the order with respect to filipin. Log–log plots for data obtained in the region of filipin concentration between 3 and 13 μM give slopes of 1.5–2.0 for liposomes and about 3 for vesicles that were obtained after 2.5 min of sonication.) For liposomes

TABLE I: Effect of Sonication Time on Initial Rate of Association of Filipin with Vesicle-Bound Cholesterol.[a]

Time of Sonication (min)	dA/dt (sec^{-1})
0.00	0.32 ± 0.01
0.25	0.89 ± 0.03
0.50	0.97 ± 0.05
1.50	1.35 ± 0.06
15.00	1.16 ± 0.10

[a] The concentration of filipin was 5.5 μM. The concentration of cholesterol was 80 μM. The total lipid concentration was 0.165 mM.

TABLE II: Dependence of Initial Rate of Filipin–Cholesterol Association on State of Lipid Packing in Liposomes.

Liposomes in		Mixed with			
KCl (M)[a]	Sucrose (M)[b]	KCl (M)	Sucrose (M)	Type of Change	dA/dt (sec^{-1})
0.06		0.06		Isotonic	0.89
0.06		0.18		Shrinking	0.64
0.06		0.02		Swelling	1.36
	0.10		0.10	Isotonic	0.61
	0.10		0.26	Shrinking	0.42
	0.10		0.00	Swelling	0.70

[a] Liposomes were subjected to an equal volume of isotonic, hypertonic, or hypotonic solution of KCl buffer. The resulting liposomes were mixed in the stopped-flow apparatus with filipin. The final concentration of filipin was 6.2 μM. The final concentration of lipid was 1.0 mM. Liposomes were prepared from an equimolar ratio of lecithin and cholesterol. [b] Same as *a* except that 0.10 M buffered sucrose was used instead of 0.06 M KCl, and the final concentrations of filipin and lipid were 5.5 μM and 0.9 mM, respectively.

containing an equimolar ratio of lecithin to cholesterol but varying total lipid concentration, the initial rate of association of filipin is linear with cholesterol concentration when the latter exceeds about 10 μM at the filipin concentration used. Liposomes were also prepared in which the total lipid concentration was maintained constant but the molar ratio of lecithin to cholesterol was varied between 1:0 and 1:1. Therefore, in such liposomes the mol % of cholesterol in the bilayer is variable. The initial rate of association of filipin with these liposomes increases as a function of cholesterol concentration, but the rates observed with these liposomes at a given concentration of cholesterol are lower than those obtained with liposomes prepared from equimolar mixtures of lecithin and cholesterol. Table III shows data that were obtained for the binding of filipin to each type of liposome preparation at various concentrations of cholesterol. The inset in Figure 2 demonstrates that the ratio of the initial rates of the two types of liposome preparations is a linear function of their ratio of % cholesterol in the bilayers. Thus, at equivalent concentrations of cholesterol initial rate data indicate that filipin exhibits a preference for reaction with liposomes having a high percentage of cholesterol in a bilayer conformation.

