STUDIES OF CHOLESTEROL IN BIOLOGICAL MEMBRANES: DISTRIBUTION AND TRANSBILAYER MOVEMENT OF CHOLESTEROL IN MYCOPLASMA MEMBRANES, AND INTERACTION BETWEEN PHOSPHATIDYLCHOLINE ANALOGS AND CHOLESTEROL IN ARTIFICIAL BILAYER MEMBRANES

by

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ABSTRACT

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by

SANDA CLEJAN

Adviser: Professor Robert Bittman

The effect of sterols on the rates of nonelectrolyte and electrolyte movement in multilayered and unilamellar liposomal membranes formed from various phosphatidylcholine (PC) analogs was studied. We measured the temperature dependence of the permeation of small, polar solutes through lipid bilayers in the liquid-crystalline state of saturated diester, diether, and alkyl analogs of phosphatidylcholines. At temperatures above the gel to liquid-crystalline phase transition of these diester-, diether-, and alkyl-phosphatidylcholines, the 3β-hydroxysteroids, cholesterol and ergosterol, decreased the permeability of glycerol, urea, acetamide, and 6-carboxyfluoresceine and decreased the rates

of ionophore-mediated release of Rb^+ and Ca^{2+}. The activation energy and activation entropy for osmotic swelling of liposomes by glycerol and urea permeation and for fluorescence change of a liposome-associated merocyanine dye upon acetamide permeation were not altered significantly by cholesterol incorporation (48 mol percent) into bilayers with diester-or diether-phosphatidylcholines we examined. The similarities between the effects of sterols on the initial rates and reflection coefficients of polar nonelectrolyte diffusion and on the rates of release of Rb^+, Ca^{2+}, and 6-carboxy-fluorescein in diester-PC bilayers and in those from our phospho-lipids lacking acyl groups indicate that carbonyl oxygens of the fatty acyl groups in the 1 and 2 positions of PC are not essential to obtain interaction with cholesterol. Thus, there is no specific requirement for hydrogen bonding between the hydrogen of the sterol hydroxyl group and a carbonyl oxygen of diester-PC.

The sterol-requiring mycoplasma, M. capricolum, was adapted to grow in a medium containing low fetal-calf serum (FCS) concentrations, providing cells in which unesterified cholesterol comprised only about 3.6% by weight of the total membrane lipids. The native strain grown with 10% FCS contained a six fold higher cholesterol concentration than the adapted strain. When an early exponential-phase culture of the adapted strain was transferred to a medium containing 10% FCS, cell growth was stimulated and the cells accumulated cholesterol into their cell membrane. Alteration of cell metabolism by treatment of

the adapted culture with chloramphenicol, valinomycin, nonactin, or gramicidin at 37°C, or transfer to 4°C, resulted in an almost complete inhibition of growth and a partial inhibition of cholesterol uptake, suggesting that the adapted cells incorporate part of their cholesterol in a growth-dependent process, and not only by a physical process involving adsorption of the sterol from the growth medium into the membrane. The rate of cholesterol translocation from the outer to the inner half of the lipid bilayer was monitored by stopped-flow kinetic measurements of the polyene antibiotic filipin with free cholesterol (FC) in intact cells and isolated membranes. The cholesterol distribution in the two halves of the bilayer was almost invariant after only one hour of incubation with medium containing 10% FCS despite a dramatic rise in cholesterol content, approaching that in the native strain. However, when growth was inhibited by chloramphenicol, valinomycin, nonactin, or gramicidin, cholesterol remained localized predominantly in the outer half of the bilayer. Therefore, FC is translocated rapidly from the external surface of the bilayer of growing M. capricolum cells at 37°C.

The phospholipid composition of the native strain of M. capricolum grown with FCS was different from that of cells grown without it; over 96% of membrane phospholipids were de novo synthesized when cells were grown in defined medium without serum. The primary phospholipids were phosphatidylglycerol (PG)

and diphosphatidylglycerol (DPG). When M. capricolum was grown with 10% FCS, 50% of the membrane phospholipids were exogenous phosphatidylcholine (PC) and sphingomyelin (SPM). A partial conversion from PG to DPG occurred in cells grown with serum. The ratio of DPG to PG was 2.0 when the medium contained 20 μg / ml of PC; the ratio was 0.5 when cells were grown without PC. Cholesterol distribution in the two halves of the membrane was not affected by the incorporation of the exogenous phospholipids we examined. When cells were grown in the absence of cholesterol oleate, more FC was found to be localized in the inner half of the membrane bilayer of the native strain of M. capricolum than when cells were grown in medium containing cholesterol oleate. The ability of various sterols to support the growth of M. capricolum was also investigated. Sterols that differed from cholesterol in the following aspects were added to defined medium as ethanolic solutions: (1) those with modifications in the position of double bonds within the steroid nucleus, (2) and those containing a side chain with either a double bond or a substituent. Growth was poorer with all of the sterols we tested compared with growth in cholesterol-containing medium. With 3β-cholestanol or 4, 6-cholestadien-3β-ol, the distribution of sterol remained the same as with cholesterol, whereas with β-sitosterol, stigmasterol, or ergosterol, almost 90% of the sterol was localized in the outer half of the bilayer. These studies establish that structural modifications in the cholesterol molecule affect the sterol uptake capacity and the distribution and the movement of the sterol between the two halves of the lipid bilayer.

To Liviu and to our son Iuval

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA, bovine serum albumin

c-C-PR, cis-1, 2-cyclohexanedioxydiacetic acid

6-CF, 6-carboxy-fluorescein

CE, cholesteryl ester

DMF, dimethylformamide

DMPC, dimyristoylphosphatidylcholine

DMS, dimethylsuberimidate

DPG, diphosphatidylglycerol

DPPC, dipalmitoylphosphatidylcholine

DSPC, distearoylphosphatidylcholine

FC, free cholesterol

FCS, fetal-calf serum

OEPC, octadecyleicosylphosphorylcholine

PA, phosphatidic acid

PC, phosphatidylcholine

PE, phosphatidylethanolanine

PC, phosphatidylglycerol

SPM, sphingomyelin

GENERAL INTRODUCTION

For more than half a century, heart and blood vessel disease have been the major causes of death in the world. It has been estimated that more than half of all deaths annually result from coronary heart disease (Fourth Report of the Director of the National Heart, Lung, and Blood Institute, 1977). Although intensive research efforts have begun to elucidate various facets of the etiology, development, and prognosis of the pathogenesis of atheriosclerosis, uncertainty remains concerning the initiating and accelerating factors such as hypercholesterolemia and hypertriglyceridemia associated with this disease process. Accumulation of cholesterol in cells is a risk factor, not only for coronary heart disease (Stanbury et al., 1974; Kimura, 1977) but possibly also for malignancy in ascites tumor cells (Alderson and Green, 1975) and leukemic cells (Inbar and Shinitzky, 1974). Therefore, investigations of the role played by cholesterol in biological membranes and of the factors controlling its incorporation (for recent review see Jain, 1975) have been stimulated.

Studies of the interactions between phosphatidylcholine analogs and cholesterol in artificial bilayer membranes. (Objectives of Part I.)

Studies with artificial membrane systems made of phospholipid-cholesterol mixtures have advanced our knowledge of the interaction of cholesterol with phospholipids (Oldfield and Chapman, 1972; Franks, 1976; Yeagle, 1978). Based on the results of such studies, it is now widely accepted that the cholesterol-phospholipid interaction involves primarily hydrophobic and Van der Waals interaction between adjacent fatty acyl chains of phospholipids and the sterol (De Kruyff, et al.,

1973; Worcester and Franks, 1976; Demel and De Kruyff, 1976; Huang, 1977).

Relatively little systematic work has been carried out toward understanding the effect of the cholesterol head group on phospholipid membranes. The conclusions are contradictory (Verma and Wallach, 1973; Brockerhoff, 1974, 1978; Huang, 1976; Jain et. al., 1979; Reiber, 1978). The first part of the thesis involves this objective. Specific subjects dealt within this part are: The effects of β-hydroxysteroids like cholesterol and ergosterol, and α-hydroxysteroids like epicholesterol and lanosterol on the rates of nonelectolyte movement in multilayered liposomal membranes. Permeation rates are compared across bilayers formed from (a) various saturated diester phosphatidylcholines, (b) the corresponding diethers which lack the carbonyl groups, and (c) alkyl analogs which lack both carboxylate ester bonds and the diacylglycerol moiety (Chapter I).

Chapter II is an extension of the above subject, namely the effects of cholesterol, epicholesterol, and lanosterol on the ionophore-mediated transport of Ca^{2+} and Rb^{+} through bilayer membranes of single-walled vesicles from phosphatidylcholines and phosphatidylcholine analogs lacking two carbonyl oxygens.

Transbilayer cholesterol movement in membranes

To gain insight into the molecular organization and function of the membrane, information of the transbilayer distribution and movement of components in a biological membrane is required. Early studies reported that the exchange of free cholesterol between erythrocyte membranes and lipoproteins was rapid (half-times less than several hours) (Murphy, 1962; Hagerman and Gould, 1951). The rate of sterol movement across phospholipid bilayer vesicles was also estimated to

be rapid (half-time of 70 min at 37°C) (Smith and Green, 1974). In contradiction, the exchange of cholesterol between dipalmitoyl lecithin-cholesterol vesicles and red blood cell ghosts (Poznansky and Lange, 1976) or influenza virus membranes (Lenard and Rothman, 1976) is very slow or non-existent. Such a slow flip-flop rate of cholesterol in erythrocyte membrane will explain the existence of two separate pools postulated from (a) studies of exchange of cholesterol from erythrocytes to serum proteins or to vesicles (Basford et al., 1964; Bruckdorfer and Green, 1967; Bell and Schwartz, 1971; d'Hollander and Chevallier, 1972; Bjorson et al., 1975), (b) permeability studies with erythrocytes having altered cholesterol content (Deuticke and Zöllner, 1972; Deuticke and Ruska, 1976); and (c) stopped-flow kinetic measurements of filipin-cholesterol association in red blood cell ghosts (Blau and Bittman, 1978).

However, a recent study (Lange et al., 1977) utilizing a new technique to determine specifically the cholesterol localized on the cytoplasmic surface of the erythrocyte membrane indicates that the rate of movement of cholesterol has a half-life of less than 50 min. A very high rate of cholesterol translocation was also postulated in vesicular stomatitis virus (Patzer et al., 1978) where no growth or macromolecular synthesis occurs. The discrepancy between the results obtained in the different exchange experiments has not been clarified yet. Mycoplasmas, the only prokaryotes that require cholesterol for growth, are unique and useful models for such studies, and this is the scope of the second part of the thesis. (See reviews by Razin, 1978a; Razin and Rottem, 1978).

Properties of mycoplasmas

Mycoplasmas are minute prokaryotic microorganisms approaching the larger viruses in size. Unlike viruses, mycoplasmas are fully capable of autonomous growth and reproduction. Unlike all other prokaryotes mycoplasmas have no cell walls nor intracellular membranous structures. The cell is bounded by a single membrane--the plasma membrane. This membrane, once isolated, is surely not contaminated with other membrane types. Mycoplasmas are sensitive to osmotic lysis or sonication (Razin, 1974), and membrane preparations of high purity can be obtained (Razin, 1975). Cholesterol is an essential component of the cell membrane (Rottem, 1978), and is incorporated exclusively into this membrane.

Cholesterol in mycoplasmas

The total dependence of mycoplasmas on an external supply of cholesterol may be utilized to introduce controlled alterations in the cholesterol content of the membrane. The successful adaptation of M. capricolum to grow with very little cholesterol (Rottem et al., 1973a) provides an ideal model system. The most remarkable difference between the membranes of the normal and adapted strains is the existence of a thermal phase transition in cholesterol--poor membranes only (Rottem et al., 1973b). Cholesterol affects (a) the physical state of membrane lipids, (b) the osmotic fragility, (c) the permeability of the cells, and (d) the activity of membrane-associated enzymes (Rottem, 1978; Cirillo, 1978). Mycoplasma cells are susceptible to cholesterol-binding agents such as polyene antibiotics (Kinsky, 1970; Bittman and Rottem, 1976; Bittman, 1978), digitonin (Rottem and Razin, 1972), and oxygen-labile bacterial hemolysins (Rottem et al., 1976). Cholesterol also reduces the rates of passive

diffusion of solutes into Acholeplasma laidlawii (De Kruyff et al., 1972, 1973; McElhaney et al., 1973), which does not require cholesterol for growth and seems to incorporate sterols through a physical absorption process (Gershfeld et al., 1974; Razin et al., 1974). The amount of cholesterol that can be incorporated into the membrane of growing A. laidlawii cells appears to be subject to an unknown control mechanisms. The amount of cholesterol incorporated into membranes of intact cells is lower than that incorporated into isolated membranes even after a 5-hour incubation period in medium containing labeled cholesterol and Tween 80, implying that the outer half of the lipid bilayer may be enriched in the sterol relative to the inner surface (Razin et al., 1974). Evidence that cholesterol is distributed in both halves of the lipid bilayers in mycoplasmas species has been obtained by measuring the changes in the spectrum of the polyene antibiotic filipin on its interaction with membrane cholesterol (Bittman and Rottem, 1976). The initial rates of filipin-cholesterol association were significantly lower with intact cells than with isolated membranes. The rate constants indicate a symmetrical distribution of cholesterol in the two leaflets of the M. gallisepticum membrane, whereas in M. capricolum about two-thirds of free cholesterol is localized externally. In confirmation with this, exchange studies of ^{14}C-cholesterol between resting M. gallisepticum cells and high density lipoproteins shows the existence of two different cholesterol environments with approximately 50% in the inner half of the bilayer (Rottem et al., 1978).

Alteration of membrane composition of mycoplasmas

The protein to phospholipid ratios of the mycoplasma membrane can be changed dramatically by the selective inhibition of the

synthesis of membrane protein with chloramphenicol or valinomycin (Razin, 1974) or by manipulation of physiological conditions such as aging of the culture (Rottem and Greenberg, 1975). In aged cultures of M. hominis the increase in protein to lipid ratio in the membrane is accompanied by a continuous increase in the packing density of proteins on the inner surface of the cell membrane (Amar et al., 1976). Proteolytic digestion with trypsin and papain modified the cell surface moderately, whereas with pronase the membrane permeability was damaged (Amar et al., 1974).

Another parameter subject to experimental variation is the phospholipid content. Mycoplasma cells were demonstrated to incorporate phosphatidylcholine and sphingomyelin from the medium up to the amount of 35% of the total lipid phosphorus, increasing the phospholipid to protein ratio of the membranes (Gross and Rottem, 1979). Mycoplasma membranes are unusual in that in some strains cholesterol esters are present in appreciable amounts. Esterified cholesterol cannot be synthesized by mycoplasmas, and the incorporated cholesterol esters cannot be hydrolyzed (Rodwell, 1963; Argaman and Razin, 1965; De Kruyff et al., 1972; Razin, 1975). While cholesterol is an essential component of the membrane, cholesterol ester is not. Cholesterol esters are supposed to be localized in the hydrophobic interior of the membrane (Razin,1975). Experiments carried out some time ago (Smith, 1960; De Kruyff et al., 1973; Rottem et al., 1971) established that in order to promote mycoplasma growth, the sterol must possess a planar steroid nucleus, a free hydroxyl group at the 3β-position, and a hydrocarbon side chain--the exact structural features required for a sterol to exert a regulatory effect on the fluidity of

membranes. Some sterols like epicholesterol and lanosterol are incorporated into the cell membrane without affecting the growth, while some other like epicoprostanol inhibit the growth (Smith, 1964).

Studies of the distribution and transbilayer movement of cholesterol in mycoplasma membranes (objectives of Part II)

An intensive study was carried out in order to (1) elucidate the localization of cholesterol between the two halves of the bilayers in different strains of mycoplasma, (2) determine the rate of movement of cholesterol from the outer to the inner half of the bilayer, and (3) to analyze the factors affecting these processes.

Specific subjects dealt with in this part are: The rate of cholesterol flip-flop in the membrane of growing cells, the relative sizes of externally oriented and internally oriented cholesterol, and the distribution of cholesterol upon inhibition of the growth (Chapter III). Chapter IV answers the questions: (a) to what extent are transbilayer cholesterol distribution and the rate of flip-flop maintained upon aging of the cells? (b) Does addition of exogenous proteins or crosslinking agents influence cholesterol incorporation? Chapter V is a study of whether systematic changes in composition of phospholipids and cholesterol esters affect cholesterol distribution and the rate of transbilayer movement. In Chapter VI, structural modification in the sterol molecule are made in the M. capricolum strain and the sterol-poor strain in order to analyze how these changes affect the molecular organization and the rates of movement of sterols.

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Part I, Chapter I

Permeability Properties of Sterol-Containing Liposomes from Analogs of Phosphatidylcholine Lacking Acyl Groups

The synthesis of the phosphatidylcholine analogs was performed by Dr. P. W. Deroo (Deroo, et al., 1976) and the analysis of the efflux of trapped ^{14}C-glucose was done by Y. A. Isaacson and is also described in his Ph.D. dissertation (Ph.D. Program in Biochemistry, The City University of New York, 1978).

This manuscript was published in Biochemistry 18,2118,(1979). Preliminary accounts of this work have been presented at the 174th American Chemical Society National Meeting, Chicago, Illinois, August 1977 (Abstracts of Papers,p.160) and at the New York Academy of Sciences Conference "Liposomes and their Uses in Biology and Medicine," New York, September 1977 [Ann. N.Y. Acad. Sci. 308, 441, (1978)].

ABSTRACT: The effect of sterols on the rates of nonelectrolyte movement in multilayered liposomal membranes formed from various phosphatidylcholine (PC) analogs was studied. We measured the temperature dependence of the permeation of small, polar solutes through lipid bilayers in the liquid-crystalline state of the following saturated phosphatidylcholines: diesters containing 14, 16, or 18 carbon atoms in each acyl chain; the corresponding diethers, which lack the carbonyl groups; and alkyl analogs (such as octadecyleicosylphosphorylcholine (OEPC), which have no carboxylate ester bond and no diacylglycerol moiety. In addition, a diether-PC having one saturated and one unsaturated chain was used, and analogs of OEPC were synthesized in which the choline group is modified. At temperatures above the gel to liquid-crystalline phase transition of these diester-, diether-, and dialkyl-phosphatidylcholines, the 3β - hydroxysteroids cholesterol and ergosterol decreased the permeability of glycerol, urea, acetamide, and glucose, whereas epicholesterol and lanosterol exerted little effect on the reflection coefficient for acetamide penetration. Cholesterol reduced solute permeability in liposomes from OEPC analogs having increased separation between the phosphoryl and quaternary ammonium groups of the phosphorylcholine moiety or increased steric bulk in the choline moiety and in the quaternary ammonium group. The activation energy and activation entropy for osmotic swelling of liposomes by glycerol and urea permeation and for fluorescence change of a liposome-associated merocyanine dye upon acetamide permeation were not altered significantly by

cholesterol incorporation (48 mol percent) into bilayers with diester- or diether-phosphatidylcholines we examined.

The similarities between the effects of sterols on the initial rates and reflection coefficients of polar nonelectrolyte diffusion in diester-PC bilayers and in those from our phospholipids lacking acyl groups (diether-PC and alkyl-PC analogs) indicate that carbonyl oxygens of the fatty acyl groups in the 1 and 2 positions of PC are not essential to obtain interaction with cholesterol. Thus, there is no specific requirement for hydrogen bonding between the hydrogen of the sterol hydroxyl group and a carbonyl oxygen of diester-PC. The studies with OEPC analogs suggest that no rigid structural specificity in the choline moiety of phosphorylcholine must be met for lecithin-cholesterol interaction.

INTRODUCTION

The most extensively investigated lipid-lipid interaction in membranes is that of sterols with phospholipids. Sterols that have a planar steroid nucleus, a nonpolar side chain at C-17, and a 3β - hydroxyl group restrict the mobility of phospholipids in bilayers when the phospholipid is in the liquid-crystalline phase, resulting in a decrease in the permeability of the membrane (see review articles by Jain, 1975; Chapman, 1975; Demel and deKruyff, 1976). The importance of van der Waals forces in stabilizing the phospholipid - sterol interaction must be stressed, since X-ray and neutron diffraction measurements in hydrated multilayers indicate that the steroid nucleus penetrates into the fatty acid region of the bilayer (Lecuyer and Dervichian, 1969; Franks, 1976; Worcester and Franks, 1976). Since 3α-hydroxysteroids exert diminished effects on the permeability properties of liposomes and Acholeplasma laidlawii cell membranes (deKruyff et al., 1972; Bittman and Blau, 1972) in comparison with cholesterol, the stereochemical orientation of the sterol hydroxyl group at the aqueous-lipid interface must be considered in addition to the apolar forces between the steroid nucleus and the phospholipid fatty acyl chains. Studies with isosteric and nonisosteric analogs of phosphatidylcholine (PC)[1] bearing C-P (phosphonate) and C-P-C (phosphinate) linkages indicated that strict demands at the PC polar head group near the phosphorous-oxygen ester bonds must be met for PC-cholesterol interaction to occur (Bittman and Blau,1972.)

The motions of the choline methyl groups of PC and of the phosphorus atom are largely unaffected by incorporation of cholesterol into PC bilayers, as judged by C-13 (Keough et al., 1973), deuterium (Stockton et al., 1974; Gally et al., 1976), and phosphorus-31 nmr studies (Yeagle, et al., 1975; Cullis, et al., 1976; Brown and Seelig, 1978). These studies suggest that no direct interactions occur between cholesterol and the phosphocholine group in PC-cholesterol bilayers. Cholesterol appears to act as a spacer molecule, increasing the separation between PC head groups (Yeagle, et al., 1977; Oldfield, et al., 1978; Yealge, 1978). It should be noted, however, that a direct interaction between cholesterol and the phosphate oxygen of PC has been proposed on the basis of infrared dichroism measurements in oriented multibilayers (Verma and Wallach, 1973) and nmr data obtained from aqueous dispersions (Darke, et al., 1972). On the other hand, from measurements of force-area curves in monolayers and of calorimetric properties of liposomes from phospholipid analogs, deKruyff, et.al., (1973) concluded that cholesterol does not form hydrogen bonds with any parts of the phospholipid molecule.

Neutron and X-ray diffraction data have been interpreted to give the location of the cholesterol hydroxyl group in egg PC bilayers near the water interface, in the vicinity of the phospholipid carbonyl groups (Worcester and Franks, 1976; Franks, 1976). To minimize the contact of the steroid nucleus with the polar aqueous phase, hydrogen bonding between the cholesterol hydroxyl groups

and a PC carbonyl oxygen atom has been postulated (Brockerhoff, 1974; Huang, 1976, 1977; Yeagle and Martin, 1976). Differences in the extent of broadening of the nmr linewidths of the fatty acyl methylene protons of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 2,3-dipalmitoyl-sn-glycero-1-phosphocholine upon addition of cholesterol were suggested to provide support for a model involving hydrogen bonding between the cholesterol hydroxyl group and the carbonyl oxygen at the 2 position of PC (Chatterjie and Brockerhoff, 1978). The model proposed by Huang (1976, 1977) considers the complementarity of packing between the phospholipid and sterol to be diminished when the orientation of the hydroxy group is 3α rather than 3β.

The purpose of the present investigation was to examine whether this postulated hydrogen bond is an important stabilizing force between PC and cholesterol in bilayers. We tested the effects of sterols as a function of temperature on the permeability properties of liposomes prepared from synthetic PC analogs that lack the ester carbonyl oxygens of lecithin. The permeation rates of acetamide, glycerol, urea, and glucose through lipid bilayers of liposomes prepared from saturated diester, diether, and dialkyl phosphatidylcholines were measured in the presence and absence of sterols. We also made liposomes from a diether-PC bearing one unsaturated and one saturated chain and from dialkyl-PC analogs in which the choline moiety is altered.

Experimental Section

Materials

1,2-Diacyl-sn-glycero-3-phosphoryl cholines were purchased from

Sigma Chemical Co. 1,2-Dialkoxy-3-sn-phosphatidyl cholines were synthesized as described by Chen and Barton (1971). rac-1-Oleyl-2-hexadecylphosphatidylcholine ($C_{18:1}$ $C_{16:0}$ diether-PC)[1] was purchased from Serdary Research Laboratories, London, Ontario. The synthesis of the alkyl-PC, OEPC, was described previously (Deroo, et al., 1976). Analogs of OEPC in which the choline moiety was replaced (Figure 1) were synthesized by coupling of the desired quaternary ammonium alcohol tosylate salt with octadecyleicosylphosphoric acid, as described by Deroo, et al., (1976). Dicetyl phosphoric acid and cholesterol were purchased from Sigma Chemical Co.. Epicholesterol and ergosterol were obtained from Schwarz-Mann. These sterols were recrystallized twice from ethanol. Lanosterol was a gift of Dr. W.R. Nes and was purified (to about 95% purity) by the methods of Bloch and Urech (1958) and Rees, et al. (1966). The other lipids were found to be chromatographically pure on thin-layer analysis using silica gel G plates. Stock solutions of lipids in chloroform were stored at -20°C. Merocyanine 540 dye was obtained from Eastman Kodak. Phospholipase D from cabbage was purchased from Sigma Chemical Co., cholesterol oxidase (Bacillus cereus) was from Beckman Instruments, and horseradish peroxidase was from Worthington Biochemicals. ^{14}C-glucose was purchased from New England Nuclear Corp. Acetamide, Urea, and glycerol were reagent grade. Filipin complex was purified and dissolved as described elsewhere (Blau and Bittman, 1977).

