Determination of Cholesterol Asymmetry by Rapid Kinetics of Filipin–Cholesterol Association: Effect of Modification in Lipids and Proteins†

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ABSTRACT: The rapid kinetic behavior of filipin association with cholesterol was unaffected by binding of water-soluble proteins to vesicles and mycoplasma membranes and by proteolytic digestion of mycoplasma membrane proteins. The kinetic properties were, however, dependent on the membrane phospholipids, in that the initial rate of filipin association with cholesterol was enhanced by phospholipase A_2 treatment, by the incorporation of lysophosphatidylcholine, and by increasing the degree of unsaturation in phospholipid vesicles and mycoplasma membranes. The second-order rate constant was also dependent on the mol % of cholesterol in small unilamellar vesicles but not in large unilamellar vesicles. The ratio of rate constants in intact mycoplasma cells relative to isolated membranes provides an estimate of cholesterol distribution in membranes [Bittman, R., & Rottem, S. (1976) *Biochem. Biophys. Res. Commun. 71*, 318; Clejan, S., Bittman, R., & Rottem, S. (1978) *Biochemistry 17*, 4579]. This ratio was unaffected by proteolytic digestion of intact cells and by the incorporation of exogenous phospholipids into the *Mycoplasma capricolum* cell membrane. However, on cross-linking of surface proteins of *M. capricolum* by dimethylsuberimidate, cholesterol was localized predominantly in the outer half of the bilayer. On aging of mycoplasma cultures, the cholesterol distribution remained constant in membranes of *M. capricolum* cells but was enriched in the outer leaflet of the *Mycoplasma gallisepticum* cell membrane. The results of these experiments are discussed in relation to the use of the rapid kinetics of filipin binding as a probe of cholesterol distribution.

Little information is available about the organization and distribution of cholesterol in membranes, although detailed knowledge exists concerning the sterol's effects on the hydrocarbon and polar regions of membranes and its interaction with phospholipids. In several recent studies we have used filipin to probe the transbilayer distribution of cholesterol in membranes that can be prepared in both sealed and unsealed states, such as in mycoplasmas and erythrocytes, and in both right side out and inside-out states, such as erythrocyte vesicles [reviewed by Bittman (1978)]. In order to minimize filipin-induced membrane perturbation, we used stopped-flow kinetic measurements to monitor changes in the absorbance of filipin accompanying association with cholesterol. In addition to short reaction times, high cholesterol/filipin molar ratios and low temperature minimize membrane disruption. Previous kinetic studies of filipin–cholesterol binding showed that the initial rate is first order in filipin and cholesterol concentrations in vesicles prepared from synthetic saturated phosphatidylcholines (Blau & Bittman, 1977), in mycoplasmas (Bittman & Rottem, 1976), and in erythrocytes (Blau & Bittman, 1978). The second-order rate constant was not markedly sensitive to variation in the mole fraction of cholesterol up to 32 mol % in dimyristoyl-PC[1]–cholesterol vesicles (Blau & Bittman, 1977) nor to the ratio of egg PC to egg phosphatidylethanolamine in vesicles (Blau & Bittman, 1978). The rate was, however, influenced by fatty acyl chain length, but no discontinuity was observed at the lipid phase transition temperature in Arrhenius plots (Blau & Bittman, 1977). In order to investigate some of the factors that affect the organization of cholesterol, in the present study we used the filipin-binding approach in two membrane systems–unilamellar vesicles and the cell membrane of mycoplasmas. For example, we studied the effects on the rapid filipin–cholesterol association process of supplementation with exogenous phospholipids and water-soluble proteins, of alteration of endogenous membrane protein content by protease-catalyzed digestion and of endogenous phospholipid content by phospholipase-catalyzed digestion, of modification of cholesterol levels in the membrane, and of increasing the molecular weights of some membrane proteins by cross-linking. In view of the use of filipin as a probe in the study of cholesterol asymmetry and transbilayer movement, the results of these studies are relevant to the evaluation of continued stopped-flow measurements of filipin binding to free cholesterol as a reliable indication of the distribution of this sterol in the outer and inner surfaces of mycoplasma membranes.

Materials and Methods

Chemicals. Lipids were purchased from the following sources: egg PC from Makor Chemicals, Ltd., Jerusalem, Israel, and Sigma Chemical Co., St. Louis, MO; egg phosphatidylethanolamine from Makor and Cyclo Chemical, Los Angeles, CA; dimyristoyl-PC, dioleoyl-PC, dicetyl phosphoric acid, and egg lyso-PC from Sigma; diarachidonyl-PC and dilinoleoyl-PC from Applied Science Laboratories, State College, PA; myristoyl lyso-PC from Calbiochemical Corp., La Jolla, CA. Cholesterol was purchased from Sigma and was recrystallized twice from ethanol. The lipids were analyzed on silica gel G plates and were found to be chromatographically pure. Ribonuclease A, horse heart cytochrome *c* (type III), phospholipase C (*Clostridium welchii*), phospholipase D (cabbage), phenylmethanesulfonyl fluoride, fatty acid poor

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[1] Abbreviations used: PC, phosphatidylcholine; DMS, dimethylsuberimidate (dihydrochloride form); $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

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bovine serum albumin, oleic acid, and palmitic acid were from Sigma. Phospholipase A_2 from porcine pancreas was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Trypsin, chymotrypsin, lysozyme, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp., Freehold, NJ. [6-*methyl*-^{3}H]Thymidine (10 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA. Fetal calf and horse sera were from Grand Island Biochemical Co., Grand Island, NY. Dimethylsuberimidate dihydrochloride was from Pierce Chemicals, Rockford, IL. Filipin complex was purified and stock solutions were prepared as described previously (Blau & Bittman, 1977). 6-Carboxyfluorescein was purchased from Eastman Organic Chemicals, Rochester, NY, and was recrystallized from ethanol.