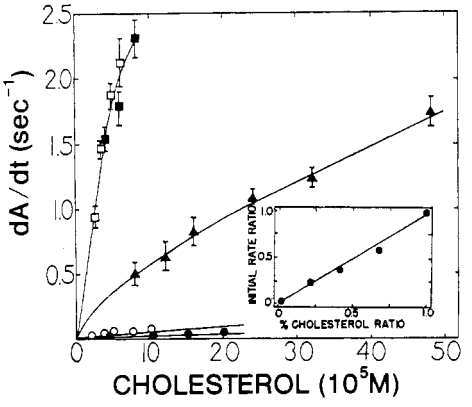

FIGURE 2: Effect of cholesterol concentration on the initial rates of association of filipin with aqueous suspensions of cholesterol, lecithin–cholesterol liposomes, and lecithin–cholesterol vesicles. Liposomes (▲) and vesicles (□, sonicated for 2.5 min; ■, sonicated for 20 min) were prepared from equimolar concentratons of lecithin and cholesterol. For aqueous suspensions of cholesterol containing a final concentration of 2% (○) DMF by volume, the filipin concentration was 6.2 μM; for those containing 10% (●) DMF by volume, the filipin concentration was 16 μM. Inset: plot of initial rate ratio, $(dA/dt)_{x\%\ \text{cholesterol}}$ divided by $(dA/dt)_{48\%\ \text{cholesterol}}$, *vs.* % cholesterol ratio in liposomal bilayers, x% cholesterol divided by 48% cholesterol. Two types of liposomal preparations were used. Liposomes having variable lecithin–cholesterol molar ratios but constant total lipid concentrations (0.75 mM) were used, in which the mol % of cholesterol in the bilayer varies (denoted as x%). Liposomes having lecithin–cholesterol molar ratio of 1.0 but variable total lipid concentrations were used, in which the mol % of cholesterol is 48. The concentration of filipin was 7.8 μM.

TABLE III: Effect of Cholesterol Concentration and Bilayer Content on Initial Rate of Association of Filipin with Cholesterol-Containing Liposomes.[a]

	Lecithin/Cholesterol Varied, Total Lipid Concn Constant[b]		Total Lipid Concn Varied, Lecithin/Cholesterol = 1.0	
Concn of Cholesterol (mM)	% Cholesterol in Bilayer	dA/dt (sec^{-1})	% Cholesterol in Bilayer	dA/dt (sec^{-1})
0.000	0	0[c]		
0.080	11	0.108 ± 0.040	48	0.490 ± 0.040
0.120	16	0.185 ± 0.050	48	0.618 ± 0.080
0.145	19	0.267 ± 0.050		
0.160			48	0.816 ± 0.100
0.180	24	0.519 ± 0.030		
0.190	19	0.478 ± 0.070		
0.240	32	0.629 ± 0.030	48	1.080 ± 0.050
0.320			48	1.250 ± 0.050
0.360	48	1.050 ± 0.050		
0.480	48	1.750 ± 0.100	48	1.750 ± 0.100

[a] The concentration of filipin was 7.5 μM in experiments using liposomes of variable lecithin/cholesterol ratio, and 7.8 μM in experiments using liposomes prepared from equimolar ratios of lecithin and cholesterol. [b] The total lipid (sum of lecithin, cholesterol, and dicetyl phosphate) concentration was 0.75 mM, except for liposomes having cholesterol concentrations of 0.190 and 0.480 mM. The final lipid concentration of the latter liposomes was 1.00 mM. [c] Liposomes having final lecithin concentrations of 0.08 and 4.0 mM were used. The concentration of filipin was 6.0 μM.

Equilibrium Binding Studies. On binding to vesicles containing cholesterol, filipin undergoes modifications in its ultraviolet absorption spectrum (Norman *et al.*, 1972; Bittman *et al.*, 1974) and its polarized fluorescence intensity (Bittman and Fischkoff, 1972). To obtain quantitative information about the binding of filipin to vesicle-bound sterols, ultraviolet absorption and fluorescence polarization measurements were conducted at constant filipin concentration and varying lipid concentrations. The ratio of bound filipin to total filipin concentration depends on the total lipid concentration of vesicles as shown in Figure 3. The concentrations of bound filipin in the presence of vesicles were calculated from the absorbance values at 358 nm using the equation