Methods

Preparation of Liposomes. The appropriate amounts of PC, sterol, and dicetyl phosphoric acid were added to a vial and the chloroform solvent was removed under nitrogen and vacuum. The thin film of lipid that coated the walls of the vial was dispersed in aqueous medium by agitation with a Vortex mixer in the presence of glass beads. Multilameller liposomes were prepared at temperatures several degrees above the lipid phase transition. (The transition temperatures were measured by Jain, et.al., 1979). Rates of solute permeation were also measured above the phase transition temperature. To ascertain the phospholipid and sterol content of the liposomes, liposomes were pelleted at 4°C by centrifugation at 20,000 rpm for 30 minutes in a Sorvall SS 34 rotor. The assay of phospholipid and sterol, using methods cited previously (Blau and Bittman, 1977) showed that liposomal concentrations were equal to the concentrations prior to centrifugation.

Swelling of Liposomes by Glycerol and Urea Permeation. A 50-μL aliquot of liposomes dispersed in 60 mM KCl was added to a 1-cm pathlength cuvette containing 0.6 mL of 0.4M aqueous glycerol in urea solution. The final total lipid concentration was 0.5 mM, except in some experiments where 1.0 mM was used. Liposomes contained 4 mol percent of dicetyl phosphoric acid. After the liposome suspension was mixed rapidly with the hypertonic glycerol on urea solution, the initial rapid decrease in light scattering from liposomes shrinking and then the slow increase in light scattering from swelling were

monitored on a Perkin-Elmer Hitachi MPF - 2A spectrofluorometer. Both monochromators were set to 450 nm. The temperature was controlled by circulating water through the cuvette holder. The initial rate of liposome swelling was measured from the tangent to the light-scattering increase. The relative intensity at which the swelling phase was completed was also measured, allowing calculation of the rate constant, k, from the quotient of the initial rate-total amplitude of the light scattering change. At least five measurements were made at each temperature. At temperatures above 35°C, glycerol and urea solutions were saturated with helium to minimize air bubbles. The activation energy was calculated according to the equation $k = A \exp(-Ea/RT)$; semilogarithmic plots of the rate constants or the initial rates vs. the reciprocal of the absolute temperature by the method of least squares were made with a Tektronix 8200 plotter coupled to a Sigma 7 computer. The value of $\Delta S^{\ddagger}$ was obtained from the relationship:

$\Delta S^{\ddagger} = 4.576(\log k - 10.753 - \log T) + E_a/T$, where k is in units of of sec^{-1}.

Efflux of Trapped ^{14}C - Glucose. A trace of [1 - ^{14}C] - glucose (2×10^7 cpm), specific activity 48.2 C_i/mol, in 0.5 mL of 75 mM KCl - 75 mM NaCl was added to a thin, dry film of the desired concentration of lipid coated on the walls of a conical centrifuge tube. The concentration of PC was 25 mM. Dicetyl phosphoric acid was added to a concentration of 10 mol percent. Liposomes were formed above 60°C. To remove untrapped glucose, the liposomes were placed in a dialysis sac and dialyzed extensively (about 5 hours) at room temperature against large volumes (1 L) of 75 mM KCl - 75 mM NaCl solution, with

frequent changes of the dialysate. To monitor the time course of efflux of trapped glucose, the liposome suspension was transferred to a small test tube containing 3 mL of 75 mM KCl - 75 mM NaCl solution and shaken in a water bath at 55° C. At intervals of 30 minutes, 100 - μL aliquots were taken from the test tube for radioactive counting in a Beckman liquid scintillation counter. An aliquot was taken after 3 hours from the dialysis bag to measure the amount of trapped glucose remaining in the liposome suspension.

Reflection Coefficients. Reflection coefficients for the rapidly permeant molecule, acetamide, were determined in liposomes prepared from PC analogs in the presence and absence of sterols with a Durrum stopped-flow spectrophotometer (Durrum-Dionex Corp., Sunnyvale, California). The light transmitted at 180° was monitored at 450 nm. Changes in liposome volumes arising from shrinking and swelling were measured beginning 150 ms after mixing. Measurements at shorter times were precluded by an artifact discussed elsewhere (e.g., Bittman et al., 1976; Owen and Eyring, 1975). The transmittance (T) following the 150 - ms disturbance period was linear with respect to time, and the oscilloscope traces were similar to those shown previously for osmotic shrinking and swelling (Bittman and Blau, 1972). The initial absorbance was calculated from the initial value of T. The initial rate of absorbance change, dA/dt, was obtained from the measured dT/dt, and then converted to d(1/A)/dt using the relationship $(A_{initial})^{-2}$ dA/dt = d (1/A)/dt. The latter quantity is proportional to relative changes

in liposome volume arising from changes in osmolarity (Bangham et al., 1967). The reflection coefficient, σ, was obtained from the ratio C_{iso}/C_s (Goldstein and Solomon, 1960), where C_{iso} is the concentration of the isosmolar electrolyte (30 mM KCl) and c_s is the concentration of the permeant molecule (acetamide) at which no volume change occurs. The latter value was determined by the "zero-time" method (Goldstein and Solomon, 1960; Lelievre and Rich, 1973). The d(1/A)/dt values were plotted vs. the acetamide concentrations, and c_s was determined by extrapolation to zero volume change.

Treatment of Liposomes with Filipin or with Enzymes. Aliquots of filipin in dimethylformamide were added to liposomes. The filipin concentration was determined spectrophotometrically at 358 nm using the extinction coefficient cited previously (Blau and Bittman, 1977). The concentration of dimethylformamide did not exceed 1.0% (v/v). In experiments involving phospholipase D and cholesterol oxidase, liposomes containing 50 mol percent cholesterol (1 mM total lipid concentration) were first incubated for 1 hour at 37° C with phospholipase D (10 µg per mL of liposome suspension) in 0.5 mM Tris-HCl buffer containing 50 mM $CaCl_2$, pH 5.6. Immediately after the phospholipase treatment, liposomes were incubated for 10 minutes at room temperature with cholesterol oxidase (0.2 units per mL); horseradish peroxidase (50 units per mL) was added to decompose hydrogen peroxide generated upon oxidation of cholesterol. Then the reflection coefficients were measured in the stopped-flow apparatus. For assay of enzymatic

action, incubations were stopped by adding chloroform-methanol (2:1, v/v), and the fraction of PC cleaved was assayed by thin-layer chromatography in a procedure similar to that of Martin et al. (1975). The cholesterol content was assayed colorimetrically.

Fluorescence Change of Liposome-Associated Merocyanine Dye. Merocyanine 540 dye dissolved in ethanol was added to liposomes containing 4 mol percent dicetyl phosphoric acid. The final dye concentration was 2.5 mM. The ethanol concentration in the liposome suspension never exceeded 0.5%. Liposomes (0.5 mM total lipid concentration) were prepared from the PC analogs in the presence or absence of cholesterol and were dispersed in 60 mM KCl solution. The fluorescence of the merocyanine dye was monitored at 580 nm (with excitation at 558 nm) with a Perkin-Elmer Hitachi MPF - 2A Spectrofluorometer. The fluorescence intensity of the dye was lower in the presence of liposomes than in aqueous solution; a steady level of fluorescence was reached within about 2 minutes after the addition of dye to liposomes stirred in a cuvette. The addition of a small volume (e.g., 25 μL) of an aqueous stock solution of acetamide (final concentration of 57 mM) to liposomes (0.5 mL) that had reached the steady level of fluorescence caused an initial rapid drop in fluorescence intensity of approximately 8 to 12 percent per sec. A new steady level of fluorescence was reached several minutes after the addition of

acetamide. The initial rate of fluorescence decrease and the steady-state fluorescence intensity were temperature dependent. The rate constant was calculated at various temperatures between 30° and 55° C, and activation parameters were obtained as stated above for nonelectrolyte permeation. No significant change was found in the wavelength of the emission peak upon swelling of the liposomes by acetamide addition. The decrease in fluorescence intensity of the dye observed on liposome swelling may arise from an increase in the amount of liposome-associated dye relative to the amount in the external medium, from permeation of the dye into the liposome bilayer, or from formation of dye aggregates.

Results

Osmotic Behavior of Liposomes from PC Analogs. The volume change experienced by an ideal osmometer exposed to a change in the osmotic strength of the suspending medium is given by the Boyle - van't Hoff law, $\underline{V} = \underline{K}\ (1/\underline{c}) + \underline{b}$, where $\underline{V}$ is the volume change (volume is inversely proportional to absorbance for liposomes), $\underline{K}$ is a constant independent of wavelength and osmometer concentration, $\underline{c}$ is the osmotic pressure gradient across the membrane in terms of concentration,and $\underline{b}$ is the osmotic dead space (Bangham et al., 1967). Liposomes prepared in KCl solution above the phase transition temperatures of the diester, diether, and dialkyl phosphatidylcholines gave linear Boyle - van't Hoff plots when exposed to an osmotic pressure gradient of KCl

(Figure 2A, B) or acetamide (Figure 2C). This indicates that liposomes formed from these PC analogs are ideal osmometers under these conditions. Negative-staining electron microscopy of OEPC liposomes showed the usual multilamellar appearance (O. R. Anderson, personal communication). When liposomes were mixed with 0.025 - 0.3 M acetamide solutions in the stopped-flow apparatus, the initial rates of swelling and shrinking were found to vary with the osmotic gradient in a similar manner in bilayers of diester, diether, and dialkyl phosphatidylcholines (Figure 3). Figure 3 also shows that the initial rate of volume change is markedly reduced in the presence of cholesterol.

Glycerol and Urea Permeation. The initial rates of glycerol and urea permeation into liposomes are decreased upon incorporation of cholesterol at various temperatures above the PC gel to liquid-crystalline phase transition. Since cholesterol decreases hydrocarbon chain mobility, reduced rates of solute permeation are expected. Figure 4 shows the temperature dependence of the rate constant for liposome swelling by glycerol permeation. A similar dependence was observed for urea permeation. Tables I and II present the activation parameters obtained from the Arrhenius plots for liposome swelling by glycerol and urea permeation. The activation energy for solute permeation through diester-PC bilayers was similar to that of the

corresponding diether-PC bilayers, but the initial rates were lower in diether-PC bilayers. Cholesterol incorporation to 48 mol percent did not cause a significant change in the activation energies and entropies.

Leakage of ^{14}C - Glucose. Cholesterol decreased the rate and extent of release of trapped glucose from OEPC liposomes, whereas epicholesterol did not (Figure 5A). This indicates that the 3β-hydroxyl group of cholesterol is required for reduction of glucose permeability of these dialkyl-PC bilayers. Cholesterol also reduced the rate and extent of leakage of glucose from liposomes formed from analogs of 2-octadecyleicosyl phosphate. Despite the substitution of a methyl group alpha to the phosphorus in the choline moiety (Me-OEPC, Figure 5B) and of a N-ethyl group in place of one N-methyl group (N-Et,N,N-diMe-OEPC, Figure 5C), the increase in steric bulk at the head group region did not hinder the ability of the phospholipid to interact with cholesterol. Similar results were obtained with Propyl-OEP liposomes. Figure 5D shows that cholesterol affects glucose permeation across di-C_{16} diether-PC bilayers in a time course similar to that observed in bilayers formed from phospholipids lacking oxygen atoms at the 1 and 2

positions.

Effect of Sterols on Reflection Coefficients. The reflection coefficient, σ, is an indication of the ability of a membrane to discriminate between solvent and solute molecules. The typical range of σ is between zero (for solutes that are as permeable as water) and 1.0 (for solutes that are impermeant); however, if the solute is more permeant than the solvent, σ can be negative (Grim, 1953). The ability of cholesterol and ergosterol to increase the rigidity of bilayers above the lipid phase transition temperature results in an increase in σ of a permeant solute (acetamide in the present experiments) relative to the cholesterol-free bilayers (Figure 6). Lanosterol and epicholesterol failed to affect σ significantly compared to sterol-free PC bilayers.

Addition of the polyene antibiotic, filipin, to PC bilayers containing 50 mol percent cholesterol resulted in the reappearance of the PC gel to liquid-crystalline phase transition; this calorimetric behavior is compatible with a filipin-induced, reversible withdrawal of cholesterol from the PC (Norman et al., 1972). We sought to use low doses of filipin to perturb the interaction of the various PC analogs with cholesterol, as indicated by the σ values for acetamide penetration. The data in Table III show that under conditions where filipin causes no alteration of the σ value of pure PC bilayers, σ of cholesterol-containing PC bilayers is reduced by 35 - 40%. Interaction of filipin with cholesterol

in liposomes from each PC examined reversed the increase observed in σ in PC - cholesterol bilayers relative to pure PC bilayers. An additional type of experiment to examine the effect on σ of a specific perturbation of the bilayer was to subject liposomes to the action of phospholipase D and cholesterol oxidase prior to measurement of the rate of acetamide penetration. (In the absence of pretreatment with phospholipase D, little or no cholesterol was oxidized by cholesterol oxidase.) The decrease in σ upon oxidation of about 15% of the cholesterol (after phospholipase D action) in DPPC and di-C_{16} diether-PC liposomes was similar (about 25 - 30%). The response of σ to perturbation by the polyene antibiotic or cholesterol oxidase suggests that the PC analogs we examined interact with cholesterol in a qualitatively similar manner.

Merocyanine Fluorescence. The activation parameters for the rate of fluorescence decrease of merocyanine on acetamide permeation are identical, within experimental error, in cholesterol-free and cholesterol-containing liposomes for the three phosphatidylcholines tested (Table IV). The incorporation of cholesterol to 48 mol percent in the PC bilayers depressed the initial rate of fluorescence change by about 20 - 25% at each temperature. The activation data in Table IV show that the rate-limiting step in the fluorescence change arising from acetamide permeation is not influenced by the presence of (a) cholesterol, (b) a carbonyl oxygen atom in the PC, or (c) unsaturation in the alkoxy chain of the PC.

Discussion

Our studies of nonelectrolyte permeability in liposomes from PC analogs that lack the carbonyl group show that hydrogen bonding of the type cholesterol-OH ··· O=C-PC is not the determining force stabilizing this interaction at the polar interface of the bilayers. The current studies corroborate the calorimetric data of Jain et al. (1979), who reported a decrease in the enthalpy of the phase transition and lateral phase separation in these diether-PC and alkyl-PC analogs and in the corresponding diester phosphatidylcholines at cholesterol or ergosterol mole proportions up to approximately 25%. Our observation that epicholesterol failed to cause marked changes in the reflection coefficient for acetamide permeation and in glucose efflux across bilayers of diester phosphatidylcholines and PC analogs (Figures 5 and 6) agrees with other measurements of nonelectrolyte permeability in diester-PC bilayers (Demel et al., 1972). At the high mole proportions of sterol we used (30-32 mol percent), it is likely that rates of solute permeability reflect the average mobility of the fatty acyl chains in the bilayer, since lateral phase separations appear to be absent and the size of the cooperative unit is small. In contrast to the significant difference between cholesterol and epicholesterol in permeation processes, differential scanning calorimetry measurements of Jain et al. (1979) showed that low mole proportions of these sterols induced a lateral phase separation in bilayers of diester phosphatidyl-

cholines and PC analogs. The decrease in the enthalpy of the phase transition of the PC-rich phase caused by cholesterol incorporation was similar to that caused by epicholesterol. The extent of steric contact between PC and sterol molecules in the sterol-rich phase may be sufficiently great so as to minimize the dependence of lipid-sterol interaction on sterol structure relative to regions where more steric freedom exists.

Our results contrast with those of Tirri et al. (1977), who calculated a reduction of about 5 kcal mol^{-1} in activation energy and -16 cal mol^{-1} $degree^{-1}$ in activation entropy for glycerol and urea permeation into diether-PC bilayers upon incorporation of cholesterol. Although the condensation effect of cholesterol was observed in monolayers of PC analogs lacking the carbonyl group (Fong et al., 1977), it was speculated that hydrogen bonding between the phospholipid carbonyl oxygen atom and the hydrogen of the cholesterol hydroxyl group is important in bilayers (Tirri et al., 1977). Schwarz and Paltauf (1977) reported that the rates of diffusion of Na^+, Cl^-, and glucose across vesicles prepared from a diether-PC were not affected by incorporation of cholesterol to 30 mol percent, and suggested that the carbonyl oxygens may be required for PC-cholesterol interaction; however, cholesterol reduced the freedom of motion of a fatty acid

spin probe to a similar extent in liposomes formed from each PC (Schwarz and Paltauf, 1977), and X-ray diffraction patterns for the organization of diether-PC and diester-PC bilayers were similar (Schwarz and Paltauf, 1976).

The lower initial rates of nonelectrolyte permeation we observed in diether-PC liposomes compared with diester-PC liposomes (e.g., Figure 4) indicate that van der Waals interactions between hydrocarbon chains are stronger in diether-PC than in the corresponding diester-PC. This is also reflected in the slightly higher phase transition temperatures of the diethers (Jain et al., 197).

The activation energies we report for glycerol penetration of diester-PC and diether-PC bilayers (Table I) are in the middle of the range reported previously for glycerol permeation across diester-PC bilayers [19 kcal mol^{-1} by deGier et al. (1971); 18 kcal mol^{-1} by McElhaney et al. (1973); 11 kcal mol^{-1} by Cohen (1975); 16 kcal mol^{-1} by Tirri et al. (1977)]. Our finding of no significant change in activation energy upon incorporation of cholesterol to 50 mol percent (Tables I, II, and IV) agrees with studies of glycerol permeation in diester-PC liposomes (deGier et al., 1971; McElhaney et al., 1973; Tirri et al., 1977), although there are reports of small increases in activation energy on addition of cholesterol (Cohen, 1975; Blok et al., 1977). Small, polar solutes are believed to penetrate through bilayer membranes as single, anhydrous species (e.g., Stein, 1967; deGier et al., 1971; McElhaney et al., 1973). The rate-limiting step is thought to involve dehydration of the hydrophilic solute at the interface region; part of the

activation process may also involve solute movement from the polar interface toward the hydrophobic phase of the membrane, where conformational isomerizations in the hydrocarbon chains may be considered (Blok et al., 1977). The data we present suggest that the transition-state structure corresponding to the rate-limiting step in liposome swelling from the passage of small, polar solutes across bilayers of phosphatidylcholines bearing diacyl, dialkoxy, and dialkyl chains is not altered significantly upon the addition of cholesterol. Permeation of the anhydrous solutes through the hydrophobic interior of the membrane is slower in the presence of cholesterol than in the presence of epicholesterol, where intermolecular packing between neighboring PC and sterol molecules is apparently weaker and the increase in lateral ordering of the paraffin phase is not so high.

Our conclusion that in unsonicated liposomes the ester groups of PC do not represent a primary site for interaction with cholesterol supports the study of de Kruyff et al. (1973), in which the effects of cholesterol on the force-area characteristics of phospholipid monolayers and on the calorimetric properties of liposomes were found to occur without any specific requirement for acyl groups in the phospholipid. It remains to be determined whether in other bilayers systems (e.g., those containing charged lipids, membrane proteins, or low sterol to phospholipid ratios) the phospholipid-sterol interaction may be stabilized by associations involving the acyl carbonyl group.

TABLE I:

Effect of Cholesterol on the Activation Parameters for Glycerol Permeation in Liposomes from Diester and Diether Phosphatidylcholines

	Without Cholesterol		With Cholesterol	
	Ea	$\Delta S^{\ddagger}$	Ea	$\Delta S^{\ddagger}$
	(kcal mol^{-1})	(cal mol^{-1} deg^{-1})	(kcal mol^{-1})	(cal mol^{-1} deg^{-1})
DMPC	14.5 ± 1.8	-19.3 ± 4.5	15.5 ± 1.5	-18.4 ± 4.4
Di- C_{14} Diether-PC	15.1 ± 1.1	-18.0 ± 3.7	13.9 ± 1.1	-24.0 ± 3.8
DPPC	15.2 ± 1.9	-19.4 ± 5.0	14.6 ± 1.6	-21.0 ± 4.9
Di- C_{16} Diether-PC	14.4 ± 1.3	-23.5 ± 4.4	11.6 ± 1.5	-28.6 ± 5.0
$C_{18:1}$ $C_{16:0}$ Diether-PC	14.6 ± 1.6	-18.6 ± 4.5	11.9 ± 1.9	-26.5 ± 4.5

Liposomes were subjected to hypertonic glycerol solution as described under Methods. The temperature ranges were 35 to 55° C for liposomes prepared from DMPC and di-C_{14} diether-PC, 42 to 58° C for DPPC and di-C_{16} diether-PC, and 25 to 52° C for $C_{18:1}$ $C_{16:0}$ diether-PC. Three separate liposome preparations were made from DMPC and di-C_{14} diether-PC, and two preparations were made from the other lipids. The data represent the average of at least four analyses at each temperature with each preparation. The errors represent the standard error of the mean. $\Delta S^{\ddagger}$ is reported at 318 °K for DMPC and di-C_{14} diether-PC liposomes, 323 °K for DPPC and di-C_{16} diether-PC, and 312° K for $C_{18:1}$ $C_{16:0}$ diether-PC. The molar ratio of PC to cholesterol was 1:1. The following are examples of the initial rates of liposomes swelling (in arbitrary fluorescence intensity

TABLE I (Cont'd.)

units per sec) in the absence and (in parentheses) presence of cholesterol: DMPC at 318 °K, 2.74 ± 0.07 (2.30 ± 0.01); di-C_{14} diether-PC at 318 °K, 2.63 ± 0.03 (2.41 ± 0.03); DPPC at 323 °K, 2.92 ± 0.05 (2.63 ± 0.05); $C_{18:1}$ $C_{16:0}$ diether-PC at 312 °K, 2.39 ± 0.04 (1.72 ± 0.03).

TABLE II:

Effect of Cholesterol on the Activation Parameters for Urea Permeation in Liposomes from Diester and Diether Phosphatidylcholines

	Without Cholesterol		With Cholesterol	
	Ea	$\Delta S^{\neq}$	Ea	$\Delta S^{\neq}$
	(kcal mol^{-1})	(cal mol^{-1} deg^{-1})	(kcal mol^{-1})	(cal mol^{-1} deg^{-1})
DPPC	15.3 ± 1.4	-17.8 ± 5.0	14.6 ± 1.7	-20.2 ± 5.2
Di-C_{16} Diether-PC	13.4 ± 1.4	-22.2 ± 4.5	13.0 ± 1.5	-24.9 ± 4.6
$C_{18:1}$ $C_{16:0}$ Diether-PC	12.8 ± 2.1	-24.5 ± 4.9	11.5 ± 2.0	-26.9 ± 4.2

The temperature dependence of urea permeation was examined over the ranges of 42 to 58° C for DPPC and Di-C_{16} diether-PC liposomes and of 25 to 52° C for $C_{18:1}$ $C_{16:0}$ diether-PC Liposomes. Two liposome preparations were made from each phospholipid, with or without cholesterol. $\Delta S^{\neq}$ is reported at 323 °K for DPPC and di-C_{16} diether-PC liposomes and at 312 °K for $C_{18:1}$ $C_{16:0}$ diether-PC liposomes. The molar ratio of PC to cholesterol was 1:1. The following are examples of the initial rates of liposome swelling (in arbitrary fluoresence intensity units per sec) in the absence and (in parentheses) presence of cholesterol: DPPC at 323 °K, 2.75 ± 0.01 (2.34 ± 0.05); di-C_{16} diether-PC at 323 °K, 2.81 ± 0.02 (2.42 ± 0.05); $C_{18:1}$ $C_{16:0}$ diether-PC at 312 °K, 2.20 ± 0.03 (1.63 ± 0.04).

TABLE III:

Reflection Coefficients for Acetamide Penetration into Liposomes from Diester, Diether, and Alkyl Phosphatidylcholines[a] and the Effect of Filipin Treatment.

	Without Cholesterol	With Cholesterol[b]	
		Without Filipin	With Filipin
DMPC	0.30	0.51	0.31
Di-C_{14} Diether-PC	0.20	0.60	0.38
DPPC	0.31	0.46	0.30
Di-C_{16} Diether-PC	0.29	0.57	0.38
DSPC	0.30	0.49	0.30
Di-C_{18} Diether-PC	0.31	0.60	0.36
OEPC	0.34	0.52	0.32
Propyl - OEP	0.37	0.49	0.30
$C_{18:1}$ $C_{16:0}$ Diether-PC	0.27	0.48	0.28

[a] Liposomes containing 4 mol percent dicetyl phosphoric acid and a total lipid concentration of 2 mM were dispersed in 30 mM KCl solution and mixed with an equal volume of acetamide solution. The initial rates of shrinking or swelling were measured from the stopped-flow oscilloscope traces, allowing calculation of reflection coefficients from the zero-time tangents as described in the Experimental Section. The average error in σ was about ± 0.03 or less. Experiments were conducted at the following temperatures: 30 °C for

TABLE III. (Cont'd.)