Growth of Mycoplasma gallisepticum and Mycoplasma capricolum and Isolation of Membranes. M. gallisepticum (strain A5969) and *M. capricolum* (California kid) were grown to midexponential phase in a modified Edward medium (Razin & Rottem, 1975) containing 10% fetal calf serum. The procedure for the adaptation of *M. capricolum* to grow in a cholesterol-poor medium was previously described (Clejan et al., 1978). In the present study, the adapted strain was grown on a modified Edward medium supplemented with 1.25 μg/mL oleic acid, 10 μg/mL cholesterol oleate (each added as an ethanolic solution), and 0.5% bovine serum albumin. When the absorbance of the culture at 640 nm reached 0.10, the adapted strain was transferred to a medium containing 10 μg/mL free cholesterol. The organisms were harvested and the cells were washed as described previously (Clejan et al., 1978). The extent of cell lysis was monitored by measuring the release of [^{3}H]thymidine-labeled components, NADH oxidase, and/or K^+ into the supernatant obtained after centrifugation for 20 min at 12000*g* (Clejan et al., 1978). Unless noted otherwise, membranes were isolated from aliquots of diluted cell suspensions by sonication at 0 °C in a Heat Systems Model W-375A sonicator (Heat Systems-Ultrasonics, Plainview, NY) for four 30-s periods at power level 6. The absorbance at 500 nm of membrane preparations was 80–95% lower than that of cell suspensions of the equivalent membrane mass per milliliter.

Preparation of Unilamellar Lipid Vesicles. Appropriate volumes of stock solutions of PC and cholesterol in chloroform were mixed. The chloroform was evaporated under a stream of nitrogen, and, for preparation of large unilamellar vesicles, absolute ethanol was added to the dry lipid film to give a concentration of 35 μmol of lipid/mL of ethanol. The ethanolic lipid solution was injected into 0.06 M KCl solution at ~20 °C at a rate of 1.2 mL/h by using a Zero-Max Model E1 power block and perisaltic pump. The vesicle suspension that was formed contained no more than 30% ethanol by volume. For removal of the ethanol, the vesicles were passed through a column (1 × 40 cm) of Sephadex G-50 (medium) and were eluted with a 0.06 M KCl solution. The vesicles were centrifuged at 20000 rpm for 10 min at 0 °C in a SS34 rotor to remove large lipid suspensions. This procedure for preparation of large unilamellar vesicles by slow ethanol injection is similar to that of Nordlund et al. (1980). For preparation of small unilamellar vesicles, a 0.06 M KCl solution was added to the lipid film, and the suspension was agitated by vortex mixing and was then sonicated under nitrogen in an aluminum cooling cell for a total of 15 min at power level 3 in an ice bath with a Heat Systems W-375 sonicator. [For the experiments with vesicles prepared from unsaturated phospholipids (Figure 1), the suspensions were subjected to ultrasonic irradiation until no further decrease in the absorbance at 600 nm was observed.] The sonicated preparations were centrifuged at 20000 rpm for 20 min in a SS34 rotor to sediment any metal released from the titanium tip and remaining large multilayered vesicles. For preparation of the protein-containing vesicles, solutions of ribonuclease A, cytochrome *c*, and human serum albumin were added to vesicle suspensions prepared by sonication. After incubation of protein and vesicles for 40–60 min at 37 °C, the excess protein not associated with the vesicles was removed by chromatography on a Sephadex G-50 column (1 × 25 cm). Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Vesicle Characterization. To estimate the trapped volume, we prepared vesicles in the presence of carboxyfluorescein. For vesicles made by the slow-injection procedure, the ethanolic solution of lipids (0.7 mL) was injected into 2 mL of a 0.06 M KCl solution containing 28 mM carboxyfluorescein. For vesicles made by sonication, the thin lipid film was dispersed in a carboxyfluorescein–0.06 M KCl solution. Untrapped carboxyfluorescein was removed by passing the vesicles through a Sephadex G-50 column (1 × 40 or 1 × 25 cm). A 100-μL aliquot of the eluted vesicles was diluted with 0.9 mL of 0.2% Triton X-100, and the absorbance was measured at 490 nm. Similarly, the absorbance of the vesicle suspension was measured prior to chromatography. The concentration of carboxyfluorescein was calculated by using an extinction coefficient of 6.1×10^4 M^{-1} cm^{-1}. Phospholipid and cholesterol were measured by the procedures of Taussky & Shorr (1953) and Zlatkis & Zak (1969), respectively. The volumes of the trapped aqueous spaces were as follows: 0.98 ± 0.08 L/mol of total lipid for vesicles made by the slow-injection procedure with molar ratios of egg PC/cholesterol ranging from 5.9:1 to 0.57:1; 0.31–0.37 L/mol of total lipid for vesicles made by sonication with egg PC/cholesterol molar ratios ranging from 3.6:1 to 1.2:1. The accessibility of phosphatidylethanolamine to 2,4,6-trinitrobenzenesulfonate was determined by the procedure of Roseman et al. (1975) in order to examine whether vesicles were leaky. On incorporation of 10 mol % phosphatidylethanolamine into preparations of each type of vesicles containing molar ratios of PC/cholesterol of 2:1–0.8:1, ~65% of the phosphatidylethanolamine was derivatized by trinitrobenzenesulfonate in vesicles that were prepared by sonication. The amount of phosphatidylethanolamine exposed in the outer monolayer of vesicles prepared by the slow-injection procedure was lower. These measurements demonstrate that the phosphatidylethanolamine was present in intact vesicles. To determine whether vesicles treated with phospholipases remained intact, we prepared vesicles in buffer containing 0.5 M K_2CrO_4. Untrapped chromate was removed by dialysis. The extent of leakage of trapped chromate from phospholipase-treated vesicles was monitored in the dialyzate by using the absorbance at 370 nm. The concentration of the total chromate ion trapped was measured by disrupting the vesicles with Triton X-100.

Treatment of Membrane with Trypsin and Chymotrypsin. Suspensions of intact mycoplasma cells or isolated membranes in a 400 mM sucrose–10 mM sodium phosphate–20 mM $MgCl_2$ solution (pH 7.4) were treated with 50 μg/mL trypsin or chymotrypsin for 2 h at 37 °C. Trypsin-catalyzed digestion was stopped with trypsin inhibitor (50 μg/mL); chymotrypsin-catalyzed digestion was stopped with phenylmethanesulfonyl fluoride (20 μg/mL). The reaction mixture was diluted with 8 volumes of a cold 0.25 M NaCl solution and centrifuged at 12000*g* for 20 min at 4 °C. The pellets were suspended in and washed twice with a 0.25 M NaCl solution.