$$C_B = \frac{\epsilon_F C_T - A/l}{\epsilon_F - \epsilon_B}$$

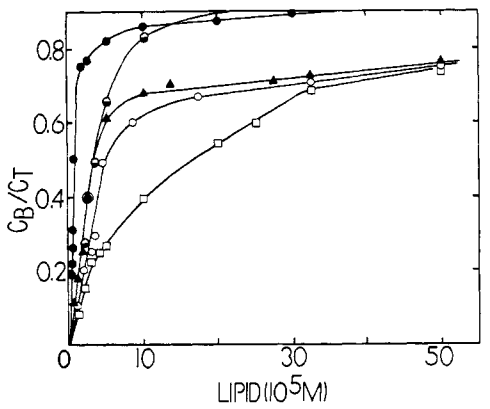

FIGURE 3: Dependence of the ratio of bound filipin/total filipin on the concentration of total lipid in vesicles. The total concentration of filipin was 12.5 μM. The concentration of bound filipin was determined from the absorbance at 358 nm as described in the text. Filipin and vesicles were allowed to stand overnight at 4° in the dark. The vesicles were sonicated for about 30 sec at 4° and were prepared from: (□) lecithin; (○) lecithin–epicholesterol in 7:3 molar ratio; lecithin–cholesterol in (▲) 7:1 molar ratio, and in (●) 7:3 molar ratio; and (◒) lecithin–ergosterol in 7:3 molar ratio.

where ϵ_F is the extinction coefficient of free filipin, C_T is the total concentration of filipin, A is the measured absorbance, l is the cell path length, and ϵ_B is the extinction coefficient of bound filipin. The extinction coefficients of the bound form of the polyene were calculated from the intercepts of double-reciprocal plots of the absorbances of filipin in the presence of high lipid concentrations. Values of ϵ_B (in the presence of various vesicles) were 4.5×10^4 (lecithin), 1.9×10^4 (lecithin–epicholesterol), 1.1×10^4 (lecithin–ergosterol), and 1.0×10^4 M^{-1} cm^{-1} (lecithin–cholesterol). Figure 3 shows that at low lipid concentrations filipin binds to lecithin–cholesterol and lecithin–ergosterol vesicles, but higher lipid concentrations are required to obtain appreciable conversion of filipin to the bound form in the presence of vesicles prepared from lecithin or from lecithin and epicholesterol. A similar trend is observed in fluorescence polarization studies (Figure 4). The binding data shown in Figures 3 and 4 can be used to construct binding isotherms. Figure 5 shows the analysis of the binding of filipin to vesicles from lecithin alone and from mixtures of lecithin and cholesterol, ergosterol, or epicholesterol by the method of

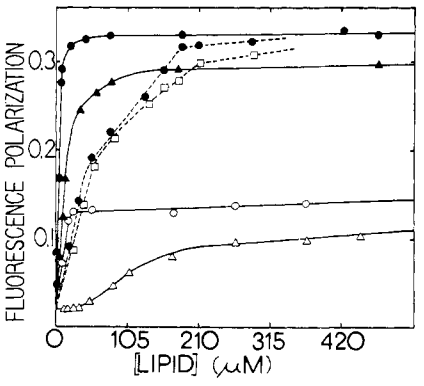

FIGURE 4: Dependence of fluorescence polarization of filipin on the concentration of total lipid in vesicles. The concentration of filipin was 5.2 μM. Filipin and vesicles were incubated for a minimum of 2 hr at room temperature in the dark. Vesicles were sonicated for about 30 sec at 4° and were prepared from: (△) lecithin; (○) lecithin–epicholesterol in 7:3 molar ratio; and lecithin–cholesterol in (▲) 7:1 molar ratio, and in (●) 7:3 molar ratio. The effects of aqueous suspensions of (□) epicholesterol and (●) cholesterol are shown in dashed curves (data taken from Bittman and Fischkoff (1972)).