DMPC and di-C_{14} diether-PC liposomes; 45° C for DPPC and di-C_{16} diether-PC liposomes; 58° C for DSPC, di-C_{18} diether-PC, OEPC, and Proply-OEP liposomes; and 25° C for $^{C}18:1$ $^{C}16:0$ diether-PC liposomes.

b The molar ratio of PC to cholesterol was 1:1. Liposomes were treated with filipin for 10 minutes at room temperature in the dark at a molar ratio of cholesterol to filipin of 8:1. Addition of dimethylformamide alone (<1% v/v) did not affect σ. Addition of filipin (0.12 mM) to pure PC liposomes did not affect σ.

TABLE IV:

Effect of Cholesterol on the Activation Parameters for Fluorescence Decrease of Liposome-Associated Merocyanine Dye on Acetamide Permeation.

	Without Cholesterol		With Cholesterol	
	Ea	$\Delta S^{\neq}$	Ea	$\Delta S^{\neq}$
	(kcal mol^{-1})	(cal deg^{-1} mol^{-1})	(kcal mol^{-1})	(cal deg^{-1} mol^{-1})
DMPC	17.2 ± 1.6	-11.6 ± 3.5	17.0 ± 2.2	-14.2 ± 4.4
Di-C_{14} Diether-PC	16.8 ± 2.0	-13.4 ± 3.8	16.4 ± 1.7	-16.1 ± 3.5
$C_{18:1}$ $C_{16:0}$ Diether-PC	16.5 ± 2.0	-16.6 ± 5.0	16.1 ± 1.6	-20.4 ± 4.6

The temperature dependence of the fluorescence decrease of merocyanine 540 dye was examined over the ranges of 30 to 55° C for DMPC and di-D_{14} diether-PC liposomes and of 25 to 52° C for $C_{18:1}$ C:16:0 diether-PC liposomes. Four liposome preparations were made from DMPC and di-C_{14} diether-PC, and two were made from $C_{18:1}$ $C_{16:0}$ diether-PC, with or without cholesterol. $\Delta S^{\neq}$ is reported at 310 °K for DMPC and di-C_{14} diether-PC liposomes and at 312 °K for $C_{18:1}$ $C_{16:0}$ diether-PC liposomes. The molar ratio of PC to cholesterol was 1:1. The following are examples of the initial rates of fluorescence decrease (in arbitrary fluorescence intensity units per sec) in the absence and (in parentheses) presence of cholesterol: DMPC at 310 °K, 2.04 ± 0.03 (1.54 ± 0.05); di-C_{14} diether-PC at 310 °K, 1.89 ± 0.05 (1.56 ± 0.07); $C_{18:1}$ $C_{16:0}$ diether-PC at 312 °K, 1.94 ± 0.05 (1.52 ± 0.04).

[1]Abbreviations used:

PC, phosphatidylcholine

DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine

DPPC, 1,2-dipalmitoyl-sn-glycerol-3-phosphorylcholine

DSPC, 1,2-distearoyl-sn-glycero-3-phosphorylcholine

di-C_{14} diether-PC, glycerol-3-sn-phosphorylcholine 1,2-bis(tetradecyl ether)

di-C_{16} diether-PC, glycerol-3-sn-phosphorylcholine 1,2-bis(hexadecyl ether)

di-C_{18} diether-PC, glycerol-3-sn-phosphorylcholine 1,2-bis(octadecyl ether)

$C_{18:1}$ $C_{16:0}$ diether-PC, rac-glycerol-3-phosphorylcholine 1-cis-9'-octadecenyl 2-hexadecyl ether

OEPC, 2-octadecyleicosylphosphorylcholine

Me-OEPC, 2-octadecyleicosyl-1-methylcholine

N-Et,N,N-diMe-OEPC, 2-octadecyleicosylphosphoryl-N-ethyl-N,N- dimethylethanolamine

Propyl-OEP, 3-hydroxypropyl-N,N,N-trimethylammonium-2-octadecyleicosyl phosphate

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FIGURE CAPTIONS

FIGURE 1: Structures of the synthetic phosphatidylcholines. The 1,2-dialkoxy - sn - glycero - 3 - phosphorylcholines (diether-PC) used are: ditetradecyl, di-C_{14}; dihexadecyl, di-C_{16}; dioctadecyl, di-C_{18}. The 1-oleyl-2-hexadecyl-3-phosphorylcholine, $C_{18:1}$ $C_{16:0}$ diether-PC, was racemic. The 2-octadecyleicosyl derivatives are:

2-octadecyleicosylphosphorylcholine, OEPC;
2-octadecyleicosylphosphoryl-1-methylcholine, Me-OEPC; 2-octadecyleicosylphosphoryl-N-ethyl-N,N-dimethylaminoethanol, N-Et, N,N -diMe-OEPC; 3-hydroxypropyl-N,N,N-trimethylammonium-2-octadecyleicosyl phosphate, Propyl-OEP.

FIGURE 2: Boyle - van't Hoff plots of the reciprocal of the absorbance at 640 nm of liposomal suspensions vs. the final concentration of KCl or acetamide at equilibrium after osmotic shrinking or swelling. Liposomes containing 4 mol percent dicetyl phosphoric acid and 2 mM final total lipid concentration were dispersed in 60 mM KCl solution. The absorbance was measured on a Cary 14 spectrophotometer before and after liposomes were subjected to varying concentrations of KCl (panels A and B) or acetamide (panel C). Lipid bilayers were formed from the following phospholipids in the absence (panels A and C) and in the presence (panel B) of 48 mol percent cholesterol: (o) di-C_{14} diether-PC and di- C_{16} diether-PC, (Δ) DMPC, (□) OEPC and Propyl-OEP. Osmotic behavior was examined at the following temperatures: 30° C for DMPC and di-C_{14} diether-PC, 45° C for di-C_{16} diether-PC, and 58° C for OEPC and Propyl-OEP. Swelling and shrinking conditions appear on the right and left sides, respectively, of the vertical dashed line

in panels A and B.

FIGURE 3: Plots of the initial rates of shrinking and swelling, d(1/A)/dt, vs. the change in osmolarity upon mixing of liposomes in the stopped-flow apparatus with an equal volume of acetamide solution (25 to 200 mM). Liposomes containing 4 mol percent dicetyl phosphoric acid and 2 mM final total lipid concentration were dispersed in 30 mM KCl solution. The transmittance arising from light scattering was measured at 450 nm. The change in reciprocal absorbance units per sec was calculated as described under Methods. Liposomes were formed from:

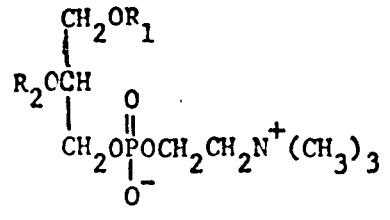
(▲) DSPC, (△) DSPC and cholesterol (1:1), (●) di-C_{18} diether-PC, (○) di-C_{18} diether-PC and cholesterol (1:1), (▬) OEPC, (▭) OEPC and cholesterol (1:1). The temperature was 58°C. Similar traces were obtained with liposomes from di-C_{14} and di-C_{16} diether-PC, DMPC, DPPC, and Propyl-OEP.

FIGURE 4: Plots of the logarithm of the rate constant for glycerol permeation into liposomes vs. the reciprocal of the absolute temperature. Liposomes were prepared from: (A) (▭) C18:1 C16:0 diether-PC, (▬) C18:1 C16:0 diether-PC and cholesterol (1:1 molar ratio); (B) (▭) DMPC, (▬) DMPC and cholesterol (1:1), (△) di-C14 diether-PC, (▲) di-C14 diether-PC and cholesterol (1:1).

FIGURE 5: Time course of efflux of trapped ^{14}C - glucose from liposomes. Liposomes were formed in the absence or presence of 30 mol percent sterol from: (A) (o) OEPC, (◐) OEPC and epi-cholesterol, (•) OEPC and cholesterol; (B) (o) Me-OEPC, (•) Me-OEPC and cholesterol; (C) (o) N-Et, N,N - diMe-OEPC, (•) N-Et, N,N - diME-OEPC and cholesterol; (D) (o) di-C_{16} diether-PC, (•) di-C_{16} diether-PC and cholesterol. The conditions are described under Methods. The temperature was 55° C.

FIGURE 6: Effect of incorporation of sterols on the reflection coefficient, σ, for acetamide penetration into liposomes. Liposomes were formed from: (A) (□) DMPC, (O) di-C_{14} diether-PC; (B) (◧) DPPC, (◐) di-C_{16} diether - PC; (C) (■) DSPC, (●) di-C_{18} diether-PC; (D) (△) OEPC, (⬭) Propyl-OEP. Reflection coefficients were measured in the absence of sterol (PC), and in the presence of 32 mol percent of lanosterol, epicholesterol, and cholesterol and of 48mol percent of cholesterol and ergosterol. The following temperatures were used: (A) 30° C, (B) 45° C, (C) 58° C, and (D) 58° C.

Diether PC



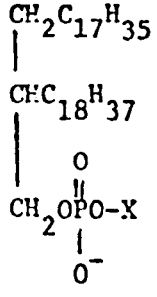
Di-C_{14} : $R_1, R_2 = C_{14}H_{29}$

Di-C_{16} : $R_1, R_2 = C_{16}H_{33}$

Di-C_{18} : $R_1, R_2 = C_{18}H_{37}$

$C_{18:1}$ $C_{16:0}$: $R_1 = \Delta^9 - C_{18}H_{35}$; $R_2 = C_{16}H_{33}$

Octadecyleicosyl Derivatives



	X
OEPC:	$CH_2CH_2N^+(CH_3)_3$
Methyl-OEPC:	$CH(CH_3)CH_2N^+(CH_3)_3$
N-Et,N,N-DiMe-OEPC:	$CH_2CH_2N^+(C_2H_5)(CH_3)_2$
Propyl-OEP:	$CH_2CH_2CH_2N^+(CH_3)_3$