Cross-Linking with DMS. Stock solutions of the bifunctional alkylating agent DMS were prepared in ethanol. Membrane suspensions were incubated with DMS (0–10 mM) for 1 h at room temperature in 0.05 M triethylamine, pH 8.5, containing 0.25 M sucrose. The ethanol concentration did not exceed 0.5%. An early exponential-phase culture of the adapted strain of *M. capricolum* was treated with DMS (5 mM final concentration) in the defined growth medium, and at the same time free cholesterol was added to 10 μg/mL. The reaction was terminated by dilution with 5–10 volumes of a cold 0.25 M sucrose (for the isolated membranes) or 0.25 M NaCl solution (for the cells). Cells and membranes were isolated by centrifugation in the usual manner. Incubations with DMS at concentrations exceeding 5 mM gave very turbid suspensions.

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. Electrophoresis in a gradient of 10–15% polyacrylamide gels was performed by the procedure described by Studier (1973). The membrane suspension (1–2 mg of protein/mL) was boiled for 2 min in 0.01 M Tris buffer, pH 6.8, containing 0.1% $NaDodSO_4$ and 0.1% 2-mercaptoethanol. The gels were stained with Coomassie blue.

Incorporation and Removal of Cytochrome c and Lysozyme. M. capricolum membranes (1.0 mg of membrane protein/mL) were treated with varying concentrations of cytochrome *c* and lysozyme in 1 mM sodium phosphate buffer, pH 7.4, containing 400 mM sucrose (no $MgCl_2$ was present) for 2 h at room temperature. Removal of cytochrome *c* and lysozyme was accomplished by washing 3 times with 5 volumes of 1 mM sodium phosphate buffer, pH 7.4, containing 25 mM Na_2EDTA and 1 M NaCl (for cytochrome *c* experiments) or 0.3 M NaCl (for lysozyme experiments).

Phospholipase A_2 Treatment of Mycoplasma Membranes. M. gallisepticum and *M. capricolum* membranes were labeled by growing cells in Edward medium containing 5% horse serum and 0.002 μCi of [1-^{14}C]palmitate (59 Ci/mol) or [1-^{14}C]oleate (53 Ci/mol) (both from the Amersham Corp., Arlington Hts., IL) per mL of medium. The cultures were incubated, cells were harvested and washed with a 0.25 M NaCl solution containing 0.5% bovine serum albumin, and membranes were prepared and isolated from cells as described above. To 1 mL of membrane suspension containing about 1–1.5 μmol/mL of total cholesterol were added 200 μL of 0.05 M Tris buffer, pH 7.3, 30 μL of 1 M $CaCl_2$, 50 μL of bovine serum albumin (0.5% initial concentration), and 75 μL of phospholipase A_2 (100 μg/mL) (or 75 μL of water for blank). The reaction mixtures were incubated at 37 °C. To terminate the reaction, we added 0.5 mL of 0.1 M Na_2EDTA, followed by 8 mL of a cold 0.25 M NaCl solution. Membranes were sedimented (16 000 rpm for 40 min) and resuspended in saline. Lipids were extracted from aliquots of the membrane suspensions by the method of Bligh & Dyer (1959). The extracted lipids were dried, dissolved in chloroform, and applied to silica gel plates, which were developed first with petroleum ether (bp 40–60 °C)–acetone (3:1 by volume) and then with chloroform–methanol–water (65:25:4 by volume). After visualization of the spots with iodine vapor, the compounds were scraped from the plate into scintillation vials containing 10 mL of toluene scintillation liquor. The radioactivity in the lyso compounds, residual undigested phospholipids, and free fatty acids was determined in a Beckman LS200 liquid scintillation counter.

Phospholipase Treatment of Vesicles. Phospholipid vesicles (~5–8 μmol) were incubated with phospholipase A_2, C, or D in a final volume of ~1.2 mL for 10 min at 25 °C. Vesicles were prepared in a 0.06 M KCl solution from egg PC or dimyristoyl-PC and cholesterol in a 3:1 molar ratio of PC to sterol and contained 2 mol % dicetyl phosphoric acid, with a total lipid concentration of 10 mM. The concentrations of the phospholipases were as follows: A_2, 6.8 units/mL; C, 1.2 units/mL; D, 2 units/mL. The following buffers were used: for phospholipase A_2, 0.05 M Tris, pH 8.0; for phospholipases C and D, 0.01 M 2-(*N*-morpholino)ethanesulfonic acid, sodium salt, pH 6.0. Calcium chloride was added to a final concentration of 8 mM for phospholipase A_2, 1.8 mM for phospholipase C, and 50 mM for phospholipase D. The incubations were stopped by addition of ethylenediaminetetraacetic acid at a concentration double that of Ca^{2+}, followed by dilution by at least 8-fold. Lipids were extracted from 50–100-μL aliquots of the phospholipase-treated vesicles with chloroform–methanol–12 M HCl (2:1:0.02 by volume). The extent of hydrolysis was determined by thin-layer chromatography of the chloroform phase on 250-μm silica gel plates by using the following developing solvents: for phospholipase A_2 treated vesicles, chloroform–methanol–4% NH_4OH, 65:35:5 by volume; for phospholipase C, benzene–ethyl acetate, 3:1 by volume; for phospholipase D, chloroform–methanol–glacial acetic acid–water, 50:25:4:2 by volume. Iodine vapor or molybdate spray reagent (Dittmer & Lester, 1964) was used for visualization. Unsprayed spots were used for phospholipid determination in the phospholipase A_2 and D treatments, as described by Martin et al. (1975). For vesicles treated with phospholipase C, the phosphorus content was measured in both the organic phase (containing unreacted PC) and the aqueous phase (containing choline phosphate).

Kinetics of Filipin-Free Cholesterol Association. Stopped-flow spectrophotometry at 360 nm was used to study the rates of filipin binding with free cholesterol. Equal volumes of filipin solution and mycoplasma suspensions (either intact cells or isolated, open membranes) or vesicles were mixed rapidly in a Dürrum-Dionex stopped-flow spectrophotometer. Filipin solutions were prepared as described previously (Bittman & Rottem, 1976). The final concentration of dimethylformamide was 0.3% (by volume). The initial rate of the association process was calculated from the increase in the transmittance of filipin, which was photographed by using a Tektronix storage oscilloscope and a Polaroid camera. The initial reaction was found to be first order with respect to each reactant. Second-order rate constants for association in intact cells and isolated membranes, designated as k_{cells} and $k_{membranes}$, respectively, were calculated as described previously (Bittman & Rottem, 1976). All rate measurements with mycoplasma cells and membranes were made at 10 °C; kinetic measurements with vesicles were conducted at 25 °C. To minimize cell shearing, we reduced the pressure used to initiate mixing with the air-actuator assembly to 30 psi.