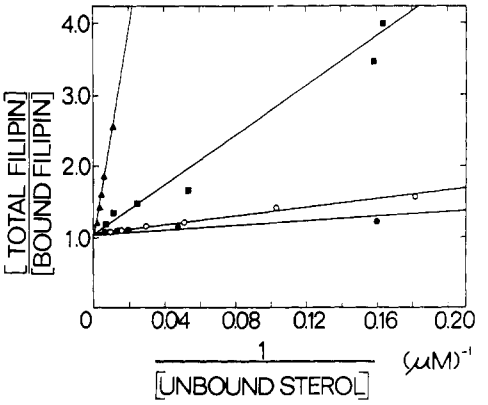

FIGURE 5: Determination of the binding constants of filipin with vesicles. The total concentration of filipin was 12.5 μM. The concentration of lipid (expressed in terms of sterol for sterol-containing vesicles and in terms of lecithin for vesicles prepared from lecithin alone) was varied. Data were obtained from Figure 3. The vesicles were prepared from: (▲) lecithin; (■) lecithin–epicholesterol; (○) lecithin–ergosterol; and (●) lecithin–cholesterol.

Steinemann and Stryer (1973). Absorbance data from Figure 3 were used. Analysis of the fluorescence polarization data (Figure 4) by this method gave similar results. The concentration of unbound sterol was obtained by subtracting the concentration of bound filipin from the total sterol concentration, under the assumption that the molar ratio of filipin to sterol in the complex is 1. The apparent dissociation constants were calculated from the slopes of the lines shown in Figure 5, such that the standard deviation was minimized by the method of least squares. The binding constants are given in Table IV.

Discussion

Results reported in Table III show that no initial rate of interaction of filipin with liposomes prepared from lecithin alone was observed in the stopped-flow apparatus. The rate of association of filipin with liposome-bound cholesterol depends on both the absolute concentration of cholesterol and the mol % of cholesterol in the bilayer (Table III, Figure 2). For a given cholesterol concentration, the rate is higher when the lecithin–sterol molar ratio is unity than when a molar excess of lecithin with respect to cholesterol is present. Several workers have reported that an equimolar ratio of cholesterol to lecithin represents the maximum amount of cholesterol

TABLE IV: Apparent Dissociation Constants (K_d) for the Binding of Filipin to Vesicles.[a]

Type of Vesicle	K_d (μM)
Lecithin	144 ± 24
Lecithin–epicholesterol	13 ± 0.5
Lecithin–ergosterol	2.47 ± 0.05
Lecithin–cholesterol	0.80 ± 0.14

[a] For lecithin–sterol vesicles, K_d values are reported in terms of binding to the sterol (not to total lipid in the vesicle). K_d values were calculated from the slopes of the lines shown in Figure 5. The value of K_d given for lecithin–cholesterol vesicles is the average of the binding constants obtained from experiments using preparations of 7:3 and 7:1 molar ratios of lecithin to cholesterol. The molar ratio of lecithin to sterol in vesicles containing epicholesterol and ergosterol was 7:3.