FIG. 1

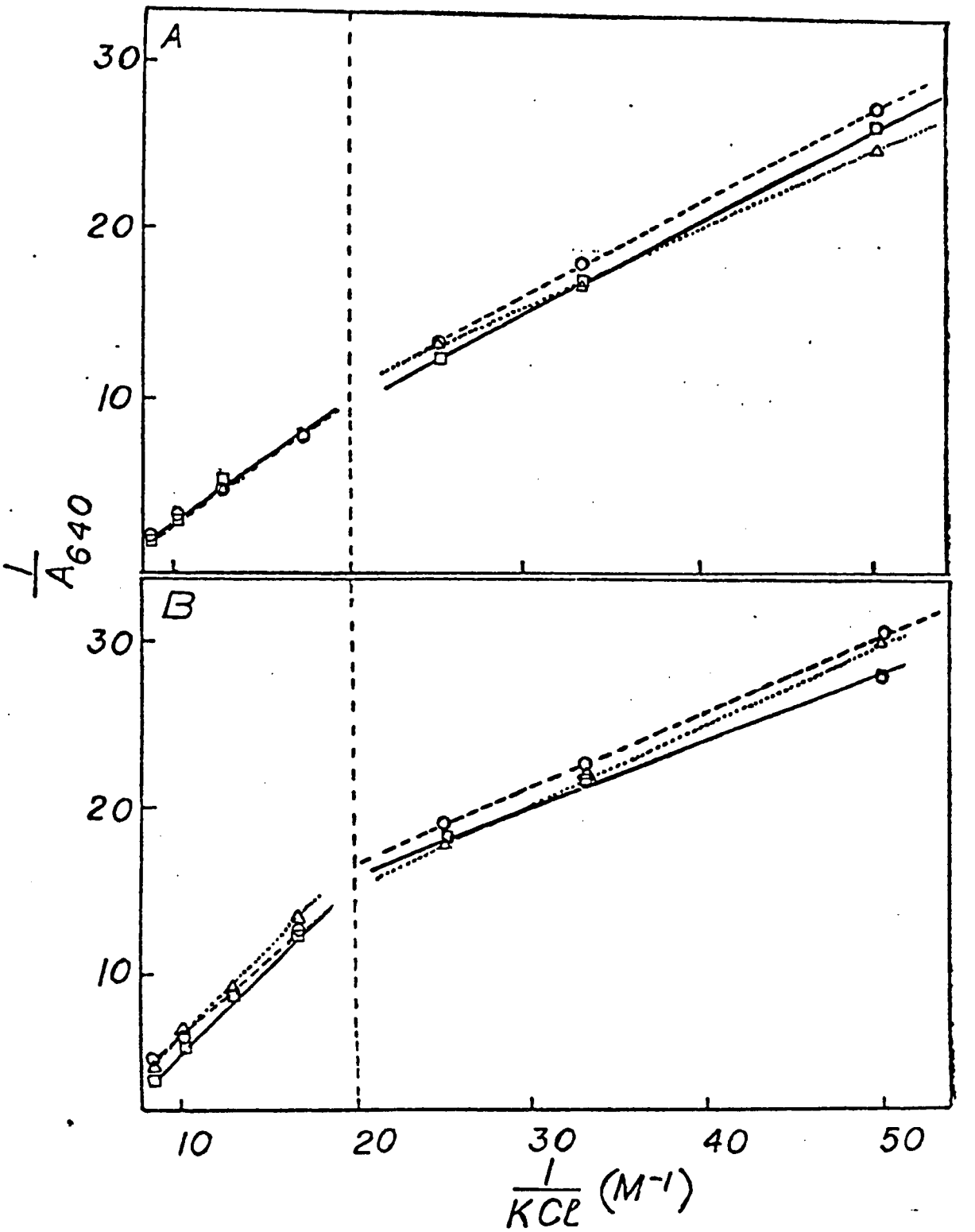


FIG. 2

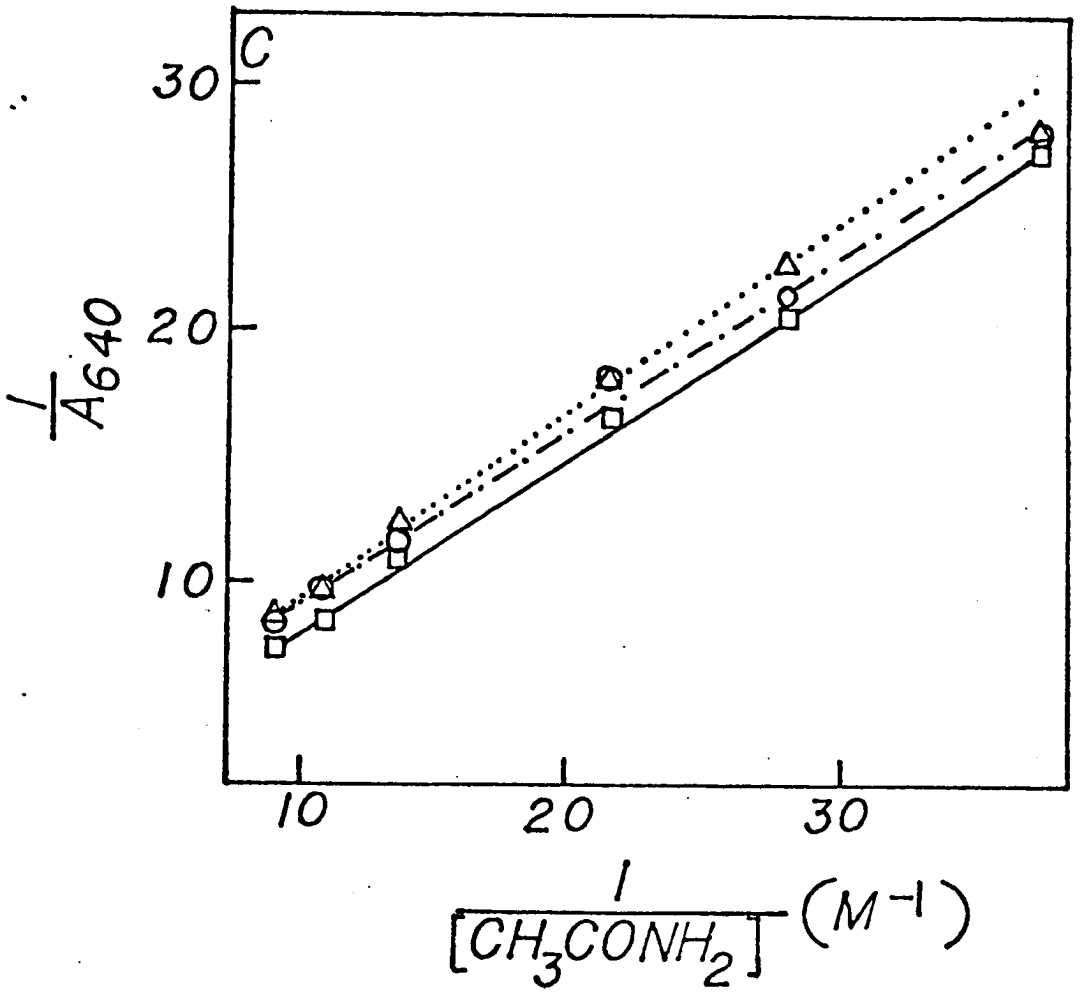


FIG. 2

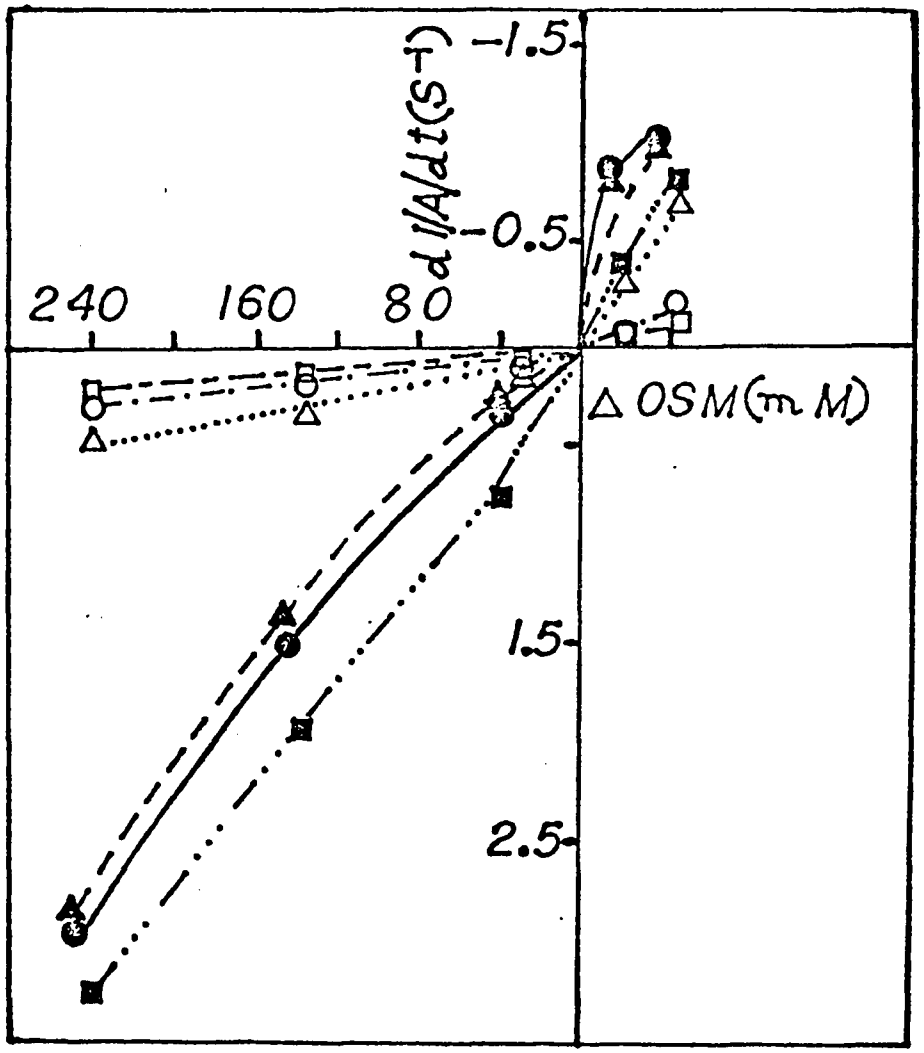


FIG. 3

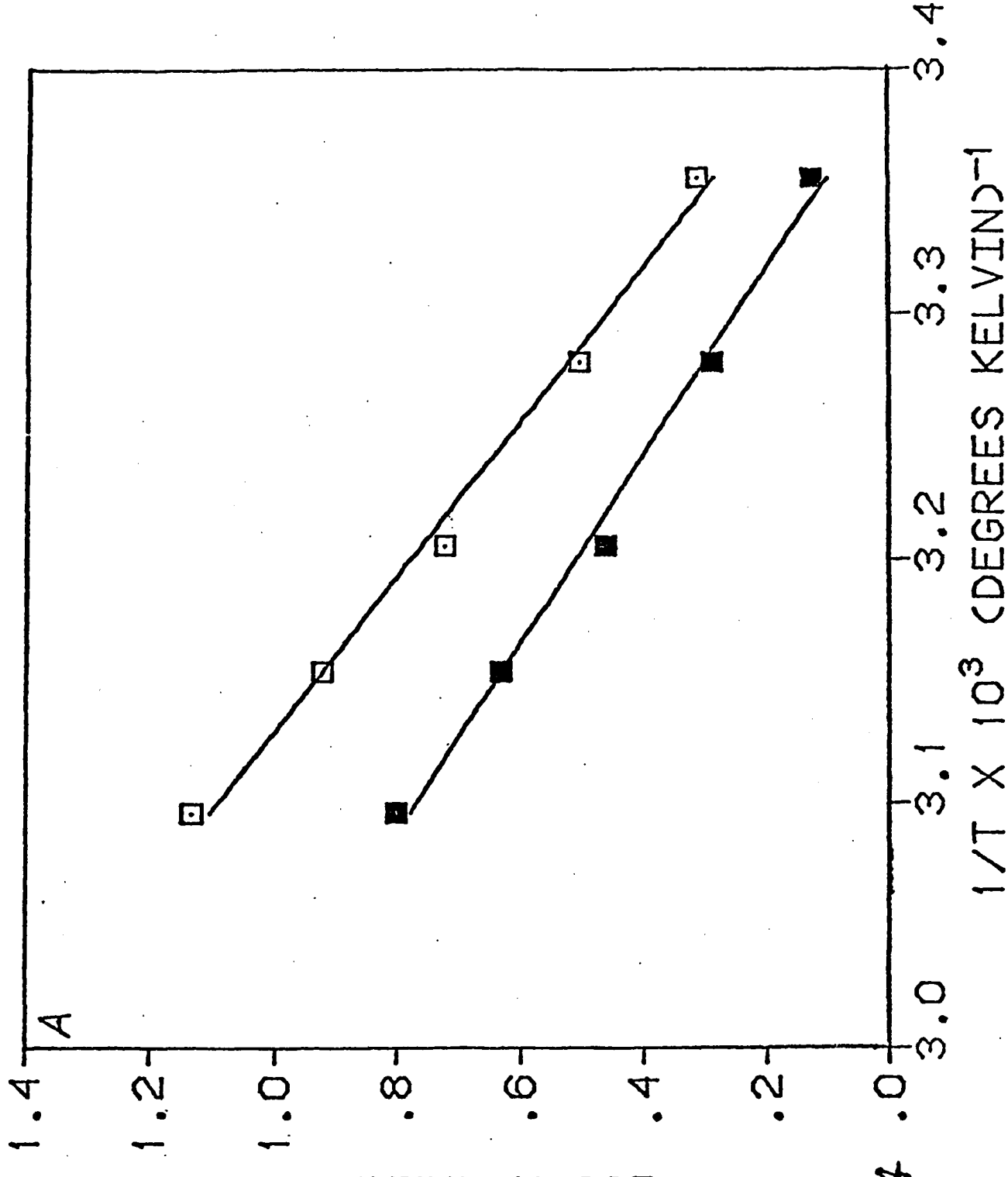


FIG.4

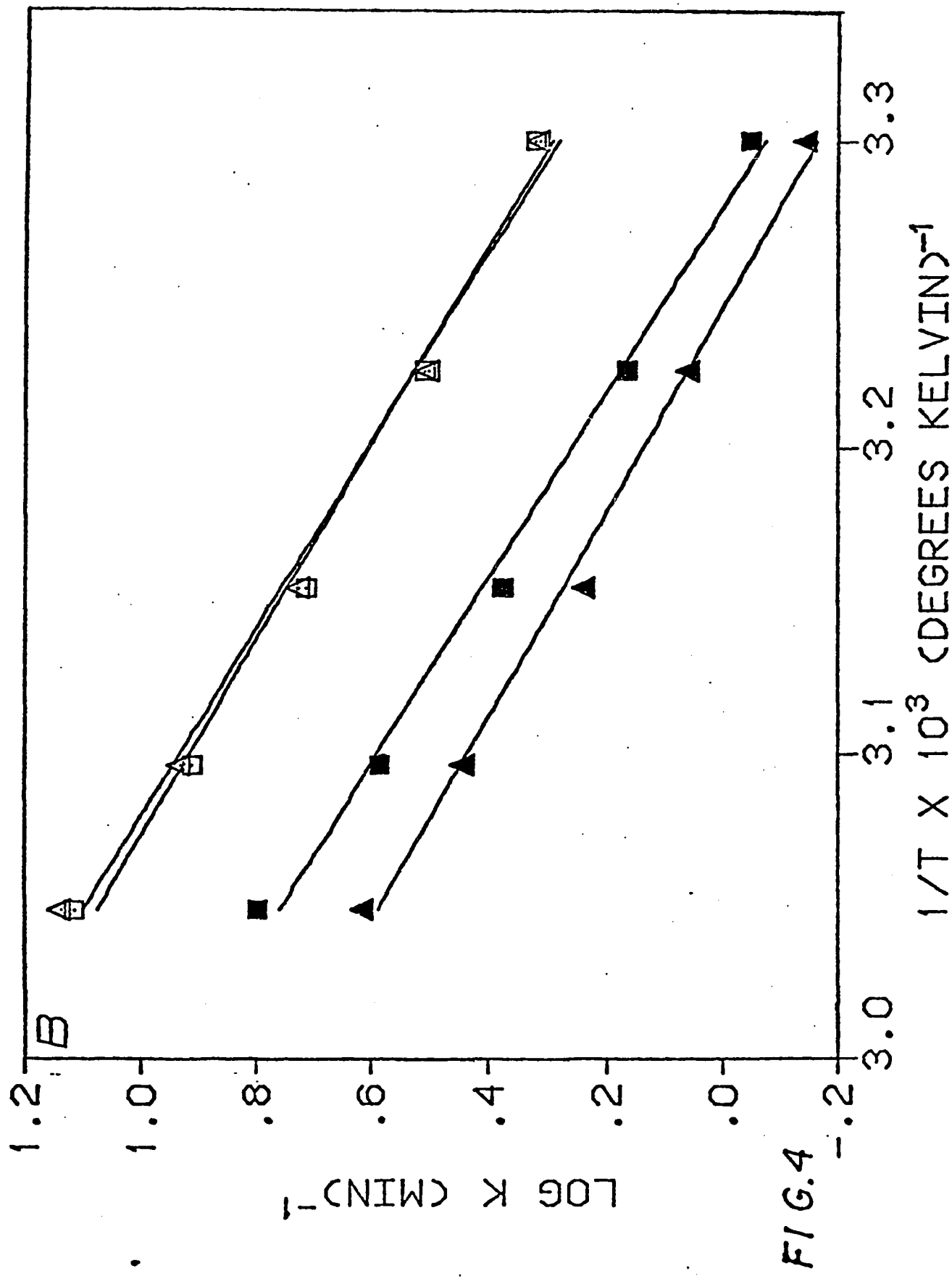


FIG.4

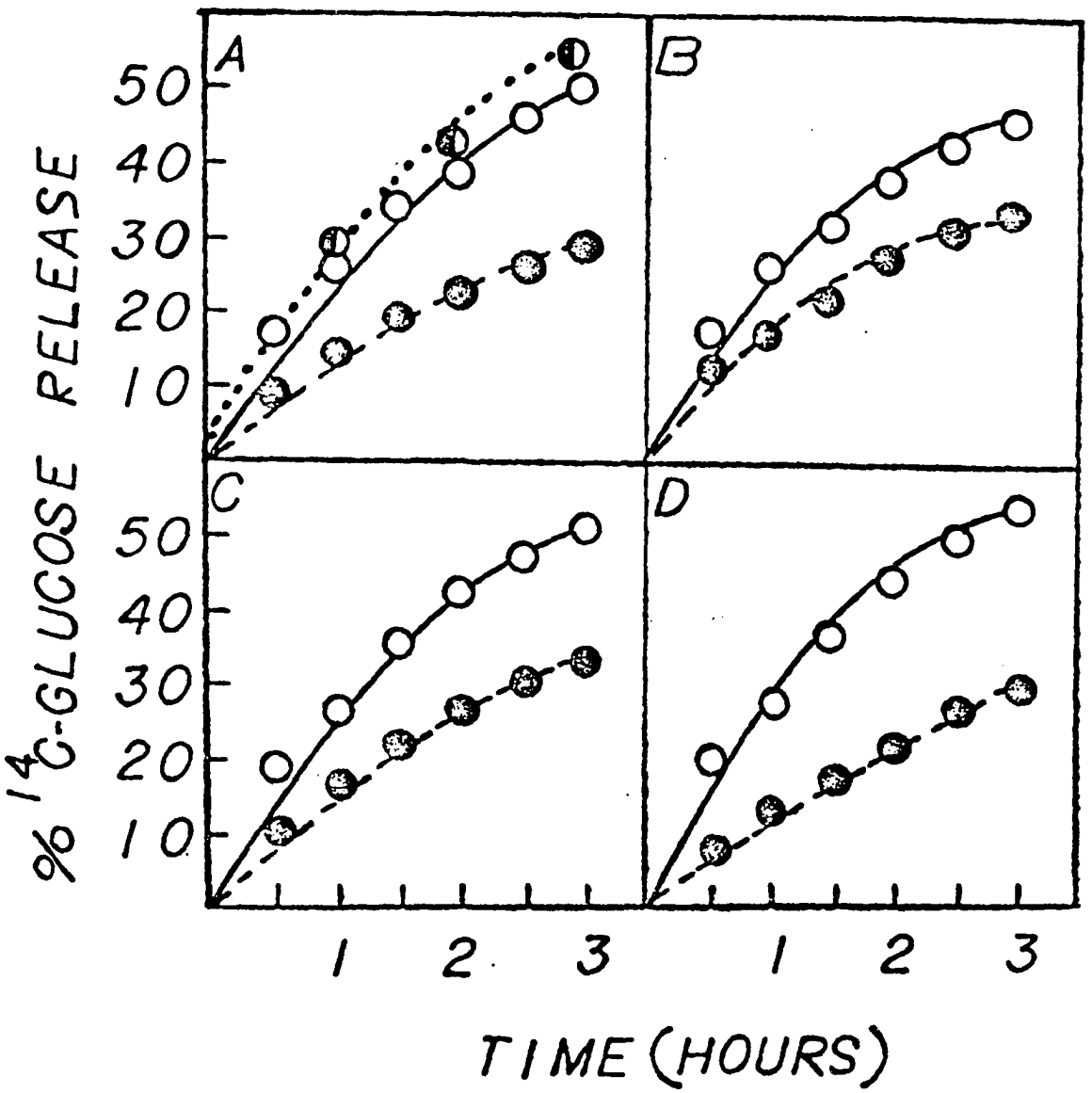


FIG. 5

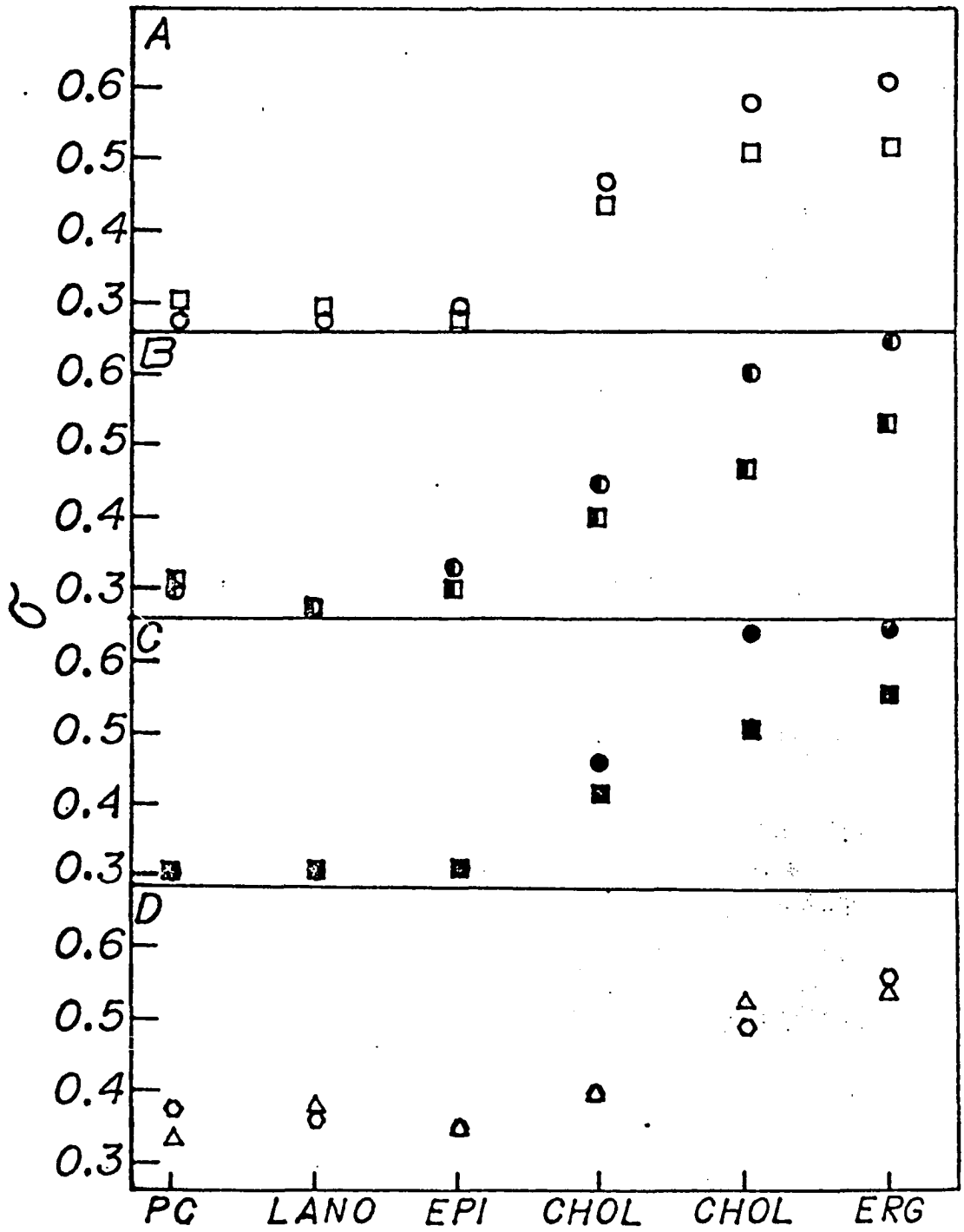


FIG. 6

Part I, Chapter II

The influence of cholesterol on the ionophore-mediated efflux of cations (Rb^+, Ca^{2+}), and on the release of the anion 6-carboxyfluorescein from vesicles of analogs of phosphatidylcholine lacking acyl groups.

ABSTRACT

The effect of sterols on the rates of ion movement in vesicles formed from various phosphatidylcholine (PC) analogs was studied. We measured the release of Rb^+, Ca^{2+}, and 6-carboxyfluorescein (6-CF) from sonicated lipid bilayers in the liquid-crystalline state of the following phosphatidylcholines: dimyristoyl and dipalmitoyl phosphatidylcholines and the corresponding diethers with 14 and 16 carbon atoms which lack the carbonyl groups, 2-octadecyleicosylphosphorylcholine (OEPC), an alkyl analog which has no carboxylate ester bond nor diacylglycerol moiety and oleyl palmityl diether PC having one saturated and one unsaturated chain.

At temperatures above the gel to liquid-crystalline phase transition of these diester-, diether-, and alkyl-phosphatidylcholines, 3β-hydroxysterols cholesterol and ergosterol decreased the initial rate of release of Ca^{2+} mediated by two ionophores, A23187 and cis-1, 2-cyclohexanedioxydiacetic acid (c-C-PR). The initial rate of Rb^+ efflux induced by valinomycin was also decreased by incorporation of cholesterol and ergosterol, as was the rate of release of the fluorophore, 6-CF, from the vesicles. Epicholesterol exerted no such effect. These results suggest that there is no specific requirement for hydrogen bonding between the hydrogen of the sterol hydroxyl group and a carbonyl oxygen of diester-PC.

INTRODUCTION

The interaction of sterols with phospholipids has been studied extensively and the structural requirements of a planar ring, an intact side chain at C_{17} and a 3β-hydroxyl group for sterols have been demonstrated (De Kruyff et al., 1973). These steric features are necessary in order to restrict the mobility of the phospholipid and decrease the permeability of the membrane. While it is clear that the phospholipid-sterol interaction is highly hydrophobic in nature, the requirement for a 3β-hydroxyl group indicates that polar interactions may also be important. A number of hypotheses have been put forward to explain how the 3β-hydroxyl group influences the interaction between lipid and sterol (Verma and Wallach, 1973; Huang, 1976, 1977; Brockerhoff, 1974). The Brockerhoff and Huang models use the α or β carbonyl group of lecithins as the site of a hydrogen bonding to the 3β-hydroxyl group of sterols. Support for this was given by some permeability and nmr studies (Yeagle et al., 1975; Tirri et al., 1977; Schwarz et al., 1977). On the other hand, force-area curves studies in monolayers (De Kruyff et al., 1973) and calorimetric studies (Jain et al., 1979) concluded that cholesterol does not form a hydrogen bond with any part of phospholipid molecule. We have recently used liposomes prepared from PC analogs in the presence and absence of sterols to study the perme-

ability of the nonelectrolytes, glycerol, urea, acetamide, and glucose and the fluorescence change of a liposome-associated merocyanine dye upon acetamide permeation (Clejan et al., 1979). The sterols had the same effect on the initial rates and reflection coefficients of polar nonelectrolyte diffusion in diester-PC bilayers and in bilayers of diether- and alkyl-PC analogs.

In the light of reports that the rates of passive diffusion of cations (Na^+) and anions (Cl^-) across vesicles prepared from a diether-PC were not affected by addition of 30 mol percent cholesterol (Schwarz and Paltauf, 1977), we tried in this study to extend our experiments. We studied the mediated diffusion of Ca^{2+} by the ionophores A23187 and c-C-PR, and of Rb^+ by the ionophore valinomycin.

The ability of these lipophilic antibiotics to increase the permeability of many biological membranes has been clearly established (Pressman, 1970, 1973; Scarpa and Inesi, 1972; Reed and Lardy, 1972). These ionophores form complexes with cations and function as mobile carriers of cations in response to concentration gradients; from the binding and transport properties of these ionophores the assumption was made that their biological activity is a result of their ability to effect the equilibration of certain cations across membranes.

In addition, a technique introduced by Cohen et al. (1979) permitted us to study the diffusion across bilayers of the lipophilic fluorophore 6-CF entrapped in the aqueous compartment of the vesicles.

The structures of the synthetic phosphatidylcholines used are

shown in the preceding paper (Clejan et al., 1979).

Materials and Methods

1, 2-Diacyl-sn-glycero-3-phosphorylcholines, and cholesterol were purchased from Sigma. $C_{18:1}$, $C_{16:0}$ Diether PC was obtained from Serdary Research Laboratories. 1, 2-Dialkoxy - 3-sn-phosphatidylcholines and OEPC were synthesized as described previously (Deroo et al., 1976). The purity of the phosphatidylcholines was ascertained by chromatography on silica gel G plates in chloroform: methanol: water (65:25:4). Epicholesterol and ergosterol were supplied by Schwarz-Mann. Sterols were recrystallized twice from ethanol, and stock solutions in chloroform were stored at -20°C. Valinomycin and A23187 were from Eli Lilly. 6-Carboxyfluorescein was from Eastman-Kodak, and was purified by recrystallization from absolute ethanol by P. Kramer. $^{45}Ca^{2+}$ and $^{86}Rb^{+}$ were from New England Nuclear, and cis-1, 2-cyclohexanedioxy - diacetic acid (c-C-PR) was a gift from Dr. I. J. Borowitz.

Preparation of vesicles containing trapped Ca^{2+}, Rb^{+}, or 6-CF.

The desired amounts of phosphatidylcholines and sterol were added to vials and the chloroform was removed under N_2 and vacuum. The thin lipid film was dispersed in a medium containing 10 mM

imidazole buffer (pH 7.0), 0.15 mM $CaCl_2$ (with $^{45}Ca^{2+}$), and 135 mM NaCl for Ca^{2+} efflux; and imidazole buffer containing 0.15 M RbCl (with $^{86}Rb^{+}$) for Rb^{+} efflux. For the experiments with 6-CF, 1 ml of 50 mM Tris-HCl (pH 7.4) containing 200 mM of 6-CF (neutralized to pH 7.0 with KOH) were used. The dispersions were agitated with a vortex mixer at temperatures with approximately five degrees above the lipid phase transition for 3 min. Then the dispersions were sonicated for 40 min under N_2, with circulating cold water, in a cup horn using a Heat Systems Model W375 sonicator at power level 6 and 50% cycle. For Rb^{+} efflux, vesicles were subjected to an additional brief period of sonication. The sonicated dispersions were stored at 4°C for 2 hours (Ca^{2+}, Rb^{+} experiments) or 24 hours (6-CF experiments) prior to gel filtration. For 6-CF experiments, the vesicles were centrifuged for 5 min at 10,000 r.p.m. in a Sorvall type SS 34 rotor.

Removal of untrapped $^{45}Ca^{2+}$, $^{86}Rb^{+}$, or 6-CF from the external medium by gel filtration

The suspension (1 ml) was passed through a column (1.5 x 30 cm) of 4g of Sephadex G-50 and eluted with 10 mM imidazole buffer (pH 7.0) containing 135 mM NaCl for Ca^{2+} efflux, or 150 mM choline chloride for Rb^{+} efflux; 50 mM Tris-HCl buffer (pH 7.4) was used for 6-CF release. Gel filtration was done at room

temperatures, except for $C_{18:1}$ $C_{16:0}$ diether PC vesicles with which the temperature was 4°C.

Assay of $^{45}Ca^{2+}$ and $^{86}Rb^{+}$ release

Stock solutions of c-C-PR and valinomycin were prepared in dimethylformamide (DMF); A23187 was dissolved in ethanol. A 10 - µl aliquot of the ionophore solution (final concentration: c-C-PR, 1.4×10^{-4} M; valinomycin, 3.2×10^{-6} M; and A23187, 5.2×10^{-8} M) or only DMF or ethanol, was added to one-ml portions of $^{45}Ca^{2+}$ or $^{86}Rb^{+}$ loaded vesicles. Vesicles were then placed in bags of one-cm diameter visking tubing. The bags were knotted with air bubbles trapped and placed in test tubes (1.7 x 3.5 cm) containing 4 ml of 10 mM imidazole buffer (pH 7.0) containing 135 mM NaCl for Ca^{2+} efflux or imidazole buffer containing 150 mM choline chloride for Rb^{+} efflux.

The sealed test tubes were rotated in a model G-25 Precision Instruments giratory shaker with the thermostat regulated at temperatures five degrees above Tc.

Aliquots (0.1 ml) of the dialysates were taken for radioactive counting in Bray's solution at various time intervals starting at 10 min. An aliquot was taken after 18 hours to measure the amount of trapped ion remaining in the vesicles.

Fluxes are expressed as the percentage of the initial trapped radioactivity lost as a function of time. The rate of efflux was corrected for the spontaneous leak of ions in the presence of DMF or ethanol by subtracting the percentage efflux in these solvents. The initial rate of efflux was measured from the tangent to the efflux increase with time. The maximum percentage efflux was calculated from the last three points using the extrapolation of the reciprocals of time and percentage efflux. From the quotient of the initial rate/total efflux, the first-order rate constant, k_1, was calculated.

When the kinetics of ionophore-induced ion efflux was measured in triplicates from the same vesicle preparation the percentage of efflux varied by less than 5% for Ca^{2+} and Rb^{+} efflux, and less than 10% for 6-CF release.

Assay of 6-CF release

For 6-CF fluorescence measurements, a Perkin-Elmer Hitachi MPA-2A spectrofluorometer, with excitation at 490 nm and emission at 520 nm and a slit of 4 nm, was used. Fifty microliters of vesicle dispersion was transferred to 2.5 ml buffer at the desired temperature. The temperatures were maintained by a thermoregulated water circulator, and the sample temperature was measured by a thermistor immersed in the sample cuvette. Fluorescence intensity

was recorded before and after the addition of 0.2% Triton X-100 and the results were calculated as percentage of release.

Determination of phospholipid concentration

Phospholipid determinations were done by digesting 0.1 to 0.3 ml of aliquots of vesicle suspensions with 0.1 ml of concentrated H_2SO_4 at 260° for 15 min, except for samples containing choline chloride (choline interferes with phosphate analysis). These samples were decomposed first in a Bunsen flame for 3 min, prior to H_2SO_4 digestion. The concentration of inorganic phosphate was determined by the method of Taussky and Shorr (1953).

Measurements of trapped volume of vesicles containing 6-CF

The trapped volume of vesicles containing 6-CF was calculated as ml of 6-CF / μ mol of phospholipid. Absorption measurements were made with a Cary 14 spectrophometer, using a wavelength of 490 nm and a molar extinction coefficient of 6.4×10^6 M^{-1} cm^{-1} (Cohen et al., 1979).

RESULTS AND DISCUSSION

Table I shows the ability of the ionophore A23187 to enhance the membrane permeability to Ca^{2+} ions, in comparison to the alicyclic ligand, c-C-PR. c-C-PR was chosen because it was found to be highly effective in promoting Ca^{2+} release in egg-PC vesicles, relative to other synthetic ionophores, and because incorporation of cholesterol into these bilayers depressed the rate of movement of Ca^{2+}(Wun and Bittman, 1977). Indeed, cholesterol caused a striking decrease in the amount and rate of Ca^{2+} release from our vesicles. Using A23187 the decrease was between 70 and 80% relative to the rate in vesicles prepared with pure phospholipids. This is consistent with the well-known importance of membrane fluidity in regulating permeability (De Kruyff et al., 1973). Our results show that in vesicles prepared from mixtures of diester PC and cholesterol, the decrease in Ca^{2+} release was almost the same as from vesicles obtained from mixtures of diether PC and cholesterol or OEPC and cholesterol.

These results do not agree with the experiments of Schwarz and Paltauf (1977) on Na^{+} diffusion. When c-C-PR was used as the ionophore, the initial rates of Ca^{2+} release were decreased to zero upon incorporation of cholesterol into vesicles prepared from diester PC, diether PC, or $C_{18:1}$ $C_{16:0}$ diether PC (Table I).

Table II shows the same definite trend for the valinomycin-mediated Rb^+ efflux. Under the conditions used, the first-order rate constant was 3 times higher for valinomycin-mediated Rb^+ release than for A23187-mediated Ca^{2+} release, but cholesterol decreased the rates efflux of both ions from diester PC vesicles in similar manner to that observed in vesicles formed from phospholipids lacking oxygen atoms. The same was true for 6-CF release (Figure 1A), where similar decreases in rates were found upon the addition of cholesterol to the PC vesicles.

Vesicles prepared from $C_{18:1}$ $C_{16:0}$ diether PC are more fluid and the initial rate of Ca^{2+} or Rb^+ release was increased in these vesicles. Inversely, OEPC presented a relatively high hydrophobic barrier and the inital rate of efflux was 2 - 3 times lower. Interestingly enough, this trend is not maintained in the vesicles with the trapped anion, 6-CF, where the rate of release is higher in vesicles prepared from OEPC than in the $C_{18:1}$ $C_{16:0}$ diether PC vesicles (Figures 1C and 1D). This may be related to the amount of 6-CF captured by the vesicles. Vesicles from OEPC trapped 8.4 ml 6-CF / μ mol P, whereas vesicles from $C_{18:1}$ $C_{16:0}$ diether PC trapped only 2.5 ml 6-CF / μ mol P.

The effects of cholesterol and epicholesterol are compared in Table II and Figure 1B. There is a striking difference between the behavior of cholesterol and epicholesterol in the vesicles

prepared from each PC analog we examined, and this difference was seen with vesicles containing trapped Ca^{2+} or trapped 6-CF. Conversely, ergosterol behaved in a similar manner as cholesterol. The mole fraction of cholesterol was varied between 0 and 0.5. As shown in Table III for Ca^{2+} efflux and in Figure 1B for 6-CF release, initial rates strongly decreased as the mole fraction of cholesterol was increased.