Results

Effect of Age of Culture of M. gallisepticum and M. capricolum on Transbilayer Distribution of Cholesterol. Initial rates of filipin binding to cholesterol in intact cells and isolated membranes (dA/dt) have been used to calculate the second-order rate constants, k_{cells} and $k_{membranes}$; the ratio of these rate constants provides an estimate of the transbilayer distribution of cholesterol between the inner and outer halves of the mycoplasma membrane (Bittman & Rottem, 1976; Clejan et al., 1978). Table I shows that with *M. gallisepticum* the ratio of $k_{cells}/k_{membranes}$ increased from 0.5 (in cells from the early exponential phase) to 0.6 (in stationary-phase cells). These rate ratios indicate that cholesterol is distributed symmetrically in membranes of early *M. gallisepticum* cells but is enriched

Table I: Effects of Culture Age and Partial Digestion of Membrane Proteins on Second-Order Rate Constants of Filipin Binding to Cholesterol in *M. gallisepticum* and *M. capricolum* Cells and Membranes[a]

stage of culture[b]	$10^{-3} \times k_{cells}$ (M^{-1} s^{-1})	$10^{-3} \times k_{membranes}$ (M^{-1} s^{-1})	$k_{cells}/k_{membranes}$
M. gallisepticum			
early logarithmic	4.6 ± 0.2	9.1 ± 0.2	0.51
logarithmic	3.7 ± 0.3	6.6 ± 0.4	0.56
stationary	3.3 ± 0.2	5.4 ± 0.2	0.60
M. capricolum			
early logarithmic	9.5 ± 0.3	13.9 ± 0.2	0.68
logarithmic	8.9 ± 0.5	13.3 ± 0.4	0.66
stationary	8.1 ± 0.4	11.8 ± 0.5	0.69

	protease[c]	% protein digested in cells	% protein digested in membranes	$10^{-3} \times k_{cells}$ (M^{-1} s^{-1})	$10^{-3} \times k_{membranes}$ (M^{-1} s^{-1})
M. gallisepticum					
early logarithmic	trypsin	17	34	4.9	9.8
	chymotrypsin	9	20	4.3	7.9
logarithmic	trypsin	24	45	4.7	9.6
	chymotrypsin	10	22	4.8	8.7
late logarithmic	trypsin	24	50	5.1	10.7
	chymotrypsin	11	24	3.6	7.1

[a] The initial rates of filipin-free cholesterol association were measured at 10 °C. Intact cells and isolated membranes obtained from five cell cultures of *M. gallisepticum* and three cell cultures of *M. capricolum* at four cholesterol concentrations were used. Second-order rate constants were calculated from the stopped-flow traces of the initial rates (see Materials and Methods). The second-order rate constants in this table are representative examples obtained from one culture. Error limits are standard errors of the mean. The average ratio of second-order rate constants, $k_{cells}/k_{membranes}$, has an error limit of ~5%. [b] Early logarithmic cultures were obtained after ~15 h of growth and gave an A_{640} of ~0.09 in *M. gallisepticum* and *M. capricolum*. Logarithmic cultures correspond to ~22 h of growth and had A_{640} values of about 0.25 in *M. gallisepticum* and 0.30 in *M. capricolum*. Stationary cultures were obtained after ~26 h of growth and had A_{640} values of about 0.3 in *M. gallisepticum* and 0.5–0.6 in *M. capricolum*. [c] The extent of cell lysis after trypsin or chymotrypsin treatment was estimated from the release of [*methyl*-^{3}H]thymidine-labeled components from the cells. The cells underwent lysis of 8–12% on trypsin treatment and 6–8% on chymotrypsin treatment.

Table II: Rate Constants for Filipin–Cholesterol Association as a Function of Binding of Soluble Proteins to *M. capricolum* Membranes[a]

soluble protein added (mg/mL)	cytochrome *c* bound (mg/mg of membrane protein)	$10^{-3} \times k_{membranes}$ (M^{-1} s^{-1})	lysozyme bound (mg/mg of membrane protein)	$10^{-3} \times k_{membranes}$ (M^{-1} s^{-1})
0	0	12.2	0	11.8
0.25	0.14	12.0	0.16	11.3
0.75	0.66	10.9	0.56	11.3
1.00	0.70	10.0	0.76	11.3
2.00	0.78	9.7	1.06	10.5
3.00	0.81	9.0	1.16	9.2

[a] *M. capricolum* membrane suspensions containing 1.0 mg of membrane protein/mL (145 μg of cholesterol/mg of membrane protein) in 1 mM phosphate buffer, pH 7.4, containing 0.4 M sucrose were incubated with equal volumes of various concentrations of the soluble proteins for 2 h at room temperature. The amounts of soluble protein bound and the rate of filipin–cholesterol association were determined as described under Materials and Methods.

in the outer half of the bilayer upon aging of the culture. These results agree very closely with data obtained by studies of exchange of cholesterol between *M. gallisepticum* and high-density lipoprotein (Rottem et al., 1978). Table I also shows that with *M. capricolum* the ratio of $k_{cells}/k_{membranes}$ is unaffected by the age of the culture, indicating that the distribution of free cholesterol between the two halves of the *M. capricolum* membrane remained constant throughout the growth cycle. Upon aging of *M. capricolum*, the content of unsaturated fatty acyl chains in the membrane increases (Z. Gross, unpublished results). In *M. gallisepticum*, *M. capricolum*, and other mycoplasma cultures, aging is accompanied by a marked decrease in membrane lipid/protein ratio without substantial changes in the free cholesterol/phospholipid molar ratio (Rottem & Greenberg, 1975; Amar et al., 1976). Partial removal of membrane proteins by proteolytic digestion resulted in the disappearance of the age-dependent decrease in the values of k_{cells} and $k_{membranes}$ in *M. gallisepticum* (Table I). It therefore appears that in *M. capricolum* the increased content of unsaturated fatty acyl chains of membrane phospholipids on aging may compensate for the decreased lipid/protein ratio, resulting in no change in the $k_{cells}/k_{membranes}$ ratio.