that can be incorporated into aqueous dispersion with lecithin (Bourgès *et al.*, 1967; Kellaway and Saunders, 1967; Ladbrooke *et al.*, 1968). For a given molar ratio of lecithin to cholesterol, *e.g.*, 1 (Figure 2 and Table III), the rate of binding increases with the concentration of cholesterol. To the extent that the transition state resembles the equilibrium process, the observation that the initial rate of association of filipin with liposome-bound cholesterol increases with cholesterol concentration is consistent with ultraviolet absorption and fluorescence polarization studies that show that the degree of binding increases with cholesterol concentration. At a given total lipid concentration, more filipin is bound to lecithin–cholesterol vesicles containing 30 mol % of cholesterol than to those containing 10 mol % of cholesterol (Figures 3 and 4). This is a consequence of the fact that filipin binds approximately 180 times more tightly to cholesterol in lecithin–cholesterol vesicles than to lecithin in lecithin vesicles (Table IV). Our results showing that the rate and degree of binding increase as the amount of cholesterol relative to lecithin increases support the suggestion that the phospholipid–sterol ratio may be an important parameter in determining whether filipin interacts with membranes (Kinsky, 1970). The results are in agreement with reports that filipin, at low concentrations, (a) does not undergo marked spectral changes in the presence of liposomes having very low cholesterol content (Norman *et al.*, 1972); (b) has no effect on monolayers (Demel *et al.*, 1965), single bilayer membranes (Van Zutphen *et al.*, 1966), and biological membranes (Kinsky *et al.*, 1965) having low cholesterol content; (c) interacts with cholesterol-containing monolayers but not with cholesterol-lacking monolayers (Demel *et al.*, 1968); and (d) increases the rate and extent of release of trapped ions and solutes in cholesterol-containing liposomes (Kinsky *et al.*, 1968; Sessa and Weissmann, 1968). The kinetic and binding data presented in this paper provide support for the hypothesis that at low concentrations filipin interacts specifically with membrane-bound cholesterol in a manner that is sensitive to the molar ratio of phospholipid to sterol. Furthermore, binding of filipin to cholesterol incorporated into bilayers seems to be preferred to binding to cholesterol present in the form of microparticles. Figure 2 shows that very slow initial rates of association are observed with aqueous suspensions of cholesterol; at equivalent sterol concentrations, much faster rates are observed with liposomes and vesicles. The interaction of filipin with aqueous suspensions of sterols may be mechanistically different from that with lecithin–sterol vesicles. The order with respect to cholesterol in aqueous suspension is different from that in lecithin–cholesterol liposomes and vesicles. Furthermore, in aqueous suspension epicholesterol interacts with filipin almost as strongly as cholesterol (Figure 4), but in liposomes with lecithin epicholesterol interacts more weakly than cholesterol (Figures 3 and 4).

The marked increase in initial rate of association of filipin with vesicles relative to that with liposomes (Table I and Figures 1 and 2) suggests that sonication leads to an increase in the accessibility of cholesterol for reaction with the antibiotic. This is expected since small sonicated vesicles are believed to have a looser, inherently more disordered structure than larger ones (Sheetz and Chan, 1972; Marsh *et al.*, 1972). Furthermore, sonication increases the surface area and decreases the number of multiconcentric bilayers, causing more cholesterol molecules to be exposed in the outermost bilayer where they have maximal accessibility to the antibiotic.

Initial rate studies reveal that the rate law for the interaction of filipin and cholesterol is complex. The finding that the reaction with liposome- and vesicle-bound cholesterol has an order with respect to filipin of more than 1 suggests that terms involving filipin species other than monomers play a role in determining the rate. Each of these higher order terms, *e.g.*, dimer, trimer, tetramer, contains both a rate constant for reaction with cholesterol and a binding constant for aggregate formation. Since data concerning the kind and distribution of filipin aggregates present in aqueous media have not been reported, further speculation about the participation in the rate law of various kinds of filipin species is not warranted.

The binding of filipin to lecithin–epicholesterol vesicles is weaker than that of lecithin–cholesterol vesicles (Figure 5, Table IV). This result is consistent with the observations that mixed lecithin–epicholesterol monolayers gave only small surface pressure increases (although monolayers of epicholesterol alone gave significant pressure increases) after addition of filipin (Norman *et al.*, 1972), lecithin–epicholesterol vesicles were not lysed by treatment with filipin at concentrations that lysed lecithin–cholesterol vesicles (Bittman *et al.*, 1974), and qualitatively less filipin appears to be bound to vesicles and *Acholeplasma laidlawii* membranes containing epicholesterol than to those containing cholesterol (Bittman and Fischkoff, 1972; Norman *et al.*, 1972; Bittman *et al.*, 1974). That filipin binds somewhat more strongly to lecithin–cholesterol vesicles than to lecithin–ergosterol vesicles (Table IV) is consistent with the report that cholesterol antagonized the inhibitory action of filipin on *Candida albicans* more effectively than ergosterol (Zygmunt and Tavormina, 1966). The protection of fungi against the action of filipin by exogenous sterol has been ascribed to result from a reaction between the antibiotic and sterol *in vitro*, thus reducing the concentration of the antibiotic available for interaction with the fungal membrane (Lampen *et al.*, 1960).