Following the reasoning of Brockerhoff (Brockerhoff, 1974; Tirri et al., 1977), vesicles prepared from cholesterol and diether phosphatidylcholines or OEPC should exhibit higher rates of cation or anion efflux above T_c than vesicles prepared from cholesterol and diester phosphatidylcholines. Tables I, II, and II and Figure 1 do not support this hypothesis, since cholesterol-containing vesicles from diether PC or OEPC yielded similar reduced permeabilities for Ca^{2+}, Rb^{+}, or 6-CF, as did vesicles prepared from diester phosphatidylcholines and cholesterol. Therefore, it cannot be concluded that there is any requirement for hydrogen bonding between the hydrogen of the sterol hydroxyl and the carbonyl oxygen of the diester-PC.

TABLE I. A23187 and c-C-PR-Mediated Ca^{2+} Efflux from Vesicles Prepared from Diester, Diether, and Alkyl PC Analogs

Composition of Vesicles	Temperature (°C)	Amount of Ca^{2+} captured (nmol/μmol PC)	Phospholipid (mM)	Initial Rates of Ca^{2+} efflux (pmol Ca^{2+}/μmol ionophore/hr)	
				A23187	c-C-PR
DMPC	32	0.66	2.0	1845	2.08
DMPC:Cholesterol	32	0.34	1.3	361	0
Di-C_{14} Diether PC	32	0.60	2.0	1686	1.83
Di-C_{14} Diether PC: Cholesterol	32	0.38	1.3	506	.0
DPPC	45	0.63	1.9	1639	1.97
DPPC:Cholesterol	45	0.31	1.3	310	0
Di-C_{16} Diether PC	45	0.62	1.9	1592	1.96
Di-C_{16} Diether PC: Cholesterol	45	0..36	1.3	392	0
$C_{18:1}$ $C_{16:0}$ Diether PC	26	0.61	2.4	2061	2.12
$C_{18:1}$ $C_{16:0}$ Diether PC: Cholesterol	26	0.35	1.6	382	0
OEPC	60	0.62	3.0	1604	1.80
OEPC:Cholesterol	60	0.30	2.0	343	0

Vesicles with trapped Ca^{2+} were prepared as described in the Experimental section. The molar ratio of PC to cholesterol was 1:1.

The observation that cholesterol-containing vesicles are smaller than those prepared from PC alone merits further investigation.

TABLE II. Rate of A23187- Mediated Ca^{2+} Efflux and Valinomycin - Mediated Rb^{+} Efflux from Diester, Diether, and Alkyl PC Analogs

	Rb^{+} Efflux		Ca^{2+} Efflux	
PC in Vesicle	-Cholesterol k x 10^{-4} (s^{-1})	+Cholesterol k x 10^{-4} (s^{-1})	-Cholesterol k x 10^{-4} (s^{-1})	+Cholesterol k x 10^{-4} (s^{-1})
DMPC	7.91	4.07	2.46	1.78
Di-C_{14} Diether PC	7.17	6.08	2.40	2.06
DPPC	8.15	4.67	3.04	1.66
Di-C_{16} Diether PC	7.33	5.91	2.92	2.08
$C_{18:1}$ $C_{16:0}$ Diether PC	8.83	6.04	3.72	2.32
OEPC	2.80	2.30	1.96	1.01

The molar ratio of PC: cholesterol was 1:1.

The ionophore concentrations were valinomycin, 3.2×10^{-6} M and A23187, 5.2×10^{-8} M.

TABLE III. Effect of Sterols on Initial Rates of A23187-Mediated Ca^{2+} Efflux from DMPC and Di-C_{14} Diether-PC Vesicles

Lipid Composition (molar ratio)	Initial Rate of Ca^{2+} Efflux (% of trapped Ca^{2+}/sec)		$k \times 10^{-4}$ (s^{-1})	
	DMPC	Di C_{14} Diether PC	DMPC	Di C_{14} Diether PC
PC:Cholesterol 1:1	0.0090	0.0108	1.76	2.06
" " 2:1	0.0090	0.0109	1.79	2.06
" " 3:1	0.0096	0.0122	1.89	2.18
" " 5:1	0.0145	0.0150	2.41	2.36
PC only	0.0156	0.0153	2.46	2.40
PC:Ergosterol 2:1	0.0094	0.0105	1.43	2.42
PC:Epicholesterol 3:1	0.0158	0.0155	2.48	2.42

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Figure Caption

Rate of 6-CF release from vesicles of different lipid composition

A. Δ-.-ΔDMPC; o-o Di C_{14} diether PC; ▲...▲DMPC: Cholesterol 3:1 (molar ratio) ●--● Di C_{14} diether PC: cholesterol 3:1. The volumes of 6-CF trapped were: DMPC vesicles, 7.4×10^{-4} ml/μ mol P; Di C_{14} diether PC vesicles, 6.5×10^{-4} ml/μ mol P; DMPC: cholesterol vesicles, 4.8×10^{-4} ml/μ mol P; Di C_{14} diether PC: cholesterol vesicles, 3.9×10^{-4} ml/ μmol P.

B. O DPPC: Epicholesterol 3:1

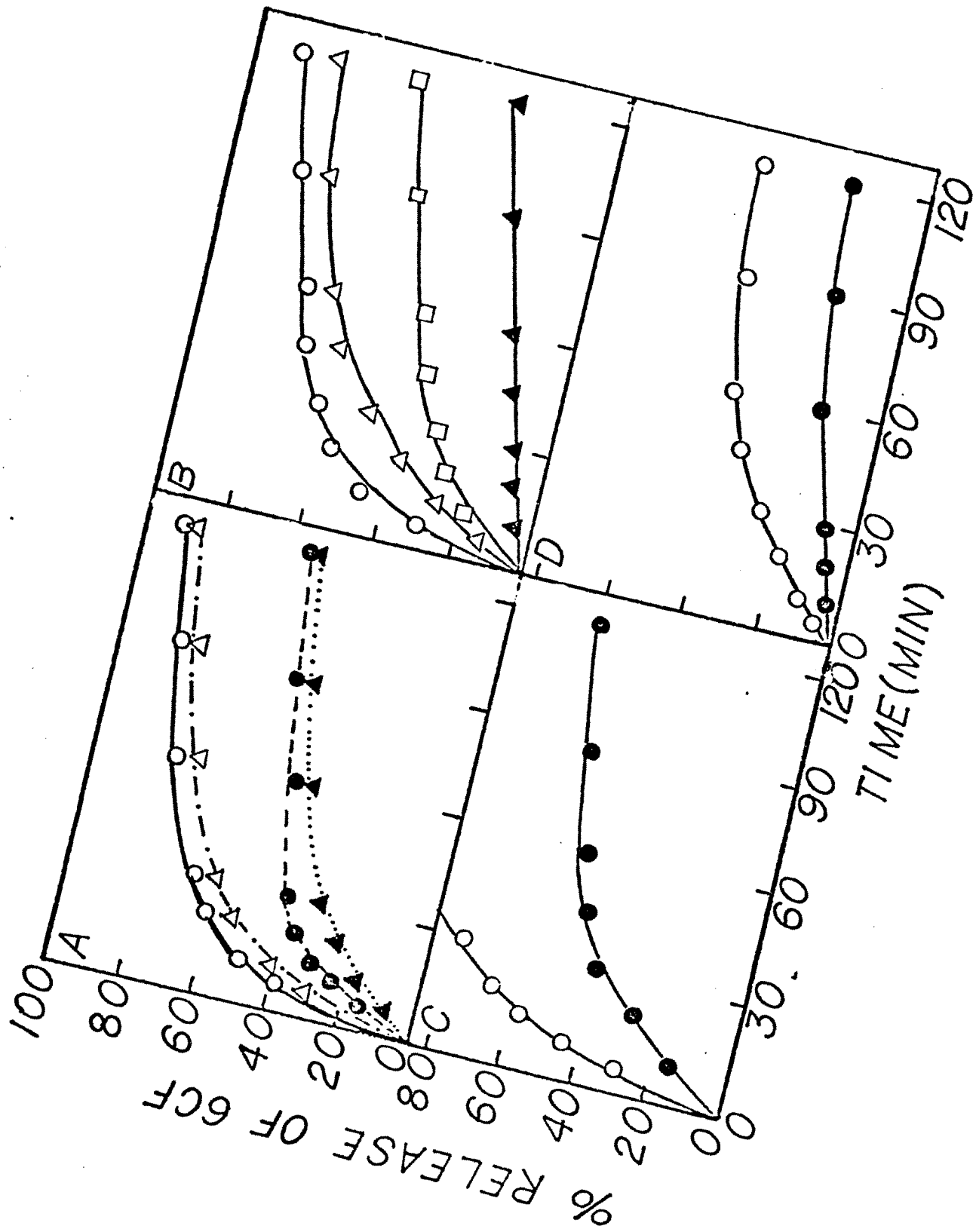
Δ DMPC: Cholesterol 5:1

□ DMPC: Ergosterol 3:1

▲ DMPC: Cholesterol 1:1

C. oOEPC;●OEPC: Cholesterol, 3:1. The volumes of 6-CF trapped were: OEPC vesicles, 8.4×10^{-4} ml / μ mol P;OEPC: cholesterol vesicles, 3.9×10^{-4} ml / μ mol P

D. o$C_{18:1}$ $C_{16:0}$ diether PC ; ● $C_{18:1}$ $C_{16:0}$ diether: cholesterol, 3:1. The volume of 6-CF trapped were: $C_{18:1}$ $C_{16:0}$ diether PC vesicles, 2.5×10^{-4} ml / μ mol P; $C_{18:1}$ $C_{16:0}$ diether PC: cholesterol vesicles, 1.1×10^{-6} ml / μ mol P.



Part II, Chapter III

Uptake, transbilayer distribution and movement of cholesterol in growing Mycoplasma Capricolum Cells*

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ABSTRACT

The sterol-requiring mycoplasma, M. capricolum, was adapted to grow in a medium containing low fetal-calf serum (FCS) concentrations, providing cells in which unesterified cholesterol comprised only about 3.6% by weight of the total membrane lipids. The native strain grown with 10% FCS contained a six-fold higher cholesterol concentration than the adapted strain. When an early exponential-phase culture of the adapted strain was transferred to a medium containing 10% FCS, cell growth was stimulated and the cells accumulated cholesterol into their cell membrane. Alteration of cell metabolism by treatment of the adapted culture with chloramphenicol, valinomycin, nonactin, or gramicidin at 37°C, or transfer to 4°C, resulted in an almost complete inhibition of growth and a partial inhibition of cholesterol uptake, suggesting that the adapted cells incorporate part of their cholesterol in a growth-dependent process, and not only by a physical process involving adsorption of the sterol from the growth medium into the membrane. The rate of cholesterol translocation from the outer to the inner half of the lipid bilayer was monitored by stopped-flow kinetic measurements of the polyene antibiotic,filipin, with free cholesterol in intact cells and isolated membranes. The cholesterol distribution in the two halves of the bilayer was almost invariant after only one hour of incubation with medium containing 10% FCS despite a dramatic rise in cholesterol content, approaching that in the native strain. However, when growth was inhibited by chloramphenicol, valinomycin, nonactin, or gramicidin, cholesterol remained localized predomi-

nantly in the outer half of the bilayer. These studies establish that free cholesterol is translocated rapidly from the external surface of the bilayer of growing M. capricolum cells at 37°C.

Mycoplasmas are unique among prokaryrotes in requiring cholesterol for growth (Razin, 1973). Mycoplasmas lack the ability to synthesize cholesterol and therefore require an exogenous source of cholesterol, usually supplied by serum lipoproteins present in the growth medium (Slutzky, et al., 1977). The cholesterol taken up from the lipoproteins or from any other exogenous source is incorporated exclusively into the cell membrane. There is no doubt that the sterol is first incorporated into the outer half of the lipid bilayer. Kinetic analysis of the association of the polyene antibiotic, filipin, with cholesterol in intact cells and isolated membranes of Mycoplasma gallisepticum showed that cholesterol is distributed symmetrically between the inner and outer halves of the lipid bilayer of intact M. gallisepticum cells (Bittman and Rottem, 1976). This suggests that translocation of the cholesterol from the outer to the inner half of the bilayer takes place. The presence of about 50% of the membrane cholesterol in the inner half of the lipid bilayer of M. gallisepticum has also been demonstrated by experiments measuring the exchange of labeled cholesterol between resting cells or isolated membranes with unlabeled cholesterol in high-density lipoprotein (Rottem, et al., 1978). In these studies, the rate of movement of cholesterol from the inner to the outer half of the lipid bilayer was found to be very slow or nonexistent, resembling that recorded in influenza virus membranes (Lenard and Rothman, 1976). These findings imply that the translocation of cholesterol across the lipid bilayer may be much faster in growing cells than in resting cells. The ability to alter the cholesterol content of some

mycoplasma species by adapting the cells to grow with very low cholesterol (Rottem, et al., 1973) provided a most useful model for establishing the rate of cholesterol translocation in growing mycoplasma cells. In this report, we show that the rapid reaction between filipin and cholesterol in intact cells and isolated membranes of M. capricolum can be analyzed to lead to conclusion concerning the localization of cholesterol at intervals during growth stimulation. The advantages of using the cholesterol-depleted M. capricolum cells to define the factors controlling the uptake of cholesterol from the growth medium are discussed.

MATERIALS AND METHODS

Growth of the Organism and Isolation of Membranes. Mycoplasma capricolum (California kid) was grown in 200-400 ml volumes of a modified Edward medium (Razin and Rottem, 1975) containing 0.6-10% of fetal calf serum (FCS[1], Grand Island Biological Co.), 1% fatty acid-poor bovine serum albumin (Sigma Chemical Co.), and 25 µg per ml each of palmitic and oleic acids (Sigma Chemical Co.). To test for cell leakiness, in some experiments 0.1 µCi/ml of 6-methyl-^{3}H-thymidine (10 Ci/mmol, New England Nuclear Corp.) was added. Growth was determined by measuring the absorbance of the culture of 640nm. The organisms were harvested after 15-20 hr of incubation in FCS, by centrifugation at 12,000 x g for 10 min at 4°C. The cells were washed once in 5 ml of 0.25 M NaCl colution, treated at 37°C for 10 min with deoxyribonuclease (50 µg/ml, Sigma Chemical Co.) in the presence of 20 mM $MgCl_2$, then washed once and re-suspended in 4 ml of

0.25 M NaCl solution. Membranes were prepared from portions of the diluted cell suspensions by ultrasonic disruption for four 15-s periods at 0°C in a Branson Model S-110 sonicator. The absorbance values of membrane suspensions at 500 nm were 80-90% lower than those of cell suspensions containing the equivalent mass per ml.

Protein and Lipid Analysis. Protein concentrations were determined by the method of Lowry, et al., (1951), using bovine serum albumin as the standard. Lipids were extracted in chloroform-methanol (9:1, v/v). The solvent was evaporated under nitrogen, and the dried lipid was dissolved in 1 ml of glacial acetic acid. The total cholesterol concentration in the extracted lipids was measured colorimetrically (Zlatkis and Zak, 1969). Unesterified cholesterol was separated from esterified cholesterol by thin-layer chromatography on silica gel G plates using benzene-ethyl acetate (5:1, v:v). The free and esterified cholesterol were scraped off the plates, extracted, and their relative concentrations were determined as described previously (Bittman and Rottem, 1976). Phospholipid phosphorus was measured by the method of Taussky and Shorr (1953).

Kinetic Measurements. Filipin complex (lot no. 8393-DEG-11-8) was obtained from the Upjohn Co. and was purified as described previously (Bittman and Rottem, 1976). Stock solutions of filipin were prepared in dimethylformamide. The final concentration of filipin, after mixing with cells or membranes, was 16 μM, and the final concentration of dimethylformamide was 0.3% (v/v.).

Initial rates of filipin-cholesterol association were measured at 358 nm at 10°C with a stopped-flow spectrophotometer (Durrum Instrument Corp.) equipped with a Tektronix storage oscilloscope and a Polaroid camera. A signal of 1.6 V corresponded to 100% transmittance when buffer was in the stopped-flow cuvette. Cells or membrane preparations were diluted with 0.40 M sucrose - 10 mM sodium phosphate - 20 mM $MgCl_2$ solution (pH 7.0) to give a total cholesterol concentration ranging from about 1 to 4 μM. At least six measurements of the initial rates of filipin binding were made at each cholesterol concentration. The initial rates of disappearance of free filipin and the second-order rate constants for filipin-cholesterol association were calculated as described previously (Bittman and Rottem, 1976). The pressure used to charge the air actuator assembly was reduced to 30 psi to minimize cell shearing. No transmittance changes were detected after the initial 100-150-ms disturbance period had elapsed when cells or membranes were mixed with buffer solution in the stopped-flow apparatus.

Chloramphenicol and Ionophore Treatment. Chloramphenicol, valinomycin, or nonactin (all obtained from Sigma Chemical Co.) and gramicidin (Mann Research Laboratories) were added to the growing cultures as a solid (chloramphenicol) or as an ethanolic solution (valinomycin, nonactin, and gramicidin). Ethanol did not exceed a final concentration of 0.05% (v/v). Serum was added to the growth medium after the time intervals indicated in Tables III and IV. The leakage of K^+ from the valinomycin-, nonactin-, and gramacidin-treated cells was determined by monitoring the K^+ concentration in the super-

natant from the 12,000 x g centrifugation. A model 303 Perkin-Elmer Elmer atomic absorption spectrometer equipped with an automatic null recorder was used to measure K^+. The extent of ionophore-mediated K^+ efflux after 30 min of incubation with FCS and valinomycin (10 μM) or nonactin (10 μM) was approximately 30% of the total K^+ content of the cells, and about 42% when treated with 3 μM gramicidin. The ionophore-mediated K^+ efflux did not exceed 65% after 4 hr of incubation. The total trapped K^+ concentration, determined by lysis with 1-propanol (1:1, v/v) and comparison with standards in the same medium, ranged from 130-165 mM. To determine whether the chloramphenicol - and ionophore-treated cells remained intact, the percent efflux of ^{3}H-thymidine-labeled components and of NADH oxidase in the supernatant obtained after centrifugation at 12,000 x g for 20 min was determined. Total ^{3}H-thymidine-labeled components and NADH oxidase activity were found by disrupting intact cells by prolonged sonication. Furthermore, no transmittance changes at 450 nm were observed after the initial rapid disturbance period when filipin was mixed with ionophore-treated cells in the stopped-flow apparatus. Filipin does not absorb at this wavelength, but turbidity changes corresponding to membrane disruption could have been detected. The initial rate of filipin-cholesterol association in ionophore-treated cells remained first order in each reactant, and the second order rate constants were similar in magnitude to those in untreated cells. The viability of the cells, as determined by the colony-count technique (Butler and Knight, 1960) was reduced by approximately three orders of magnitude by incubation with 10 μM valinomycin for three hours at 37 °C.

RESULTS

Adaptation. Serial passages of the sterol-requiring mycoplasma, M. capricolum, in a modified Edward medium containing bovine serum albumin, palmitic and oleic acids, and decreasing concentrations of FCS were made in order to adapt the cells to grow in a cholesterol-poor medium. The FCS concentration was decreased from 10% to 0.62% by growing the cells in media containing one-half of the FCS concentration of the previous medium. At each FCS concentration two passages were made. Cells adapted to a cholesterol-poor medium containing 0.62% FCS grew more slowly than cells maintained in a cholesterol-rich medium containing 10% FCS, and their cultures reached the stationary phase of growth at a lower turbidity than cultures of the native strain. The native strain grown with 10% FCS contained about six times more cholesterol and had a cholesterol-to-phospholipid molar ratio four-fold higher than the adapted cells (Table I). Poor growth was obtained in a medium containing 0.3% FCS. Since the kinetic experiments require membranes with a cholesterol content sufficient to produce a change in absorbance when complexed with filipin, we carried out experiments with the adapted strain grown with 1.25% FCS.

Transfer to Cholesterol-Rich Medium. When an early logarithmic culture of M. capricolum was transferred to the cholesterol-rich medium (containing 10% FCS), the growth rate was markedly enhanced (Figure 1). This was accompanied by a marked increase in the free cholesterol content from about 28 µg per mg membrane protein at zero times, to about 127 µg per mg membrane protein after five hr of growth (Figure 1; Table II). The free to esterified cholesterol molar ratio

was 1.1, and it was maintained throughout the experiment. When the culture was transferred to the cholesterol-rich medium, but maintained at 4°C, neither growth nor an increase in cholesterol content occurred. In order to examine whether active cell growth is necessary for the incorporation of cholesterol into the cell membrane, chloramphenicol (100 μM), nonactin (10 μM), or gramicidin (3 μM) were added to the adapted cells, and the cells were transferred to the cholesterol-rich medium. Cell growth and protein synthesis at 37°C were completely inhibited under these conditions, but cholesterol accumulation into the cell membrane was only partially inhibited (Table III). The inhibitory effect of chloramphenicol on cholesterol incorporation was more more pronounced when the inhibitor was added to the cells 30 min before the transfer to the cholesterol-rich medium. Phospholipid biosynthesis was, however, inhibited to varying degrees. The order of inhibition was valinomycin > nonactin > gramicidin > chloramphnicol, resulting in the increase in phospholipid to protein ratio of membranes from the treated cells (Table III). Since the leakage of ^{3}H-thymidine-labeled components and NADH oxidase from chloramphenicol-, valinomycin-, and nonactin-treated cells was only about 10% higher than that from untreated cells, and about 15% higher in gramicidin-treated cells, the permeability barrier was retained essentially intact. These experiments demonstrate that cholesterol uptake, which was previously shown in Acholeplasma laidlawii to be a physical process (Gershfeld, et al., 1974; Razin, et al., 1974), is affected in M. capricolum by cell growth. As will be shown below, in cells where growth is inhibited, cholesterol translocation from the outer half to the inner half of the

bilayer is much slower than in growing cells, resulting in the accumulation of the sterol in the outer half of the bilayer and a decrease in the total unesterified cholesterol content of the cell membrane.

Distribution of Cholesterol between the Inner and Outer Halves of the Bilayer. The distribution of free cholesterol in the two halves of the lipid bilayer of the adapted cells was probed at various time intervals after the transfer to the cholesterol-rich medium (Table II). Approximately three-fourths of the free cholesterol was estimated to be localized in the outer half of the bilayer in cells that were isolated immediately after transfer to 10% FCS. After 1 hr of incubation, cholesterol was distributed about equally between the two halves of the bilayer, and at longer times of incubation roughly 55% of the total free cholesterol was present in the inner half of the bilayer. In the chloramphenicol- or ionophore-treated cells 66-92% of the free cholesterol remained localized in the outer half of the bilayer (Table IV).

To determine whether a correlation exists between the rise in the cholesterol content of the membrane and the translocation of the sterol into the inner half of the bilayer, we incubated the adapted cells in media containing various concentrations of FCS. The localization of cholesterol in the two halves of the bilayer was probed by measuring the initial rates of filipin-cholesterol association in intact cells and isolated membranes. The analysis of the cholesterol distribution after a 4-hr incubation in cholesterol-rich media (Figure 2) shows that the cholesterol content of the inner leaflet can be correlated with FCS concentrations between 3 and 10%.

DISCUSSION

The present study demonstrates that the cholesterol content of adapted M. capricolum cells increased by more than two-fold between the first and third hours of incubation in FCS-rich medium, and approached the cholesterol content of the native strain. The increase in cholesterol content appears more pronounced when expressed as µg of cholesterol per mg of membrane protein rather than as µg of cholesterol per mg of phospholipid because the cells incorporate phosphatidylcholine and sphingomyelin from the FCS-rich medium up to the amount of 35% of the total lipid phosphorous, increasing the phospholipid to protein ratio of the membranes (Z. Gross and S. Rottem, unpublished data). The influence of the exogenous lipid components on cholesterol uptake and translocation remains to be established. The distribution of free cholesterol in the two halves of the bilayer was almost invariant after the first hour of incubation at 37°C, despite the dramatic increase in cholesterol content, indicating that the rate of translocation of free cholesterol in growing M. capricolum is very rapid. Indeed, at a low temperature (4°C) where growth rate was decreased dramatically, cholesterol uptake and translocation were inhibited. One cannot exclude the possibility that such inhibition arose because membrane lipid may be in the gel state at that temperature, as was found in the adapted strain of the closely related M, mycoides subsp. capri (Rottem, et al., 1973). The localization of cholesterol predominantly in the outer half of the bilayer in chloramphenicol-, valinomycin-, nonactin-, and gramicidin-treated cells (Table IV) indicates that translocation is

facilitated in actively growing cells where a membrane potential gradient is maintained and/or macromolecular synthesis takes place. We wish to emphasize that in these experiments the translocation is from the external surface, which is in contact with the cholesterol of the medium, to the internal surface, which is initially low in cholesterol content. In contrast, the inner-to-outer transbilayer movement of cholesterol in resting M. gallisepticum cells is an slow process, having a half-time of at least 18 days at 37°C (Rottem et al., 1978). Although a direct comparison cannot be made since different organisms were used, these experiments suggest that the rate of transbilayer cholesterol movement may depend on a number of factors, e.g., active cell growth, direction of movement, sterol concentration gradient in each half of the lipid bilayer, and others. However, in red blood cells, where no growth or macromolecular synthesis occurs, the rate of cholesterol translocation appears to be very high (Lange et al., 1977; Kirby and Green, 1977).