Effects of Changes in Membrane Protein Composition and Content on dA/dt and Transbilayer Cholesterol Distribution. The influence of hydrolysis of membrane proteins in intact cells and isolated membranes on the initial rate was investigated. When isolated membrane preparations of *M. capricolum* were treated with trypsin, the second-order rate constant for filipin–cholesterol association did not change, despite the removal of ~35% of the membrane protein (data not shown). Table I shows that the values of k_{cells} and $k_{membranes}$ remained almost constant in *M. gallisepticum* upon removal of ~20% of the membrane proteins from intact cells and between 34 and 50% of the membrane proteins from isolated membranes by tryptic digestion. Chymotrypsin treatment resulted in less hydrolysis than trypsin digestion, and again no marked changes in initial rates were noted when membrane proteins were hydrolyzed (Table I). Cytochrome *c* and lysozyme are thought to interact by electrostatic bonding with phospholipid head groups in vesicles and *Acholeplasma* membranes, without penetrating extensively into the hydrocarbon phase of the membrane (Razin et al., 1973; Rottem et al., 1973). In order to further examine the effects of surface components, we measured the initial rate of filipin-free cholesterol association

Table III: Effect of Dimethylsuberimidate on Cell Growth and Transbilayer Distribution of Cholesterol in the Adapted Strain of *M. capricolum* Transferred to a Cholesterol-Rich Medium[a]

time of incubation in high-cholesterol medium (h)	A_{640}		free cholesterol/phospholipid (molar ratio)		$k_{cells}/k_{membranes}$	
	−DMS	+DMS	−DMS	+DMS	−DMS	+DMS
0	0.10	0.10	0.20	0.20	0.73 ± 0.08	0.75 ± 0.06
1	0.14	0.12	0.27	0.25	0.53 ± 0.04	0.80 ± 0.06
2	0.17	0.14	0.58	0.34	0.45 ± 0.06	0.70 ± 0.05

[a] Cells were grown as described under Materials and Methods. When the culture reached an A_{640} value of 0.10 (after ~20 h of growth), 10 μg/mL free cholesterol in ethanol and 5 mM DMS were added. After the cultures were incubated for the indicated period of time, cells were harvested and washed. The extent of cell lysis was estimated to be 10–15% after a 2-h incubation period in the medium containing 5 mM DMS. The free cholesterol content was calculated by using a molar ratio of free cholesterol to cholesterol esters of 1.1. The average molecular weights assumed for the cholesterol esters and phospholipids were 626 and 750, respectively.

in *M. capricolum* membranes containing various concentrations of cytochrome *c* and lysozyme (Table II). The initial rate was not altered by incorporation of ~0.6 mg/mg of membrane protein but was decreased upon more extensive incorporation of these proteins. The removal of these extrinsic proteins caused $k_{membranes}$ to return to the original value (12×10^3 M^{-1} s^{-1}), except for the membrane preparations that had been treated with 2.0 and 3.0 mg of lysozyme/mL. (These preparations gave $k_{membranes}$ values of 10.5×10^3–11×10^3 M^{-1} s^{-1}.) The decreased rates of filipin–cholesterol association may result from the decreased freedom of motion of the hydrocarbon chains in the membrane interior on incorporation of cytochrome *c* and lysozyme (Rottem & Samuni, 1973) or from the restricted accessibility of filipin to free cholesterol.

The effect of incorporating extrinsic proteins on filipin binding to cholesterol was also examined in vesicles. The initial rate was unaffected by incorporation of ribonuclease into small and large vesicles. For example, for binding of filipin (11.6 μM) to cholesterol (50 μM) in egg PC–cholesterol (1:1 molar ratio) vesicles prepared by sonication, dA/dt was 1.10 ± 0.14 absorbance units s^{-1} at 25 °C when the ribonuclease concentration in the vesicles was varied between 0 and 25 mg/μmol of lipid; dA/dt was 1.01 ± 0.06 absorbance units s^{-1} when comparable amounts of ribonuclease were incorporated into vesicles prepared by slow ethanol injection. The rate was also not changed by incorporation of human serum albumin or cytochrome *c*.

Another compositional change in membrane proteins was introduced by the reaction of *M. capricolum* membranes with the bifunctional alkylating agent, DMS. The initial rate of filipin-free cholesterol association was lowered by DMS treatment. The following values of $k_{membranes}$ were determined after treatment for 1 h: untreated membranes, 8.4×10^3 M^{-1} s^{-1}; 1 mM DMS, 7.6×10^3; 2 mM DMS, 6.9×10^3; 4 mM DMS, 6.2×10^3; 5 mM DMS, 6.0×10^3; 10 mM DMS, 4.6×10^3. The Coomassie blue stained gels revealed the absence of at least two polypeptide bands from membranes treated with 5 and 10 mM DMS and the appearance of two or three bands of higher molecular weight (90 000–93 000). Although the identities and molecular weights of the cross-linked components are not known, membrane proteins in mycoplasmas have been found to be distributed predominantly at the inner membrane surface (Amar et al., 1974, 1976). It is probable that the reduction in the initial rate of association in the open, isolated membranes caused by DMS treatment arises because of the decreased availability of free cholesterol in the inner half of the membrane bilayer.