Added in Proof

After this manuscript had been submitted for publication, Schroeder *et al.* (1973) reported that freshly prepared aqueous solutions of filipin *complex* exist in a form that does not bind cholesterol, but the antibiotic is converted to a cholesterol-binding form on heating for about 2 hr at 50°. In contrast to the studies reported by these authors, we find that freshly prepared aqueous solutions (at 25°) of both filipin complex and filipin III interact rapidly with cholesterol in aqueous suspension and liposomes, as evidenced by changes in the absorption peak ratios of the antibiotic on mixing with the sterol-containing preparations. Furthermore, the absorption peak ratio (A_{338}/A_{306}, which we find to be about 0.9–1.0 for filipin complex and about 2.3–2.5 for filipin III), and the molecular ellipticities of the freshly prepared antibiotic solutions are not altered significantly when the polyene solutions are heated at 50° for 2 hr.

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Properties and Activity of the Lipopolysaccharide–Receptor from Human Erythrocytes†

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ABSTRACT: We have isolated from human erythrocyte membranes a physicochemically homogeneous lipoglycoprotein with a molecular weight of 256,000. It is rich in *N*-acetylneuraminic acid, galactose, and hexosamines. The intact substance prevented attachment to erythrocytes of unheated and heated, smooth and rough lipopolysaccharide and protein–lipopolysaccharide of all gram-negative bacteria tested. It did not interact with other bacterial antigens and therefore is referred to as "lipopolysaccharide–receptor." The receptor physically and reversibly blocked those groupings on lipopolysaccharide which attach to red cells. Both citraconylation and dissociating polyacrylamide gel electrophoresis under standard conditions produced 1 large and 1 small fragment. Citraconylation-inactivated decitraconylation of the large fragment restored high activity but the small fragment remained inactive. Only the large fragment had a composition similar to the intact receptor. The light fragment had significantly less apolar amino acids and carbohydrate. Prolonged incubation of the small fragment in sodium dodecyl sulfate resulted in its further fragmentation. Circular dichroism spectra of the intact lipopolysaccharide–receptor showed α-helix, β-conformation and some flexible aperiodic conformation. Removal of lipid resulted in extensive disorganization. Receptor activity was destroyed by proteases; lipid and carbohydrate were not involved in the receptor's activity.

Endotoxin (=lipopolysaccharide, or O antigen) of gram-negative bacteria in minute quantities produces numerous noxious effects (Braude *et al.*, 1955; Microbial Toxins, V, 1971) for which attachment to host tissue is a prerequisite. Lipopolysaccharide has the ability to fix to human red cells *in vitro* (*cf.* Neter, 1956) and under extreme conditions *in vivo* (Boyden, 1953; Buxton, 1959; Springer and Horton, 1964). A receptor for lipopolysaccharide attachment has been isolated from the membrane of erythrocytes (Springer *et al.*, 1966b, 1973). It interacts with all lipopolysaccharide preparations tested and with the related Kunin antigen but not with other antigens of gram-negative or gram-positive bacteria and hereafter is referred to as lipopolysaccharide–receptor. Other compounds such as glycolipids, lipoproteins, and basic proteins also combine with lipopolysaccharide but are less active and, so far as investigated, nonspecific (Whang *et al.*, 1970).

Serological procedures indicated that lipopolysaccharide–receptor prevented lipopolysaccharide attachment to red cells by blocking sites on the lipopolysaccharide and not receptors on erythrocytes (Springer *et al.*, 1970). The lipopolysaccharide–receptor was obtained in apparently homogeneous form. It is a *N*-acetylneuraminic acid rich glycoprotein which also contains some lipid. Preliminary physicochemical analysis indicated the receptor to possess a molecular weight of about 230,000 (Springer *et al.*, 1973).

The present paper reports on detailed chemical, physicochemical, activity, and immunochemical analyses of the receptor and of fractions obtained from it by mild procedures.

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