Isolated membranes of Acholeplasma laidlawii incorporate cholesterol by a physical adsorption process (Gersfeld et al., 1974). However, the results that we report with cells incubated with 10% FCS at 4°C and with chloramphenicol, valinomycin, nonactin, and gramicidin at 37°C show that intact M. capricolum cells incorporate much less cholesterol under conditions where growth is inhibited. The decrease in cholesterol incorporation under these conditions suggests that growing mycoplasmas may possess a mechanism that catalyzes cholesterol incorporation into the inner half of the bilayer. That this mechanism is not operative in nongrowing cells is

supported by the high $^{k}_{cell}/^{k}_{membrane}$ ratios obtained in the chloramphenicol- and ionophore-treated cultures (0.7-0.9), indicative of a preferential localization of the newly acquired cholesterol into the outer half of the lipid bilayer. The adapted strain offers advantages in the study of cholesterol movement in mycoplasmas, and further experiments may lead to a better understanding of the factors that influence the lipid distribution in this model biological membrane.

TABLE I: Growth and Lipid Composition of Native and Adapted M. capricolum Cells

strain	% FCS in medium	A_{640}[a]	phospholipid (μg/mg of cell protein)	free cholesterol[b] (μg/mg of cell protein)	free cholesterol/phospholipid[c] (mol/mol)
native	10.00	0.21	80.2	42.3	1.02
adapted	1.25	0.12	50.0	7.5	0.29
adapted	0.62	0.10	48.0	6.2	0.25

[a] A_{640} was measured after 15 hr of growth.

[b] The content of free cholesterol was calculated based on a free cholesterol to cholesterol ester molar ratio of 1.1, which was determined colorimetrically as described in the Materials and Methods section. An average molecular weight of 626 was assumed for the cholesterol esters.

[c] An average molecular weight of 750 was assumed for the phospholipids.

TABLE II: Second-Order Rate Constants for Association of Filipin and Free Cholesterol in Intact Cells and Isolated Membranes of the M. capricolum Adapted Strain Transferred to a Cholesterol-Rich Medium[a]

time of incubation in 10% FCS (h)	A_{640}	free cholesterol (μg/mg of membr protein)	k_{cells}[b] ($M^{-1} s^{-1}$)	k_{membr}[b] ($M^{-1} s^{-1}$)	k_{cells}/k_{membr}[c]
0	0.08	33	5.0×10^4	7.1×10^4	0.73 ± 0.08 (14)
1	0.16	44	4.3×10^4	8.3×10^4	0.53 ± 0.04 (9)
2	0.24	92	4.0×10^4	8.8×10^4	0.45 ± 0.06 (16)
4	0.26	127	4.4×10^4	9.1×10^4	0.45 ± 0.07 (16)

[a]The initial rates of filipin-free cholesterol association were measured at 10°C at various cholesterol concentrations in intact cells and isolated membranes obtained from at least nine different cells cultures, each incubated with medium containing 10% FCS for the indicated period of time. The number of cell cultures investigated is indicated in parentheses in the right-hand column.

[b]A representative example of second-order rate constants analyzed from one culture.

[c]Average ratio of secon-order rate constants. Error limits of the kinetic data are standard errors of the mean.

TABLE III: Effects of Chloramphenicol, Valinomycin, Nonactin, and Gramicidin on the Membrane Composition of Adapted M. capricolum Cells Upon Transfer to Cholesterol-Rich Medium

treatment	time of incubation (h)	A_{540}	membr protein[a] (mg)	membr phospholipids (μg mg of membr protein)	free cholesterol (μg mg of membr protein)	free cholesterol phospholipid (mol mol)
no inhibitor	0	0.08	1.8	302	33	0.21
	1	0.16	3.1	309	44	0.28
	2	0.24	4.2	297	62	0.40
	4	0.26	4.6	325	127	0.76
chloramphenicol[b]	1	0.13	2.0	358	37	0.20
	4	0.14	2.3	369	74	0.30
chloramphenicol[c]	1	0.10	1.9	365	35	0.19
	4	0.11	2.0	477	52	0.21
valinomycin[b]	1	0.14	2.1	300	36	0.25
	4	0.17	2.2	307	78	0.49
nonactin[b]	2	0.12	2.2	374	69	0.36
	4	0.13	2.4	390	80	0.40
gramicidin[b]	1	0.11	1.9	342	35	0.20
	4	0.13	2.2	458	72	0.31

[a]Cells were grown in 250 ml of medium. [b]The final concentration of chloramphenicol, valinomycin, nonactin, and gramicidin are 100 μg/ml, 10 μM, 10 μM, and 3 μM, respectively. The cultures were transferred to media containing 10% FCS immediately after the addition of the inhibitor.

TABLE IV: Effect of Growth Temperature, Chloramphenicol, Valinomycin, Nonactin, and Gramicidin on Cholesterol Incorporation and Transbilayer Distribution Upon Transfer to Cholesterol-Rich Medium[a]

expt	temp (°C)	free cholesterol (μg/mg of membr protein)	k_{cells}/k_{membr}	cholesterol distribution (μg, mg of member protein) outer half	inner half
no inhibitor	37	127.0	0.45	57.2	69.8
	4	25.7	0.70	17.9	7.8
chloramphenicol	37	57.5	0.66	39.1	17.4
valinomycin	37	79.7	0.90	71.5	8.2
nonactin	37	81.5	0.78	63.1	18.4
gramicidin	37	71.3	0.92	65.5	5.8

[a]The data were obtained after a 4-hr incubation in medium containing 10% FCS. Essentially identical data were obtained when inhibitor-treated cultures were incubated for 2 hr. FCS was added to the medium (10% final concentration) immediately after the addition of chloramphenicol (100 μg/ml), valinomycin (10 μM), nonactin (10 μM), or gramicidin (3 μM). Similar ratios were found when chloramphenicol was added 15 to 45 min prior to FCS.

[b]The cholesterol distribution was calculated from $k_{cells}/k_{membranes}$ times free cholesterol, which equals the cholesterol content in the outer half of the lipid bilayer.

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FIGURE CAPTIONS

Figure 1: Time course of stimulation of cell growth (o) and increase in membrane free cholesterol content (□) upon transfer of adapted M. capricolum cells to medium containing 10% FCS. The inset shows the increase in the free cholesterol:lipid phosphorus molar ratio.

Figure 2: Plot of the free cholesterol concentration in the membrane (o) and in the outer (□) and inner (Δ) halves of the bilayer of adapted M. capricolum cells vs. FCS concentration in the medium. Cells were incubated for 4 hr in the cholesterol-rich medium.

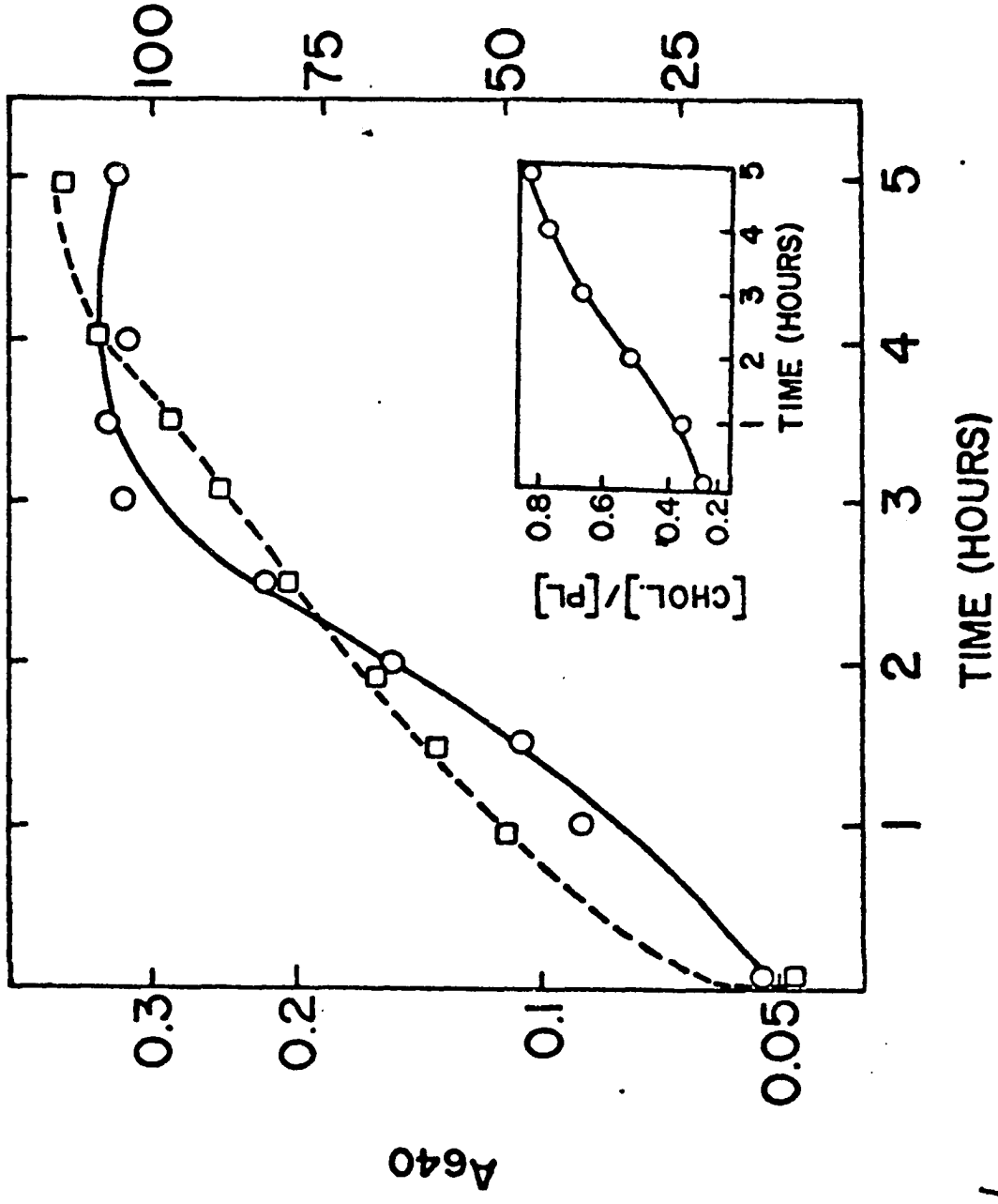

FIG. 1

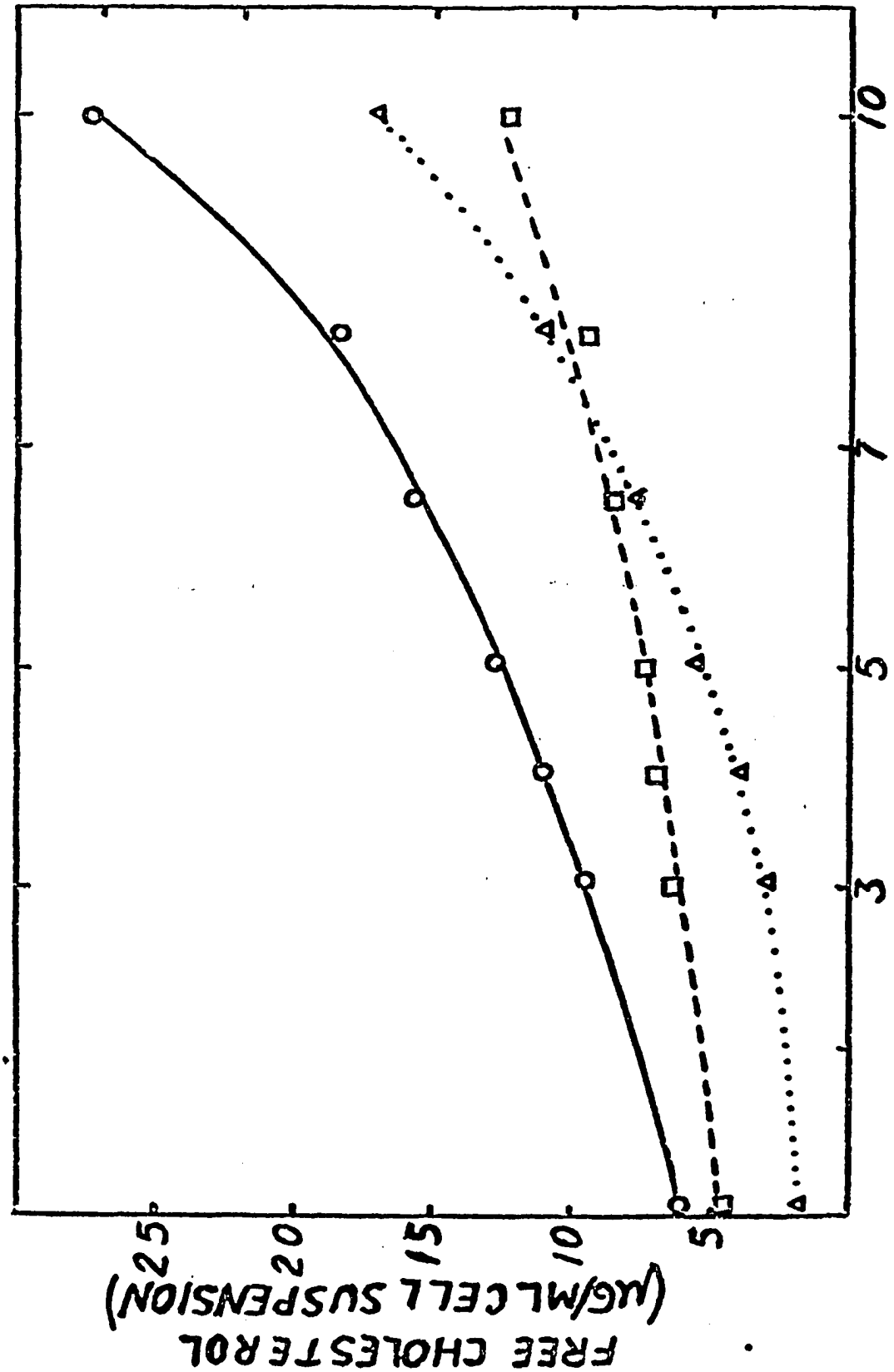

FIG. 2

Part II, Chapter IV

The effects of proteins on the rapid filipin-cholesterol association in mycoplasma cells and membranes

Parts of this chapter were presented at the 63rd FASEB Meeting, April 1979, Dallas, Texas [Abstract in Fed. Proc. 38, 795, (1979)].

ABSTRACT

The effects of the following parameters on the kinetics of filipin binding with free cholesterol were studied: ionic strength and pH alterations; treatment of the membranes with detergents; decrease in the protein content at the membrane surface by proteolytic digestion; increase in the protein content by the reversible incorporation of water-soluble exogenous proteins; and increase in the molecular weight of membrane proteins by cross-linking. The results of these studies confirmed the usefulness of stopped flow-measurements of filipin association with cholesterol as an appropriate estimate of the distribution of this sterol in the outer and inner leaflets of the mycoplasma membrane.

INTRODUCTION

The transbilayer distribution of cholesterol in membranes is a subject of considerable interest and importance. Recent studies showed that the rapid kinetics of association of filipin with free cholesterol (FC) in intact mycoplasma cells and in isolated, open membranes provide an estimate of the FC distribution in the two halves of the membrane bilayer (Bittman and Rottem, 1976). Exchange studies confirmed the conclusion drawn from the filipin complexation measurements with M. gallisepticuum cells, namely, that FC is distributed symmetrically between the inner and outer surfaces of the bilayer of this organism (Rottem et al., 1978). Previous kinetic studies of the binding of filipin with FC showed that the initial rate is dependent on the antibiotic and sterol concentration (first order in each reactant) in phospholipid vesicles prepared from synthetic saturated phosphatidylcholines (Blau and Bittman, 1977), mycoplasmas (Bittman and Rottem, 1976), and erythrocyte ghosts (Blau and Bittman, 1978). The second-order rate constant was not markedly sensitive to variation in the mol fraction of cholesterol in phosphatidylcholine (PC) vesicles, nor to the ratio of egg PC to egg phosphatidylethanolamine in PC/PE vesicles, although in pure PC vesicles the rate constant was influenced by the fatty acyl chain length (Blau and Bittman, 1977, 1978).

Many important aspects of the rapid filipin-FC association process remain to be established. Proteins are major components of biological membranes and the dependence of their lateral and rotational mobility on the physical state of membrane lipids are well established (Hidalgo et al., 1978). It was also shown that membrane proteins interact with membrane lipids, protecting them from hydrolysis by exogenous phospholipases and affecting the freedom of motion of their hydrocarbon chains (Bevers et al., 1977; Rottem and Shamuni, 1973). It was, therefore, of great interest to study the effect of controlled alteration in M. capricolum membrane protein content on the filipin-membrane cholesterol complexation. The protein content at the membrane surfaces was decreased by protease-catalyzed digestion and increased by the reversible incorporation of water-soluble exogenous proteins. The molecular weight of some of the proteins was increased by crosslinking. In addition, the effects of the following parameters on the filipin-cholesterol interaction were studied: disruption of membranes by detergent treatment, fragmentation of membranes by sonication, and alterations of the ionic strength and pH of the medium. The results of these studies provide support for the use of stopped-flow measurements of filipin binding to FC as a reliable indication of the distribution of this sterol in the outer and inner surfaces of the mycoplasma membrane bilayer.

Materials

Filipin (Lot No. 8393-DEG-11-8) was obtained from the Upjohn Co. Trypsin, chymotrypsin, lysozyme, and soybean trypsin inhibitor were from Worthington Biochemical Corp. [6-Methyl-^{3}H]-thymidine (10Ci / mmol) was purchased from New England Nuclear Corp. Fetal-calf serum was obtained from Grand Island Biological Co. Cholesterol oleate was from Serdary Research Laboratories. Dimethylsuberimidate dihydrochloride (DMS)[1] was from Pierce Chemicals. Phenylmethanesulfonyl fluoride, cytochrome c (horse heart, type III), fatty-acid-poor bovine serum albumin (fraction V), palmitic acid, and oleic acid were from Sigma Chemical Co. The detergents were from the following sources: $NaDodSO_4$ (Sigma); Triton X-100 (Rohm and Haas), Tween 80 (Sigma), and deoxycholate (Sigma).

Methods

Growth of M. gallisepticum and M. capricolum and isolation of membranes.

M. gallisepticum (A5969) and the native strain of M. capricolum

(California kid) were grown to mid-exponential phase in a modified Edward medium (Razin and Rottem, 1975) containing 10% fetal-calf serum. The procedure for the adaptation of M. capricolum to grow in a cholesterol-poor medium was described recently (Clejan et al., 1978). In the present study, the adapted strain was grown on a modified Edward medium supplemented with 1.25 μg/ml of free cholesterol, 10 μg/ml of palmitic acid, 10 μg/ml of oleic acid, 10 μg/ml of 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 of free cholesterol. The organisms were harvested and the cells were washed as described previously (Bittman and Rottem, 1976). 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 12,000 x 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 for four 30-sec 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 ml.

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) were mixed rapidly in a Durrum-Dionex stopped-flow spectrophotometer. Filipin solutions were prepared as described previously (Bittman and Rottem, 1976). The final concentration of dimethylformamide was 0.3% (v/v). The initial rate of the association process was calculated from the increase in the transmittance of filipin, which was photographed using a Tektronix storage oscilloscope and a Polaroid camera. The initial reaction was found to be first order with repsect of each reactant. Second-order rate constants for association in intact cells and isolated membranes, designated as k_{cell} and k_{membr}, respectively, were calculated as described previously (Bittman and Rottem, 1976). All rate measurements were made at 10° C. To minimize cell shearing, the pressure used to initiate mixing with the air-actuator assembly was reduced to 30 psi.

Treatment with Trypsin and Chymotrypsin.

Suspension of intact cells or isolated membranes in 0.40 M

sucrose - 10 mM sodium phosphate - 20 mM $MgCl_2$ solution (pH 7.4) were treated with 50 μg/ml of trypsin or chymotrypsin for 2 hrs at 37°C. Tryptic digestion was stopped by the addition of 50 μg/ml of trypsin inhibitor or 20 μg/ml of phenylmethanesulfonyl fluoride; the latter agent was used to stop the chymotrypsin-catalyzed digestion. The reaction mixture was diluted with 8 volumes of cold 0.25 M NaCl solution and centrifuged at 12,000 x g for 20 min at 4°C. The pellets were suspended in and washed twice with 0.25 M NaCl solution.

Crosslinking 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 hr 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 to 10 volumes of 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 on a gradient of 10-15% polyacrylamide gels were performed by the procedure described by Studier (1973). The buffer (0.01 M Tris, pH 6.8) contained 0.1% $NaDodSO_4$ and 0.1% 2-mercaptoethanol. Samples were applied at total protein concentrations of approximately 1 - 2 mg/ml. The gels were stained with Coomassie blue.

Incorporation and Removal of Cytochrome c and Lysozyme.

M. capricolum membranes (1.0 mg membrane protein /ml) were treated with varying concentrations of cytochrome c and lysozyme in 1 mM sodium phosphate buffer, pH 7.4, containing 0.40 M sucrose (no $MgCl_2$ was present) for 2 hrs at room temperature. Removal of cytochrome c and lysozyme was accomplished by washing three 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).

Analysis of Membrane Composition.

Protein, lipids, and lipid phosphorus were measured by the procedures cited previously (Clejan et al., 1978).

RESULTS AND DISCUSSION

Sonication of Membrane Suspensions.

Membranes isolated from mycoplasma cells by sonication or osmotic lysis are predominantly unsealed (Rottem et. al., 1978). Since the estimate of the fraction of outer leaflet to inner leaflet free cholesterol is based on a comparison of k_{cells} and k_{membr}, experiments were carried out to determine whether the kinetics were dependent on sonication time or method of membrane preparation. As shown in Table I, higher rate constants were obtained with membranes prepared by sonication of M. capricolum cells than with those prepared by osmotic lysis. No significant

dependence of rate on sonication time was observed when M. capricolum membranes, initially prepared by osmotic lysis, were sonicated longer than 60 sec. Similarly, the association rate did not vary when M. gallisepticum membranes (obtained by brief sonication, since the cells resist osmotic lysis by water) were subjected to prolonged sonication.

Estimation of the Fraction of Free Cholesterol Available for Rapid Reaction with Filipin

The effect of disrupting membrane organization by detergent treatment on the kinetics of filipin-free cholesterol association was studied. The initial rate increased between 0 and 0.04% detergent by about 10 to 20% (Table II), suggesting that about 80 to 90% of the total unesterified cholesterol of M. capricolum membranes is accessible to filipin in the rapid (0 - 50 ms) association reaction. The decrease in the initial rate at detergent concentrations higher than 0.04% probably results from the disruption of the filipin-cholesterol complex, which is driven by hydrophobic interactions (Bittman and Fischkoff, 1972; Bittman, 1978), and from solubilization of membrane cholesterol.

Ionic Strength and pH Dependency.

M. capricolum membranes were suspended in 10 mM sodium phosphate buffer, pH 7.2, and the ionic strength was varied by addition of sodium chloride. No change in initial rate was found between 0.15 and 0.31 M NaCl. The reaction rate was decreased by about 20% at 0.40 M NaCl, and by more than 30% at 0.47 M NaCl.

The pH dependence was also examined. No change in reaction rate was observed between pH 6 and 8 when M. capricolum membranes were suspended in phosphate buffer containing 0.40 M sucrose and 20 mM $MgCl_2$.

Influence of Age of the Culture on the Initial Rate.

With aging of the culture of M. gallisepticum, marked decreases occur in the membrane lipid-to-protein ratio and in the fluidity of the lipid phase, without substantial change in the free cholesterol/phospholipid molar ratio (Rottem et al., 1978). The rate constants for filipin binding to free cholesterol in intact cells and isolated membranes of M. gallisepticum decrease upon aging of the culture (Table III). The ratio k_{cells} / k_{membr}

increases from approximately 0.50 (early exponential phase) to 0.60 (late exponential phase). These kinetic determinations of enrichment of free cholesterol in the outer leaflet of the bilayer relative to the inner leaflet upon aging agree very closely with data obtained in exchange studies (Rottem et al., 1978).

Table III also shows that k_{cells} and k_{membr} are unaffected by the aging of M. capricolum cultures. Therefore, the distribution of free cholesterol between the two halves of the M. capricolum membrane remained constant throughout the growth cycle of the native strain. However, the decrease in phospholipid phosphorus/membrane protein ratio and in total cholesterol/membrane protein ratio were similar in M. capricolum and M. gallisepticum (data not shown). It has been proposed that the disposition of membrane proteins associated with the inner surface is altered on aging of M. hominis cultures (Rottem and Greenberg, 1975; Amar et al., 1976). It is not known, however, whether the packing density of proteins on the inner surface of M. capricolum membranes differs from that of M. gallisepticum membranes. Therefore, we cannot explain the difference in the effect of aging on cholesterol distribution in these two strains.