The influence of DMS treatment on a growing cell undergoing active cholesterol uptake and macromolecular biosynthesis was tested by using the adapted strain of *M. capricolum*. As summarized in Table III, reaction of DMS with membrane components in an early exponential-phase culture

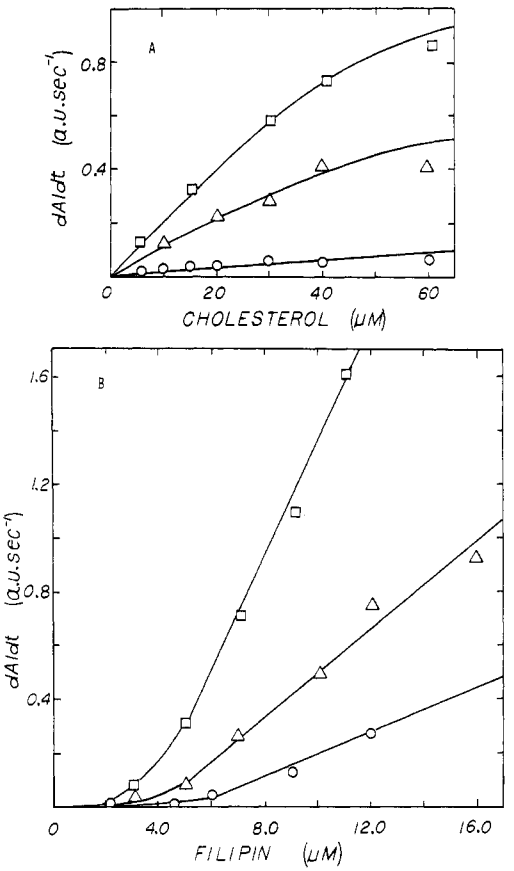

FIGURE 1: Kinetics of filipin–cholesterol association in vesicles prepared from unsaturated phosphatidylcholines and cholesterol as a function of the concentrations of (A) cholesterol and (B) filipin. The filipin concentration in (A) was 7.0 μM. The cholesterol concentration in (B) was 30 μM. The molar ratio of PC/cholesterol was 3:1. Vesicles were prepared by sonication from the following phospholipids: (○) dioleoyl-PC; (△) dilinoleoyl-PC; (□) diarachidonyl-PC.

reduced free cholesterol uptake into the membrane following the transfer of the cells to the cholesterol-rich medium. The $k_{cells}/k_{membranes}$ ratio of 0.7–0.8 during a 2-h incubation in cholesterol-rich medium indicates that free cholesterol is not translocated rapidly from the outer to the inner surface of the bilayer of DMS-treated cells. The influence of changes in membrane lipid composition and content on dA/dt was investigated in the following series of experiments.

Filipin Binding to Cholesterol in Vesicles Prepared from Unsaturated Phosphatidylcholines. The kinetics of association of filipin with cholesterol in small unilamellar vesicles was examined at various filipin and cholesterol concentrations as a function of the number of double bonds in PC. The initial rate increased at 22 °C with the number of double bonds (Figure 1). The relationship between the rate of the initial binding process and the extent of PC unsaturation indicates

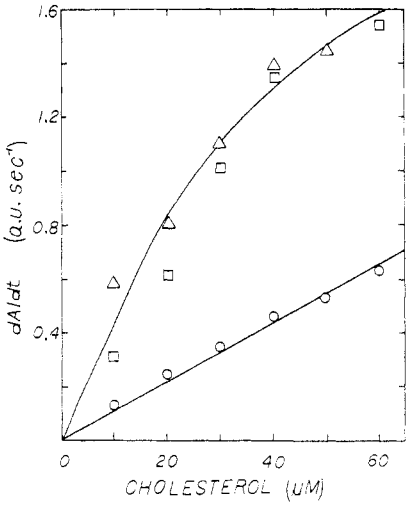

FIGURE 2: Effect of cholesterol concentration in aqueous dispersions on initial rate of filipin–cholesterol association in the presence and absence of egg lyso-PC. The filipin concentration was 7.0 μM. Sonicated vesicles were prepared from (○) egg-PC and cholesterol and from (△) egg-PC, cholesterol, and 10 mol % lyso-PC. The PC/cholesterol molar ratio was 3:1. An aqueous dispersion (□) was also formed from lyso-PC and cholesterol (3:1 molar ratio).

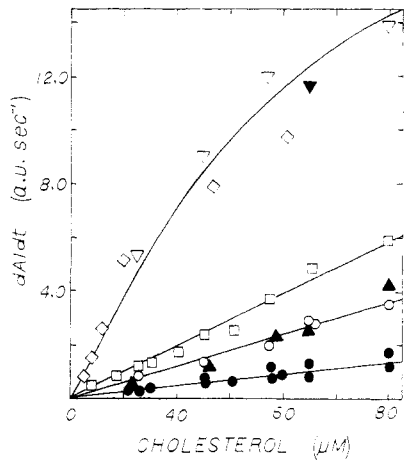

FIGURE 3: Effects of treating vesicles with phospholipase A_2 and lyso-PC on initial rate of filipin–cholesterol association as a function of cholesterol concentration. Vesicles were formed by sonication of dimyristoyl-PC/cholesterol dispersions (3:1 molar ratio of PC to sterol). Vesicles were treated with phospholipase for the following times: (●) 0 min, (○) 1 min (~10% hydrolysis), (□) 5 min (10–15% hydrolysis), (▼) 40 min, and (◊) 60 min (70–80% hydrolysis). 1-Myristoyl-2-lyso-PC was added in the following amounts: (●) 0, (▲) 10, and (▽) 30 mol %. The filipin concentration was 10 μM.

that cholesterol accessibility in unilamellar vesicles composed of 25 mol % cholesterol varies with PC unsaturation.

Effect of Incorporation of Lyso-PC on dA/dt. Aqueous dispersions were prepared by sonication of PC, cholesterol, and 1-acyllysophosphatidylcholines. Figure 2 shows that the initial rate of filipin–cholesterol association is enhanced dramatically by incorporation of 10 mol % egg lyso-PC into egg PC–cholesterol vesicles; similar rates were measured in dispersions of lyso-PC and cholesterol, which are reported to associate with each other to form a stable lamellar phase (Rand et al., 1975; Purdon et al., 1975). Dimyristoyl-PC–cholesterol sonicated dispersions containing 30 mol % 1-myristoyllyso-PC also show a large rate enhancement relative to vesicles from dimyristoyl-PC and cholesterol (Figure 3). In experiments in which cholesterol was omitted, no fast binding process was detected between lyso-PC and filipin in lyso-PC micelles or in PC dispersions containing 30 mol % lyso-PC. Thus, the rate enhancements arise from modification of the properties of the