Crosslinking of Membrane Components

Reaction of M. capricolum membranes with the bifunctional alkylating agent, DMS, reduced the initial rates of filipin-free cholesterol association. The following values of k_{membr} were determined after treatment for 1 hr: 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, 6.0×10^3; and 10 mM DMS, 4.6×10^3. The Coomassie-blue-stained gels revealed the absence of 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 crosslinked components are not known, membrane proteins in mycoplasmas have been found to be distributed predominantly at the inner membrane surface (Amar et al., 1976). It is probable that the reduction in initial rate of association 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 using the adapted strain of M. capricolum. As summarized in Table IV, reaction of DMS with membrane components in an early exponential-phase culture reduced free cholesterol uptake into the membrane following the transfer of the cells to the cholesterol-rich medium. The k_{cells}/k_{membr} ratio indicates that free

cholesterol is not translocated rapidly from the outer to the inner surface of the bilayer of DMS-treated cells. These results support the conclusion made 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; 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 internal membrane surface.

Effect of Binding of Exogenous Proteins to M. capricolum Membranes.

Based on calorimetric measurements, cytochrome c is thought to interact with phospholipid head groups in vesicles, without penetrating extensively into the hydrocarbon phase of the membrane (Papahadjopoulos et al., 1975). Cytochrome c and lysozyme are bound in large quantities to Acholeplasma ladlawii membranes; their binding is inhibited by the addition of NaCl to the medium and by removal of lipids (Rottem et al., 1973). In order to further examine the effects of surface components, the initial rate of filipin-free cholesterol association was measured in

M. capricolum membranes containing various concentrations of cytochrome c and lysozyme (Table V). The initial rate was decreased upon incorporation of these proteins. The removal of these extrinsic proteins caused k_{membr} 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 per ml. (These preparations gave k_{membr} values of $10.5 - 11 \times 10^3$ M^{-1} s^{-1}.) The decreased rates of filipin-cholesterol association may result from the decrease in the freedom of motion of the hydrocarbon chains in the membrane interior on incorporation of cytochrome c and lysozyme (Rottem and Shamuni, 1973) or from the restricted accessibility of filipin to free cholesterol.

Protease-catalyzed Hydrolysis of Membrane Proteins

The influence of depletion of proteins in intact cells and isolated membranes on the initial rate was also examined. Removal of about 20% of the membrane proteins of intact M. galliscepticum cells and of about 35 to 50% of isolated membranes by tryptic digestion did not cause a marked change in k_{cells} and k_{membr} (Table VI). Chymotrypsin treatment resulted in less hydrolysis than trypsin digestion, and no trend in initial rates was noted.

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TABLE I. Initial Rates of Filipin-Cholesterol Association in M. capricolum, and M. gallisepticum Membranes Sonicated for Various Time Periods

Time (sec)	M. capricolum[a]		M. gallisepticum[b]	
	A_{500}	dA/dt (s^{-1})	A_{500}	dA/dt (s^{-1})
0	0.27	0.049	0.18	0.075
30	0.15	0.078	0.18	0.075
60	0.12	0.089	0.17	0.075
120	0.10	0.095	-	-
240	0.10	0.094	0.16	0.077

[a] M. capricolum membranes were first prepared by osmotic lysis and then sonicated for various time periods as indicated in the table.

[b] M. gallisepticum membranes were prepared by sonication as described under Methods. The initial rates of filipin-free cholesterol association were measured at 10°C at a filipin concentration of 10 μM.

TABLE II. Effect of Detergents on the Initial Rate of Filipin-Free Cholesterol Association in *M. capricolum* Membranes

Detergent Concentration (%)	k_1 (s^{-1})	
	Triton X-100	Tween 80
None	0.83	0.83
0.02	1.04	0.82
0.04	1.03	0.94
0.06	0.96	0.83

Membranes were prepared by sonication as described under "Methods" and were treated with the detergent for 1 min at room temperature prior to reaction with filipin. Filipin (10 μM, final concentration) and membranes (50 μM, final concentration of free cholesterol) were mixed in the stopped-flow apparatus. The first-order rate constant, k_1, was calculated from $dA/dt \cdot 1/\Delta A_{total}$, where ΔA_{total} is the total amplitude change. At detergent concentrations of 0.06% or less, cholesterol and phospholipids were not detected in the supernatant obtained by centrifugation of membranes at 20,000 rpm for 10 min in a Sorvall RC-2B centrifuge.

TABLE III. Effect of Aging of the Culture on the Rate of Filipin-Cholesterol Binding in *M. gallisepticum* and *M. capricolum*

M. gallisepticum					*M. capricolum*				
Age of Culture (hr)	A_{640}	k_{cells} x 10^{-3} (M^{-1} s^{-1})	$k_{membranes}$ x 10^{-3}	$k_{cells}/k_{membranes}$	Age of Culture (hr)	A_{640}	k_{cells} x 10^{-3} (M^{-1} s^{-1})	$k_{membranes}$ x 10^{-3}	$k_{cells}/k_{membranes}$
15	0.09	4.6±0.2	9.1±0.2	0.51	15	0.08	9.5±0.3	13.9±0.2	0.68
17	0.15	4.3±0.2	8.8±0.3	0.49	16½	0.14	8.9±0.4	13.3±0.4	0.66
20½	0.33	4.0±0.3	7.4±0.4	0.54	18¼	0.31	8.7±0.3	13.1±0.3	0.66
21½	0.41	3.7±0.3	6.6±0.4	0.56	19¾	0.41	8.9±0.5	13.3±0.4	0.67
26	0.59	3.3±0.2	5.4±0.2	0.60	24	0.60	8.1±0.4	11.8±0.5	0.67

The initial rates of filipin-free cholesterol association were measured at 10°C at four cholesterol concentrations in intact cells and isolated membranes obtained from 10 cell cultures of *M. gallisepticum* and 6 cell cultures of *M. capricolum*. Second-order rate constants were calculated from the stopped-flow traces of the initial rates (see 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 approximately 5%.

TABLE IV. Effect of Dimethylsuberimidate on Cell Growth and Free Cholesterol Content and Transbilayer Distribution in Adapted M. capricolum

Time of incubation in high cholesterol medium (hr)	A_{640}		Free Cholesterol ug/mg of membrane protein		Free Cholesterol/ phospholipid (molar ratio)		$k_{cells}/k_{membranes}$	
	-DMS	+DMS	-DMS	+DMS	-DMS	+DMS	-DMS	+DMS
0	0.10	0.10	31	31	0.20	0.20	0.73±0.08	0.75±0.06
1	0.14	0.12	40	35	0.27	0.25	0.53±0.04	0.80±0.06
2	0.17	0.14	89	59	0.58	0.34	0.45±0.06	0.70±0.05

Cells were grown as described under Methods. When the culture reached an A640 value of 0.10 (after about 20 hr of growth), 10 ug/ml of 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 hr incubation in the medium containing 5 mM DMS. The free cholesterol content was calculated using a molar ratio of free cholesterol to cholesterol esters of 1.1. The average molecular weights assumed for the cholestrol esters and phospholipids were 626 and 750, respectively.

TABLE V. Rate Constants for the Association of Filipin with Cholesterol in M. capricolum Membranes Containing Cytochrome c or Lysozyme

Cytochrome c incorporated (mg)	$k_{membranes}$ $\times 10^{-3}$ (M^{-1} s^{-1})	Lysozyme incorporated (mg)	$k_{membranes}$ $\times 10^{-3}$ (M^{-1} s^{-1})
none	12.2	none	11.8
0.14	12.0	0.16	11.5
0.66	10.9	0.56	11.3
0.70	10.0	0.76	11.5
0.78	9.7	1.06	10.5
0.82	9.0	1.16	9.2

M. capricolum membrane suspension containing 1.0 mg protein/ml (115 µg total cholesterol/mg membrane protein) in 1 mM phosphate buffer, pH 7.4, containing 0.4 M sucrose was mixed with an equal volume of cytochrome c or lysozyme solution in the same buffer. The following concentrations of cytochrome c or lysozyme were used: 0, 0.25, 0.75, 1.0, 2.0, and 3.0 mg/ml. After 2 hr at room temperature, membranes were reisolated, and the pellets were suspended in 1 mM phosphate buffer, pH 7.4, containing 0.4 M sucrose. Cytochrome c and lysozyme were removed by washing as described under Methods, giving membranes that contained 1.0-1.1 mg protein/ml and 130-140 µg total cholesterol/mg protein.

TABLE VI. Effect of Proteolysis of M. gallisepticum Membrane Proteins on Rates of Filipin-Cholesterol Binding and Transbilayer Cholesterol Distribution

A. Trypsin Treatment

Age of Culture (hr)	A_{640}	Cells		Membranes	
		% Protein Cleavage	k_{cells} x 10^{-3} (M^{-1} s^{-1})	% Protein Cleavage	$k_{membranes}$ x 10^{-3} (M^{-1} s^{-1})
15	0.10	17.0	4.9	34.5	9.8
17½	0.17	18.3	5.5	39.5	10.4
18½	0.23	21.2	5.3	42.5	11.3
20	0.29	22.8	4.7	45.6	9.6
21½	0.41	23.6	5.1	50.0	10.7

B. Chymotrypsin Treatment

16	0.11	8.9	4.3	19.8	7.9
18	0.21	10.3	4.8	22.4	8.7
22	0.42	11.2	3.6	23.5	7.1

The extent of lysis of trypsin - and chymotrypsin - treated cells was estimated from the release of ^{3}H-methyl-thymidine-labeled components. Mid-phase and late-phase cells underwent lysis of about 8 and 12%, respectively, upon trypsin treatment, and 6 and 8% upon chymotrypsin treatment.

Part II, Chapter V

The effects of exogenous lipids on the transbilayer distribution of free cholesterol in the M. capricolum membrane

Parts of this Chapter will be presented at the XIth International Congress of Biochemistry, Toronto, July, 1979.

Some of the data reported in Figure 2 and Table I were obtained by Z. Gross and S. Rottem, Hebrew University - Hadassah Medical School, Jerusalem, Israel.

ABSTRACT

The phospholipid composition of Mycoplasma capricolum grown with fetal-calf serum (FCS) is different from that of cells grown without serum. When grown without serum, M. capricolum synthesizes more than 96% of its membrane phospholipids de novo, constituted primarily of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). When M. capricolum is grown with 10% FCS, 50% of the membrane phospholipids are exogenous phosphatidylcholine (PC) and sphingomyelin (SPM), which are incorporated unchanged from the medium. A partial conversion from PG to DPG occurred in cells grown with serum. The extent of conversion depended on the growth phase of the culture; the DPG/PG ratio was higher in stationary-phase cells (2.0), and lower in cells from the early logarithmic phase (0.5). A higher DPG/PG ratio was also obtained on increasing the PC concentration of the medium, but the ratio was not altered by incorporation of SPM, phosphatidic acid, or cholesteryl esters (CE). Stopped-flow kinetic measurements of the association of the polyene antibiotic, filipin, with free cholesterol (FC) in intact cells and isolated membranes revealed that the FC distribution in the two halves of the membrane is not affected by the incorporation of exogenous phospholipids or by varying the DPG/PG ratio among

de novo synthesized lipids. M. capricolum cells grown without CE showed a marked decrease in the ratio of the second-order rate constants, k_{cells} / k_{membr} (0.40). On incorporation of cholesteryl-oleate, - linoleate, or - linolenate, the ratio of k_{cells} / k_{membr} increased to 0.66. This suggests that incorporation of exogenous CE affects the transbilayer distribution of FC in the membrane of M. capricolum cells grown in media containing high FC concentrations.

INTRODUCTION

Information about the transbilayer distribution and movement of components in a biological membrane is required in order to understand the molecular organization and function of the membrane. Mycoplasmas have a number of particularly advantageous characteristics for research of this kind (Razin, 1978). The mycoplasmas require cholesterol for growth and are, therefore, well suited as models for studying cholesterol distribution and movement. This topic was recently investigated in M. capricolum (Bittman and Rottem, 1976) and M. gallisepticum (Bittman and Rottem, 1976 ; Rottem et al., 1978) by two different methods. One is the rapid kinetic study of the binding of the polyene antibiotic, filipin, with free cholesterol. The other is the exchange of [^{14}C] cholesterol between mycoplasma cells and high-density lipoproteins.

The ability to alter the cholesterol content of the M. capricolum membrane to very low concentrations made possible a study of the rate of movement of FC from the outer half of the bilayer to the inner half (Clejan et al., 1978). The rate of FC transbilayer movement is rapid in growing cellls. Although we observed that the addition of FCS to M. capricolum cultures increased the phospholipid protein ratio and that cells

incorporated PC and SPM from the serum (Gross and Rottem, unpublished), the influence of exogenous lipid components on cholesterol uptake and translocation remained to be established. In order to determine whether systematic changes in the composition of membrane lipids affect FC movement, the rapid reaction between filipin and FC was studied using cells grown with varying concentrations of exogenous phospholipids or with cholesteryl esters with different degrees of unsaturation (cholesteryl-oleate, -linoleate, - linolenate). M. capricolum was chosen because this strain is capable of incorporating a high amount of CE. A 1:1.2 molar ratio of CE to FC was reported recently in M. capricolum (Razin and Rottem, 1978). This chapter reports also changes in the composition of the polar and nonpolar lipids occurring in the presence compared with the absence of serum in the growth medium. The effects of incorporation of exogenous lipids on FC distribution and movement across the two halves of the bilayer were examined.

Materials and Methods

[1 - ^{14}C] - Palmitic acid (56 Ci / mole) and [N - methyl - ^{14}C] sphingomyelin (60 mCi / mmol) were purchased from Amersham.

[^{14}C (U)] - Phosphatidylcholine (1.765 Ci / mmol) was from New England Nuclear.

Cholesterol (recrystallized 3 times from ethanol, the cholesteryl esters, (cholesteryl - oleate, - linoleate, - linolenate), and the phospholipids, PC (egg yolk), SPM (bovine brain), and PA (egg yolk) were purchased from Sigma Chemical Co. The purity of all the phospholipids was checked by thin-layer chromatography on silica gel G plates in chloroform: methanol: water (65:25:4) using 0.005% butylated hydroxytoluene as an antioxidant.

Growth of the Organism in the Presence of Exogenous Lipids and Isolation of Membranes

Mycoplasma capricolum (California kid) was grown in a modified Edward medium (Razin and Rottem, 1975) containing 10% of FCS (Grand Island Biological Co). To label membrane lipids, 0.002 μ Ci / ml of 1 - ^{14}C palmitate was added. For incorporation of exogenous lipids, M. capricolum cells were grown in a modified Edward medium that contained 1% bovine serum albumin (fatty acid poor, Sigma Fraction V), 10 μg / ml of cholesterol, and palmitic and oleic acids (10 μg / ml each). To determine the incorporation of phospholipids, radioactive SPM or PC (0.1 μ Ci / ml)

was added to the cold SPM or PC (2.5 - 30 μg / ml). SPM, PC, and PA were added as ethanolic solutions. The ethanol concentration did not exceed 0.1% (v/v). With cultures larger than 500 ml, CE and PC visible crystals formed during the addition of solutions containing more than 20 μg / ml of PC or CE. For concentrations of more than 20 μg / ml, all the ethanolic solutions were first evaporated under N_2. To the film of phospholipids, oleic acid, palmitic acid, and cholesterol were added. The medium was warmed at 37°C, then added to the film of lipids, with vortexing in the presence of glass beads. In this way, the lipids did not precipitate during the isolation of the cells.

M. capricolum was adapted to grow in 1.25 μg / ml of cholesterol by making serial passages of the normal strain into media containing lower cholesterol concentrations (Clejan et al., 1978). For studies of the transbilayer movement of cholesterol, these cells were transferred to a cholesterol-rich medium. The growth was followed by measuring the absorbance of the culture at 640 nm. Most experiments were performed with mid-exponential-phase cells (A640 = 0.15 - 0.22). The cells were harvested by centrifugation at 12,000 g for 20 min, washed twice, and resuspended in cold 0.25 M NaCl solution. Membrane preparation was as described previously (Clejan et al., 1978).

Lipid Analysis.

Lipids were extracted from intact cells and isolated membranes by the method of Bligh and Dyer (1959). Thin-layer chromatography of total membrane lipids was performed with silica gel HR plates (Analtech). The plates were first developed at room temperature with petroleum ether (bp 40 - 60°C) - acetone (3:1, v/v/v), then at 4°C with choloroform - methanol - water (65:25:4, v/v). Lipids spots were detected by iodine vapor. The iodine was removed by evaporation. For determining radioactivity in the lipid spots, the compounds were scrapped from the plate into scintillation vials containing 5 ml of Bray's solution. The radioactivity was measured in a Beckman scintillation spectrometer. To determine the phosphorus concentration in phospholipid spots, the compounds were scrapped from the plate into test tubes and digested with 0.5 ml of 10 N sulfuric acid in the presence of silica gel. Total phosphorus was determined by the method of Taussky and Shorr (1953). Neutral lipids were separated by thin-layer chromatography on silica gel G plates. The plates were developed at room temperature with benzene: ethyl acetate (5:1, v/v). The spots were scrapped from the plates and extracted into chloroform - methanol (2:1, v/v) at 45° for one hour. Stock solutions of 10, 20, 30 µg per ml of each FC and CE were made and used as standards.

After centrifugation and evaporation of the chloroform layer, the free cholesterol and cholesterol esters separated in this way were assayed by the method of Zlatkis and Zak (1969).

Kinetic Measurements.

The filipin complex was purified as described previously (Bittman and Rottem, 1976). The final concentration of filipin, after mixing with cells or membranes, was 10 μ M for the native strain and 16 μ M for the adapted strain. Initial rates of filipin - cholesterol association were measured as described previously (Clejan et al., 1978). The initial rate of filipin association with cholesterol in cells grown in media containing PC, SPM, PA,or CE was first order in each reactant.

Miscellaneous Methods.

To determine whether cells that had incorporated PC, SPM, PA, or CE remained intact, the percent efflux of [^{3}H] thymidine - labeled components and of NADH oxidase were determined as described previously (Clejan et al., 1978).

Protein was determined according to Lowry et al. (1951).

RESULTS

Phospholipid Composition in the Presence and Absence of Serum.

When the cells were grown with [^{3}H] - palmitic acid, 10 - 12% of the label was found in the neutral lipid fraction, mainly in free fatty acids. Since the addition of the radioactive fatty acid to the growth medium failed to label the cholesterol esters, it seems as these components are not synthesized by the organism but are incorporated from the medium.

Thin-layer chromatography of the polar lipids revealed four major components: sphingmyelin, phosphatidylcholine, phosphatidylglycerol, and diphosphatidylglycerol. This identification was supported by the comigration of the four components with commercially available standards. In addition, two other spots were detected. One has a R_f value similar to that of PC, and the other has a R_f value between those of PG and DPG. These compounds are believed to be aminoacyl PG and acyl PG, respectively (Z. Gross and S. Rottem, personal communication). Table I shows that when cells are grown without serum, 96 - 98% of the membrane lipids are labeled, i.e., de novo synthesized. However, when the cells are grown with serum, SPM remained unlabeled,

suggesting that it is taken up from the growth medium.

The phospholipid content varied with the age of culture and was higher in cells from early logarithmic phase than in cells from the stationary phase. The DPG / PG ratio changed from 0.5 in cells from the early logarithmic stage to 2.0 for older cells (Figure 1). When the FCS concentration in the medium was increased the amount of de novo synthesized lipids decreased, but the exogenous phospholipid content increased (Figure 2). The incorporation of PC + SPM into the M. capricolum membrane is relatively rapid, leveling off after less than 3 hours (Figure 3).

Phospholipid Composition with the Addition of Exogenous Lipids.

The phospholipid composition was studied after addition of 25 μg / ml of either PC, SPM, PA, or CE to the growth medium (Table II). With PC supplementation, 35% of the total lipid phosphorus was found in the PC fraction; when the medium was supplemented with SPM, 19% of the total lipid phosphorus was found in the SPM fraction. Addition of CE did not change the composition of the phospholipid fractions (Table II). A higher DPG to PG ratio resulted from the addition of increasing concentrations of PC to the medium (Figure 4); however, the addition of SPM or PA did not alter the DPG to PG ratio (Table II).

Cholesterol Distribution with the Addition of Exogenous Lipids.

In order to examine how these changes in the lipid composition and the interconversion between PG and DPG affect the transmembrane distribution of cholesterol, the initial rate of filipin-cholesterol binding was studied. The ratio of k_{cells} / k_{membr} remained 0.66 $\pm$ 0.08 when the PC concentration of the medium was varied between 0 and 25 μg / ml. Similarly, the ratio of the second-order rate constants did not vary on supplementation with SPM or PA (Table IV). Moreover, no change in the ratio of rate constants was observed when the adapted strain was transferred to cholesterol-rich medium supplemented with the above exogenous lipids (data not shown). Therefore, not only is the distribution of cholesterol between the inner and outer halves of the bilayer not modified by these changes in membrane phospholipid content, but also the rapid movement of cholesterol into adapted cells transferred to cholesterol-rich medium is not changed.

The distribution of FC was probed in cells and membranes of the native strain supplemented with CE (Table V). Approximately 0.4 of the FC was localized in the outer half of the bilayer in cells not supplemented with CE. On addition of 5 μg / ml or more of CE to the medium, the localization of FC in the

outer half of the membrane was approximately 0.6 of the total cholesterol in the membrane. This latter value is essentially the same as that found when the native strain is grown with 10% serum (Bittman and Rottem, 1976). The slight increase in the extent of FC localized in the inner half of the bilayer when the CE content is low is seen also in adapted cells (Table VI) but to a smaller extent. There was not further increase in the FC content of the inner half of the bilayer during incubation longer than one hour.

DISCUSSION

The most common phospholipids synthesized de novo by M. capricolum are the acidic phospholipids, PG and DPG. These are typical microbial lipids (Razin, 1975). A similar lipid composition was found in membranes of other prokaryotes (van Deenen, 1971). PC and SPM are present in appreciable amounts only in M. capricolum grown in serum-containing media. Other mycoplasmas also incorporate PC from the medium (Smith and Henrikson, 1965; Razin et al., 1970). Labeling experiments have shown that PC and SPM are taken up from the medium and are not synthesized by the organisms (Placket et al., 1969). However,

it was shown recently that the PC of M. gallisepticum and M. capricolum has a different fatty acid composition from that of PC added to the growth medium (Rottem, personal communication). In contrast, SPM is incorporated into the M. capricolum membrane without any deacylation. This is confirmed by the observation that M. capricolum cells grown with [^{3}H] palmitic acid contain a considerable amount of radioactivity in the PC fraction (Table I). An interconversion involving PG and DPG occurs both during aging of cells grown on FCS and during supplementation with PC, but not during supplementation with SPM or PA. Therefore, these interconversions between these two acidic phospholipids are physiological processes.

The marked increase in the rate constants of filipin-cholesterol association in both intact cells and isolated membranes upon PC incorporation (Table III) suggests that the membrane fluidity is enhanced or that the accessibility of cholesterol is increased.

According to Smith (1971), some mycoplasmas are capable of esterifying cholesterol with short-chain fatty acids such as butyrate. However, data obtained with M. capricolum and in some other mycoplasmas and acholeplasmas (Rodwell; 1963; Argaman and Razin, 1965; Rottem et al., 1971; deKruyff et. al., 1972)

indicate that the incorporated cholesterol is not esterified or changed in any way. The CE detected in M. capricolum originates from the growth medium.

Incorporation of phospholipids or cholesteryl esters does not influence the FC distribution in the two halves of the membrane of M. capricolum, but absence of cholesteryl esters does. Therefore, transbilayer distribution of FC is not determined by its polar lipid content. The association of FC with other membrane components (especially proteins) must be further examined in order to gain insight into the mechanism of transbilayer movement of FC.

TABLE I. Phospholipid Composition of the Membrane of M. capricolum Cells Grown with and without Serum[a]

Tentatively Identified Lipid Compound	Cells Grown with Serum		Cells Grown without Serum	
	Lipid Phosphorus (% of total)	Radioactivity (% of total)	Lipid Phosphorus (% of total)	Radioactivity (% of total)
Sphingomyelin	22	2	2	2
Phosphatidylcholine[b]	30	8	2	2
Phosphatidylglycerol	9	15	50	45
Acyl-phosphatidylglycerol	10	12	17	21
Diphosphatidylglycerol	24	53	24	24
Phosphatidic acid[c]	5	10	7	8

[a]Cells were grown in media containing 10% fetal-calf serum or in media where the serum was replaced by 1% albumin, 10 μg/ml of each palmitic and oleic acids, and 10 μg/ml of cholesterol. Both media were supplemented with 0.001 μCi/ml of (1-^{14}C) palmitic acid.

[b]Traces of aminoacyl phosphatidylglycerol co-migrate with phosphatidylcholine.