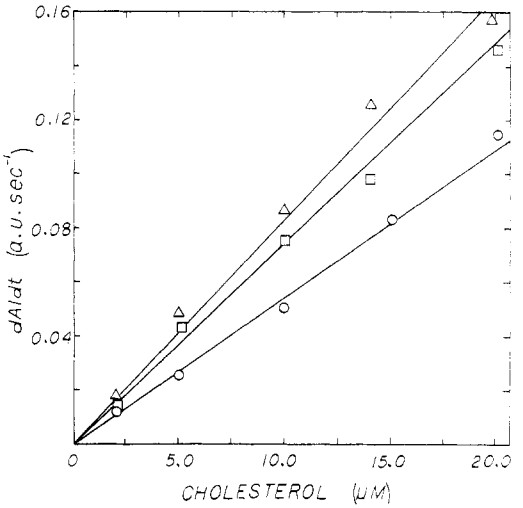

FIGURE 4: Effect of phospholipase A_2 treatment of isolated *M. gallisepticum* membranes on the initial rate of filipin–cholesterol association as a function of cholesterol concentration. Membranes containing [^{14}C]oleate (○) were treated at 37 °C with phospholipase A_2 for (□) 20 min (43% hydrolysis) and (△) 40 min (53% hydrolysis). The stopped-flow measurements were made at 25 °C. The filipin concentration was 10 μM.

bilayer rather than from direct interaction between lyso-PC and filipin.

Effect of Treatment of Vesicles with Phospholipases on dA/dt. In order to further examine the effects of modification of the bilayer on the rate of filipin binding to cholesterol, we treated vesicles with phospholipases A_2, C, and D. No significant change in the initial binding rate was found when 20% of the PC was hydrolyzed by phospholipase C. Similarly, ~10% hydrolysis of PC catalyzed by phospholipase D gave no significant change. Large increases in the rate of association of filipin with cholesterol were observed, however, in phospholipase A_2 treated vesicles under conditions where the vesicles were not leaky to chromate within the time period measured (10 min) (Figure 3). This is in agreement with the rate enhancement measured on accumulation of lyso-PC, which is a product of phospholipase A_2 action.

Effect of Treatment of M. gallisepticum Membranes with Phospholipase A_2 on dA/dt. The initial rate of filipin association with cholesterol was increased by phospholipase A_2 catalyzed digestions of 43% and 53% of the glycerophospholipids in *M. gallisepticum* membranes (Figure 4). The lack of appreciable accumulation of lysophospholipids in these membrane preparations (because of lysophospholipase activity under the conditions used) may explain the less dramatic rate enhancement in *M. gallisepticum* compared with the vesicles.

Effects of Incorporation of PC or Sphingomyelin into M. capricolum Membranes and Alteration of Phosphatidylglycerol to Diphosphatidylglycerol Ratio on Transbilayer Cholesterol Distribution. M. capricolum cells underwent pronounced changes in their lipid composition and content depending on the growth medium used. When cells were grown in a medium containing bovine serum albumin, free fatty acid, and free cholesterol, two major phospholipids (phosphatidylglycerol and diphosphatidylglycerol) and two minor phospholipids (an amino-containing and an unidentified phospholipid) comprise more than 95% of the total membrane phospholipids (Z. Gross, S. Rottem, and R. Bittman, unpublished experiments). When the growth medium was supplemented with exogenous lipids such as egg PC or brain sphingomyelin, the cells took up the phospholipid into their membrane. Incorporation of PC affected de novo phospholipid synthesis, decreasing the relative amount of phosphatidyl-

Table IV: Second-Order Rate Constants for Filipin Association with Cholesterol in Vesicles of Different Curvature[a]

type of vesicle	cholesterol (mol %)	$10^{-5} \times k_2$ (M^{-1} s^{-1})
large	21.3	1.00 ± 0.13
	23.8	1.10 ± 0.12
	37.0	0.90 ± 0.10
	50.7	0.92 ± 0.04
	51.5	0.90 ± 0.06
	64.9	0.84 ± 0.06
small	21.3	0.24 ± 0.02
	25.6	0.72 ± 0.06
	32.3	0.88 ± 0.03
	43.5	1.00 ± 0.02
	51.0	1.12 ± 0.05
	65.4	1.36 ± 0.05

[a] The second-order rate constant, k_2, was calculated from dA/dt divided by the product of the total amplitude of the reaction times the concentration of free cholesterol. The filipin concentration was 11.6 μM. The temperature was 25 °C.

glycerol in the membrane and increasing the amount of diphosphatidylglycerol. In the absence of exogenously supplied lipids, the phosphatidylglycerol to diphosphatidylglycerol phosphorus ratio was 2.1, whereas in cells grown with PC this ratio was 0.4. The relative amounts of the amino-containing phospholipid and the unidentified phospholipid remained about the same (Z. Gross, S. Rottem, and R. Bittman, unpublished experiments). The content of free cholesterol (about 130–160 μg/mg of membrane protein) and the free cholesterol to phospholipid molar ratio (0.82–0.84) were also not changed by growing cells with PC or sphingomyelin. The rate ratio, $k_{cells}/k_{membranes}$, was 0.66 in cells grown without exogenous phospholipid present and did not change when cells were grown in the presence of 50 μg/mL PC or sphingomyelin. Thus, two-thirds of the free cholesterol is localized in the outer monolayer of the bilayer of *M. capricolum* and one-third in the inner monolayer, despite a 2-fold increase in the polar lipid content of the membrane and a large increase in diphosphatidylglycerol/phosphatidylglycerol molar ratio.

Relationship between Membrane Bilayer Cholesterol Content and Kinetic Behavior of Filipin–Cholesterol Association. The initial rate increased linearly with the cholesterol concentration in the suspension when small vesicles prepared from egg PC and cholesterol (Figure 2) and large vesicles containing various egg PC/cholesterol ratios (data not shown) were used. In order to assess the influence of changes in the mole fraction of cholesterol in the bilayer on the second-order rate constant for filipin binding to cholesterol, we prepared small and large unilamellar vesicles varying in cholesterol content as described under Materials and Methods. The concentration of cholesterol relative to PC in the bilayer was increased by 3-fold. The second-order rate constants (Table IV) were measured at three or more cholesterol concentrations. In large vesicles, the apparent second-order rate constant of filipin binding does not vary significantly when the molar percentage of cholesterol in the bilayer is varied, and the average rate constant is $(0.94 \pm 0.13) \times 10^5$ M^{-1} s^{-1} at 25 °C. In small vesicles, a linear relationship was obtained between the apparent second-order rate constant and cholesterol mol % of 25–65 (Table IV). The values in Table IV are low compared with rates of elementary bimolecular steps in solution. The observed rate may actually be the product of the intrinsic bimolecular step for filipin–cholesterol binding times an equilibrium constant. The equilibrium suggested may be attributed to a filipin–cholesterol complex prior to the insertion of filipin into the vesicle bilayer.

Effect of pH, Ionic Strength, and Sonication Time on dA/dt. The initial rate of filipin–cholesterol association in *M. capricolum* membranes was unaffected by (a) varying the pH of the suspending medium between 6.0 and 8.0 in 20 mM sodium phosphate buffer containing 400 mM sucrose and 20 mM $MgCl_2$ and (b) varying the ionic strength of the membrane suspension in 10 mM sodium phosphate buffer, pH 7.2, from 0 to 0.3 M NaCl. The rate of the reaction was, however, decreased by ~20% at 0.4 M NaCl. Fragmentation of *M. capricolum* or *M. gallisepticum* membranes by prolonged sonication (15–240 s) also had no significant effect on the kinetics of filipin–cholesterol association.

Discussion

The primary purpose of this investigation was to examine how changes in membrane lipid and protein composition affect the kinetics of filipin–cholesterol association and the transbilayer distribution of cholesterol. Factors that influence molecular motion of membrane components are the length and degree of unsaturation of phospholipid acyl chains, the relative content of phospholipid classes, the presence of lysophosphatides, the ratios of cholesterol to phospholipid and of lipid to protein, and the curvature of the membrane. Changes in protein composition and content were obtained by using a cross-linking agent, proteolytic digestion, and binding of exogenous proteins. Variations in membrane lipids were obtained by phospholipase treatment of mycoplasma and vesicle membranes, by addition of exogenous lyso-PC, and by growing of *M. capricolum* cells in media containing a variety of exogenously supplied lipids. When grown in a medium containing 5% horse serum, cells in the midexponential phase had membranes with a higher content of lipid phosphorus per milligram of membrane protein than cells grown in a medium containing bovine serum albumin, cholesterol, and oleic and palmitic acids. We observed that the rapid kinetic properties of filipin association with cholesterol in mycoplasma or vesicle membranes were not influenced by (a) changes in the polar lipid content and relative amounts of de novo synthesized phospholipids, (b) incorporation of moderate amounts of extrinsic proteins, (c) removal of endogenous protein by trypsin and chymotrypsin digestion, and (d) changes in the cholesterol/phospholipid ratio in large vesicles. The above findings (a–d) suggest that filipin is a reliable probe of the transbilayer distribution of cholesterol in the mycoplasma membrane. However, important caveats about the use of rapid kinetic measurements of filipin–cholesterol association for this application have been identified; for example, the dependence of the kinetics on PC unsaturation (Figure 1) and lyso-PC content (Figures 2 and 3) indicates that an unequal distribution of these molecules between the two halves of the bilayer would lead to spurious estimates of the sizes of the cholesterol pools.

The second-order rate constants for filipin–cholesterol association in *M. gallisepticum* cells and membranes are decreased on aging of the cultures but are not altered significantly during aging of *M. capricolum* (Table I). Spin-label studies indicate an increased rigidity of the phospholipid acyl chains of *M. gallisepticum* membranes on aging, relative to membranes from cells in the early exponential phase; however, the rigidity of *M. capricolum* membranes was unaffected or even somewhat decreased by aging of the culture, presumably because of the increased unsaturated fatty acyl content in membranes of aged *M. capricolum* cells (Z. Gross and S. Rottem, unpublished results). The increase in the $k_{cells}/k_{membranes}$ ratio on aging of *M. gallisepticum* indicates that the size of the cholesterol pool in the outer monolayer of the bilayer increases (Table I). Movement of cholesterol into the inner

half of the bilayer may be impeded by the increased rigidity of the hydrocarbon chains.

The results obtained with adapted *M. capricolum* cells treated with DMS (Table III) support the conclusion we made previously with ionophore- and chloramphenicol-treated cells, i.e., that in the adapted cells part of the free cholesterol is incorporated and translocated in a growth-dependent process (Clejan et al., 1978). The mechanism of inhibition by DMS differs from the actions of ionophores and chloramphenicol. The cross-linking reagent DMS probably inhibits uptake and translocation of free cholesterol (and cell growth in these cholesterol-requiring organisms) by modulating the packing of membrane components at or within the membrane surfaces. Alternatively, we cannot exclude the possibility that a surface protein is involved in the uptake and movement of cholesterol in the *M. capricolum* membrane.

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Production and Isolation of Siderophores from the Soil Fungus *Epicoccum purpurascens*†

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ABSTRACT: A large number of iron transport agents, siderophores, which stimulated the growth of *Arthrobacter flavescens* JG-9, were isolated during a study of the antitumor activity associated with the metabolic products of the fungus *Epicoccum purpurascens*. The production of the siderophores was significantly enhanced in a variety of media by culture of the fungus in the near absence of ferric iron. A novel method of purification involving a carboxylic ion-exchange resin separated the siderophores into four subgroups. The first subgroup, which contained the majority of the activity, was subsequently resolved in a similar manner with the carboxylic resin into seven individual siderophores. Of these, two were characterized as ferricrocin and coprogen whereas the others appeared to represent new compounds. One of the latter was given the name triornicin and exhibited slight antitumor activity in mice injected with Ehrlich ascites tumor cells.

During preliminary studies of the biologically active compounds produced by a common soil fungus, both antitumor activity and siderophore activity were discovered. The purpose of this report is to describe the production, isolation, and nature of the siderophores detected among the metabolic products of the fungus identified as *Epicoccum purpurascens* Schlecht.

Siderophores are high-affinity, microbial ferric transport molecules secreted into the environment by bacteria and fungi for the purpose of sequestering iron to facilitate their growth. The criteria for preliminary assignment of a natural product to the siderophore category are (a) the repression of its formation by iron, (b) the large differential in its avidity for ferric vs. ferrous iron, the former being greatly favored, and (c) the inability of mutants or natural strains incapable of synthesizing either the particular ligand or its "permease" to take up exogenous iron (Neilands, 1973). Molecules satisfying these criteria vary in structure, but generally can be classed as secondary hydroxamic acids, or catechols. Generally, bacteria produce ligands based on catechol coordination whereas fungi

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