[c]Phosphatidic acid was tentatively identified by thin-layer chromatography as follows. To 0.5 ml of the lipid extract of M. capricolum (0.05-0.25 mg total lipid) in chloroform-methanol (2:1 v/v) was added 1 ml of 0.5 N KOH in methanol. Alkaline hydrolysis was allowed to proceed at 37°C for 30 min [J. C. Dittmer and M. A. Wells, Methods Enzymol. 14, 482 (1969)]. Glycerophosphate, the deacylated product of phosphatidic acid, was identified by thin-layer chromatography on silica gel G plates upon elution with water-acetic acid-ethanol (100:10:12, v/v/v). Preliminary two-dimensional thin-layer chromatography of lipids from M. capricolum on silica gel G plates also suggested the presence of phosphatidic acid in the membrane. The developing solvents were chloroform-methanol-water (65:25:4, v/v/v), in the first direction, and diisobutyl-ketone-acetic acid-water, (80:50:10, v/v/v) in the second direction [M. Lepage, Lipids 2, 244 (1967)].

TABLE II. Phospholipid Interconversion in M. capricolum Cells Grown with Exogenously Supplied Lipids[a]

Tentatively Identified Lipid Compound	Lipid Phosphorus (% of total)				
	Unsupplemented Medium	Medium Supplemented with 25 μg/ml of			
		Phosphatidylcholine	Sphingomyelin	Phosphatidic Acid	Cholesterol Oleate
Sphingomyelin	traces	-	19	-	-
Phosphatidylcholine[b]	traces	35	traces	3	traces
Phosphatidylglycerol	50	15	38	44	48
Acylphosphatidylglycerol	17	8	12	15	16
Diphosphatidylglycerol	24	37	24	22	25
Phosphatidic acid[c]	6	5	6	16	10

[a]Cells were grown in a media containing 1% albumin, 10 μg/ml of each palmitic and oleic acids, and 10 μg/ml cholesterol. The medium was supplemented with 25 μg/ml of either phosphatidylcholine, sphingomyelin, phosphatidic acid, or cholesterol oleate.

[b]Traces of aminoacyl phosphatidylglycerol comigrate with phosphatidylcholine.

[c]Phosphatidic acid was tentatively identified by thin-layer chromatography as follows. To 0.5 ml of the lipid extract of M. capricolum (0.05-0.25 mg total lipid) in chloroform-methanol (2:1 v/v) was added 1 ml of 0.5 N KOH in methanol. Alkaline hydrolysis was allowed to proceed at 37°C for 30 min [J. C. Dittmer and M. A. Wells, Methods Enzymol. 14, 482 (1969)]. Glycerophosphate, the deacylated product of phosphatidic acid, was identified by thin-layer chromatography on silica gel G plates upon elution with water-acetic acid-ethanol (100:10:12, v/v/v). Preliminary two-dimensional thin-layer chromatography of lipids from M. capricolum on silica gel G plates also suggested the presence of phosphatidic acid in the membrane. The developing solvents were chloroform-methanol-water (65:25:4, v/v/v), in the first direction, and diisobutyl-ketone-acetic acid-water, (80:50:10, v/v/v) in the second direction [M. Lepage, Lipids 2, 244 (1967)].

TABLE III. Second-Order Rate Constants for Association of Filipin and Cholesterol in Intact Cells and Isolated Membranes of M. capricolum Grown with Exogenous Phosphatidylcholine[a]

Media Supplemented with Phosphatidylcholine (μg/ml)	Free Cholesterol (μg/ml of cell protein)	k_{cells}[b] (M^{-1} s^{-1})	$k_{membranes}$[b] (M^{-1} s^{-1})	$k_{cells}/k_{membranes}$[c]
None	47.0	4.21×10^4	6.38×10^4	0.66±0.04(4)
5	49.4	4.47×10^4	7.21×10^4	0.62±0.06(3)
10	50.8	5.28×10^4	8.79×10^6	0.60±0.02(5)
15	50.3	7.62×10^4	11.73×10^4	0.65±0.05(4)
25	48.8	7.27×10^4	10.48×10^4	0.69±0.05(4)

[a]The initial rates of filipin-free cholesterol association were measured at 10°C at various cholesterol concentrations in intact cells and isolated membranes obtained from different cultures, each incubated with media containing different phosphatidylcholine concentrations. The number of cell cultures investigated is indicated in parentheses in the last column.

[b]A representative example of second-order rate constants analyzed from one culture.

[c]Average ratio of second-order rate constants. Error limits of the kinetic data are standard error of the mean.

TABLE IV. Effect of Exogenous Sphingomyelin or Phosphatidic Acid on Cholesterol Transbilayer Distribution in *M. capricolum*

	Media Supplemented with							
	5 μg/ml		10 μg/ml		15 μg/ml		20 μg/ml	
	SPM	PA	SPM	PA	SPM	PA	SPM	PA
Free Cholesterol (ug/mg of cell protein)	50.4	48.2	53.0	50.5	54.0	52.6	53.2	54.2
$k_{cells}/k_{membranes}$[a]	0.62±0.06	0.67±0.05	0.68±0.05	0.65±0.05	0.66±0.03		-	0.69±0.04

[a]The number of cell cultures investigated was five for SPM and two for PA.

TABLE V. Effect of Cholesterol Oleate Enrichment on Cholesterol Distribution between the Two Halves of the Bilayer in *M. capricolum*

Media Supplemented with Cholesterol Oleate (μg/ml)	Free Cholesterol (μg/mg of cell protein)	Cholesterol Ester (μg/mg of cel protein)	k_{cells}[a] (M^{-1} s^{-1})	$k_{membranes}$[b] (M^{-1} s^{-1})	$k_{cells}/k_{membranes}$[c]
None	91.8	10.0	4.65×10^4	11.34×10^4	0.41±0.09(10)
2.5	84.7	19.8	4.28×10^4	9.51×10^4	0.45±0.05(2)
5	83.0	33.4	5.95×10^4	11.90×10^4	0.50±0.06(4)
10	84.8	73.5	7.30×10^4	12.37×10^4	0.59±0.04(4)
15	88.0	75.8	7.94×10^4	12.03×10^4	0.66±0.06(2)
20	84.7	77.0	8.88×10^4	15.31×10^4	0.60±0.05(4)

[a] A representative example of the second-order rate constants analyzed from one culture

[b] Average ratio ± standard error of the mean. The number of the cell cultures investigated is indicated in parantheses.

TABLE VI. Effect of Exogeneously Added Cholesterol Oleate on Free Cholesterol Movement from the Outer to the Inner Half of the Lipid Bilayer of M. capricolum Cells Adapted to Grow on Low Cholesterol and Transferred to a Cholesterol-Rich Medium[a]

Time of Incubation in 10 µg/ml Cholesterol (hr)	$k_{cells}/k_{membranes}$ in the Presence of Cholesterol Oleate (µg/ml)				
	None		2.5	5.0	10.0
	Adapted on Cholesterol (1.25 µg/ml)	Adapted on Cholesterol (5 µg/ml)			
0	0.59	0.50	0.64	0.70	0.70
1	0.55	0.45	0.60	0.54	0.53
2	0.45	0.48	0.50	0.45	0.45
4	0.42	0.45	0.47	0.43	0.48

[a]Cells were grown in a media containing 1% albumin, 10 µg/ml of each palmitic and oleic acids, increasing concentrations of cholesterol oleate, and either 1.25 µg/ml or 5 µg/ml cholesterol. After the cells reached an absorbance of 0.1 at 640 nm, 10 µg/ml cholesterol was added for the indicated period of time. Two different cell cultures were examined.

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FIGURE CAPTIONS

Figure 1: Changes in diphosphatidylglycerol to phosphatidylglycerol ratios induced by aging of the culture of M. capricolum.

Figure 2: The effect of increasing serum concentrations on the lipid composition of M. capricolum membranes

△--△, the ratio between incorporated to de novo synthesized lipids;

○—○, de novo synthesized phosphatidylglycerol;

●···●, de novo synthesized diphosphatidylglycerol.

Figure 3: Incorporation of serum phospholipids into the M. capricolum cell membrane as a function of time.

Figure 4: Changes in the ratio of diphosphatidylglycerol to phosphatidylglycerol induced by increasing concentrations of phosphatidylcholine in M. capricolum growth medium.

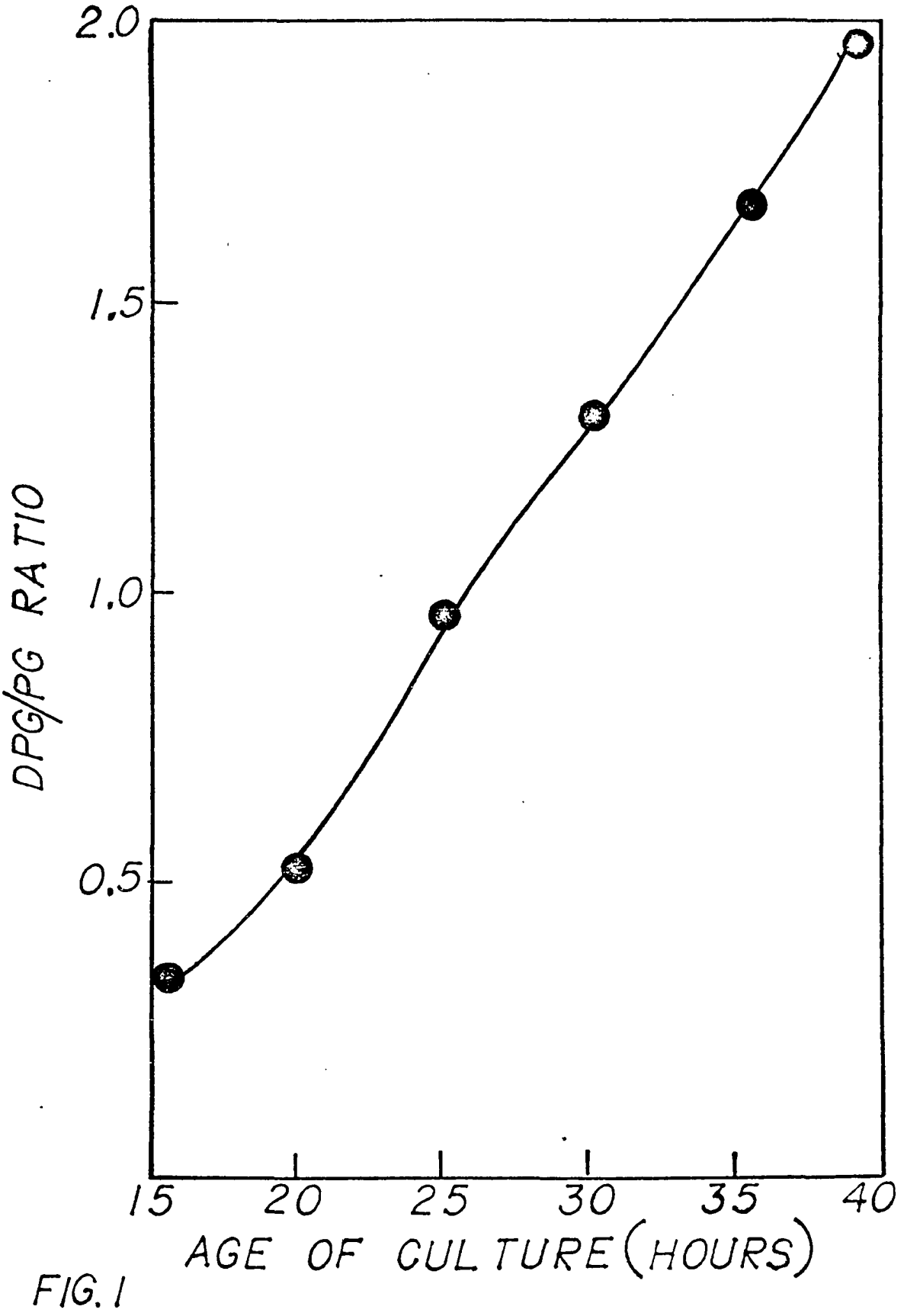

FIG. 1

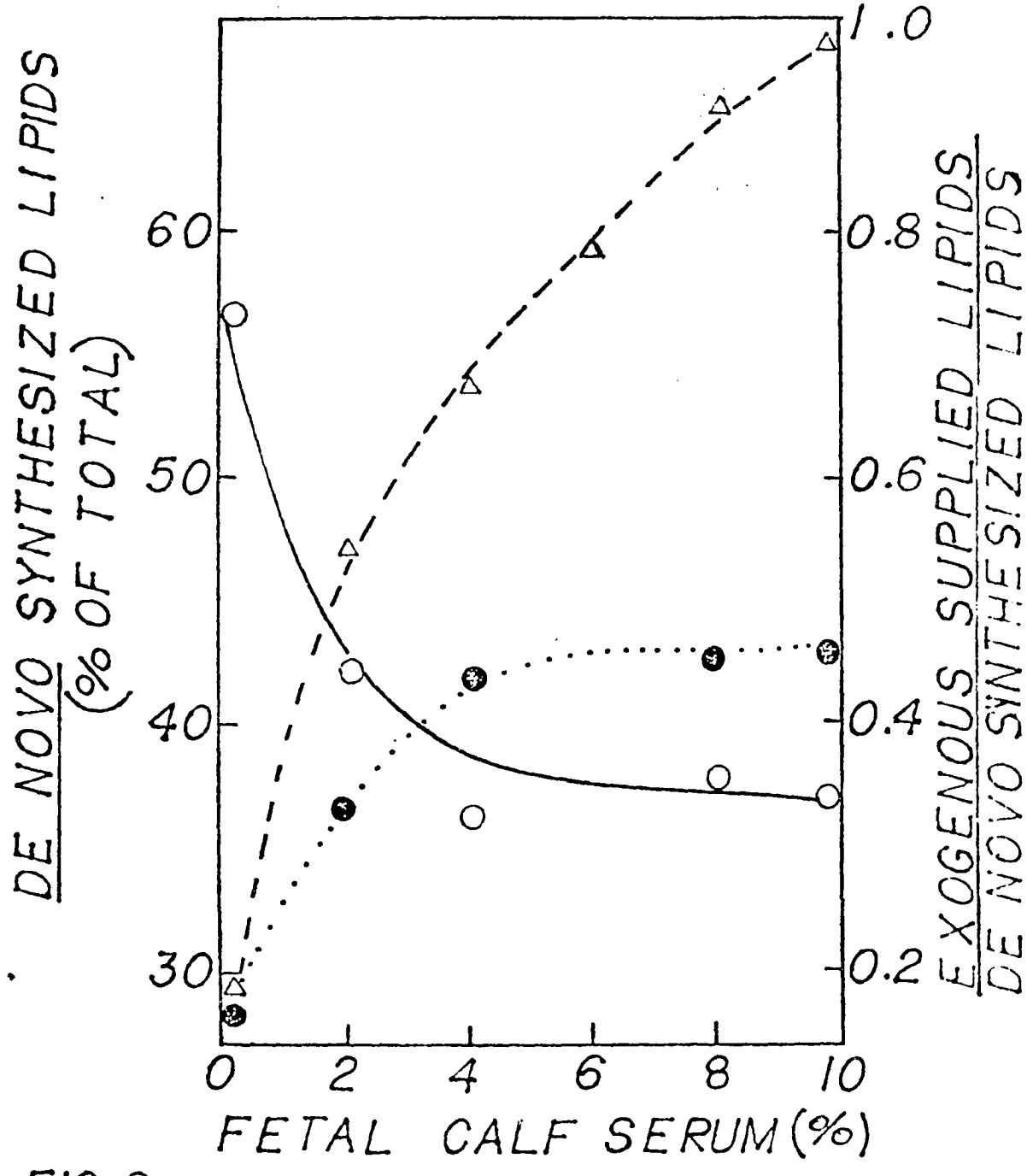

FIG. 2

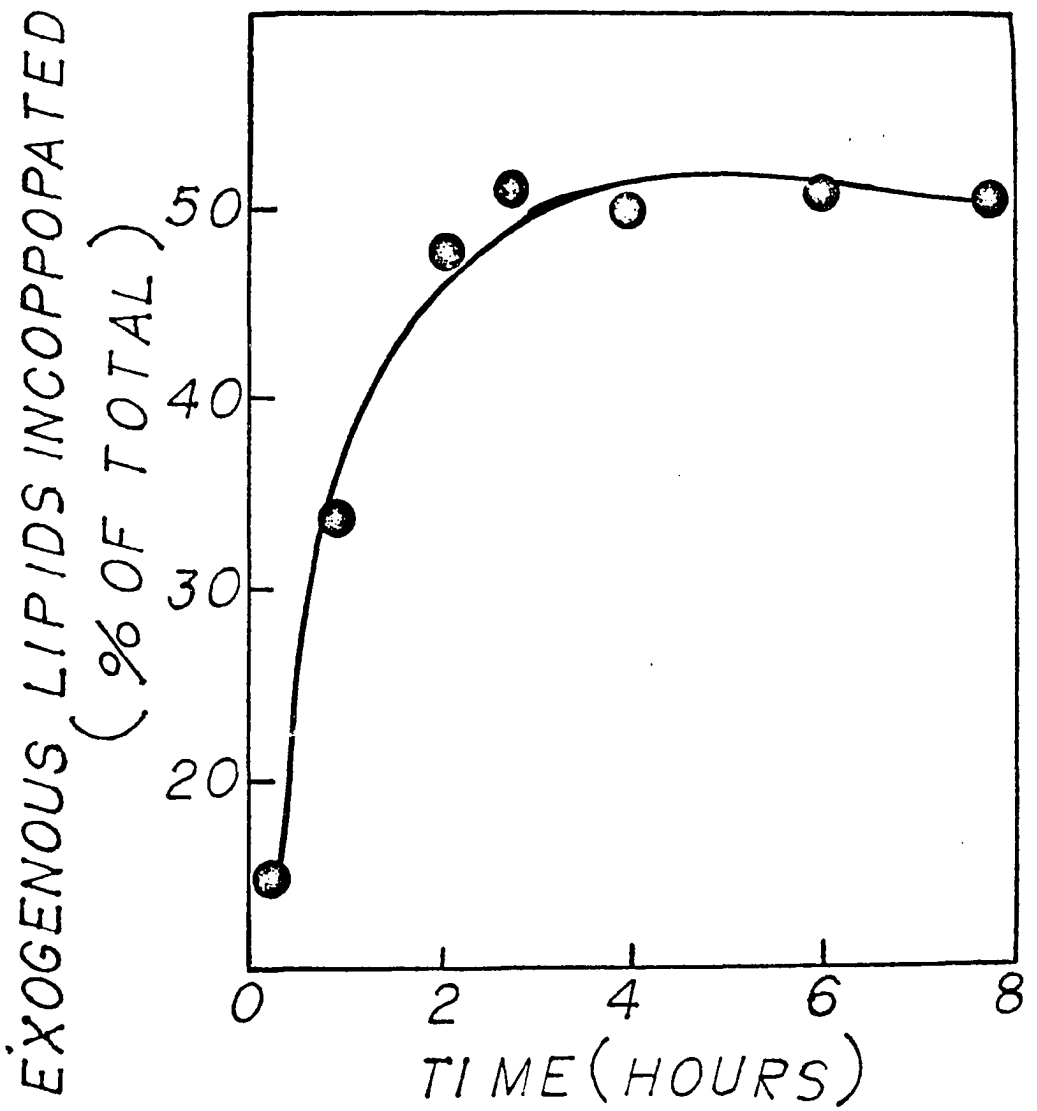

FIG. 3

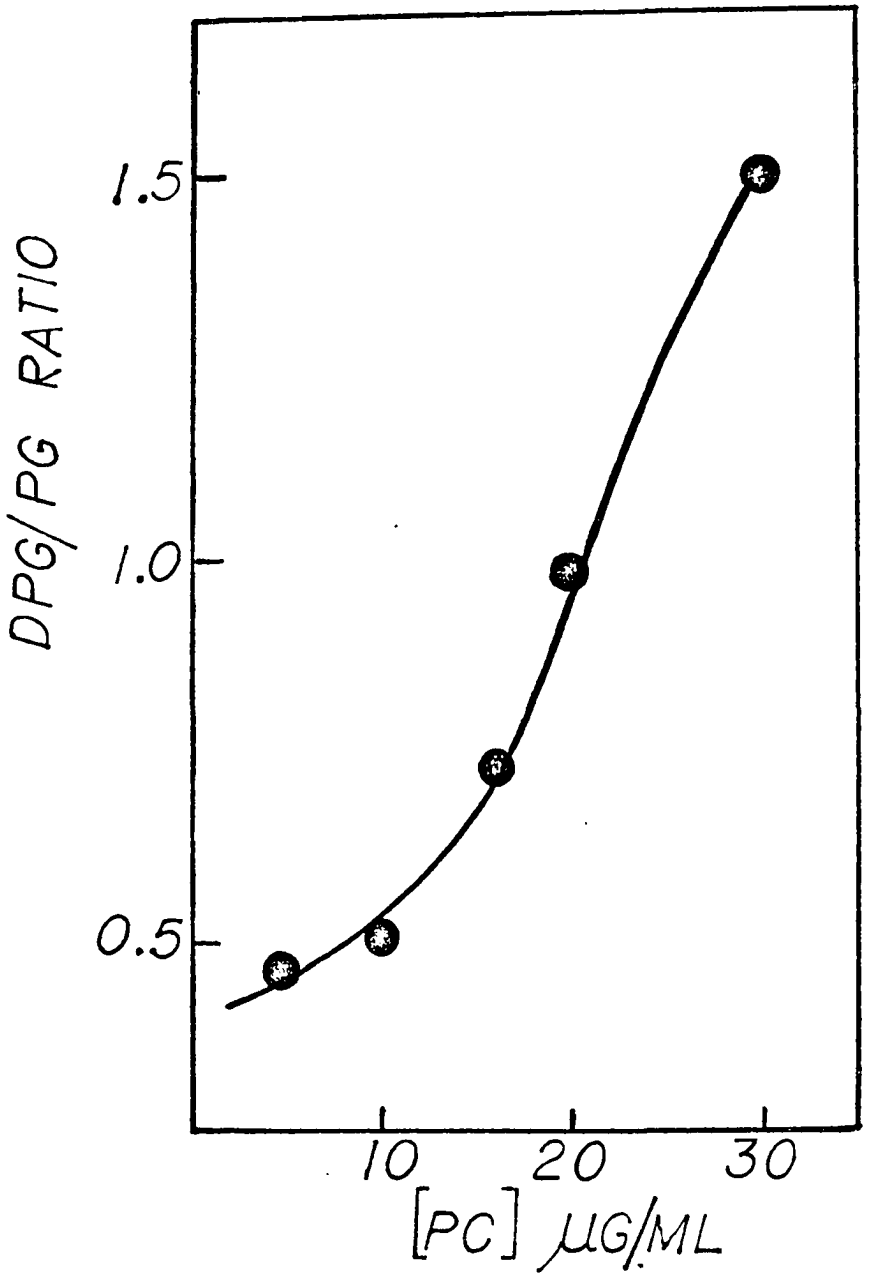

FIG. 4

Part II, Chapter VI

The effect of structural modification in the sterol molecule on growth of *M. gallisepticum* and *M. capricolum* cells and on transbilayer movement and distribution of sterols in the membrane of *M. capricolum*

ABSTRACT

The ability of various sterols to support the growth of Mycoplasma capricolum was investigated. Sterols that differed from cholesterol in the following aspects were added to defined medium as ethanolic solutions: (1) those with modifications in the position of double bonds within the steroid nucleus, (2) and those containing a side chain with either a double bond or a substituent (methyl, ethyl, or hydroxyl), or both. Growth was poorer with all of the sterols we tested compared with growth in cholesterol-containing medium. The transbilayer distribution of sterols was studied in M. capricolum membranes by stopped-flow kinetic measurements of the association of the polyene antibiotic, filipin, with sterols in intact cells and isolated membranes. Cells in cholesterol-containing medium and at mid-exponential phase of growth showed two-thirds of the cholesterol to be localized in the outer half of the bilayer (Bittman and Rottem, 1976). With 3β-cholestanol or 4, 6-cholestadien-3β-ol, the distribution of sterol remained the same as with cholesterol, whereas with β-sitosterol, stigmasterol, or ergosterol, almost 90% of the sterol was localized in the outer half of the bilayer. In order to study the transbilayer movement of sterols, M. capricolum was adapted

to grow in a medium containing low β-sitosterol or stigmasterol concentrations. Subsequent transfer to a medium containing 10 μg/ml of β-sitosterol or stigmasterol resulted in incorporation of the sterol from the medium, but the rate of the sterol translocation from the outer to the inner halves of the membrane was low. To follow the simultaneous incorporation of two sterols from the medium into the membrane of the adapted strain, adapted M. capricolum cells were transferred to different proportions of [^{3}H] cholesterol and [^{14}C] β-sitosterol. Cholesterol was taken up preferentially from the medium. These studies establish that structural modifications in the cholesterol molecule affect the sterol uptake capacity and the distribution and the movement of the sterol between the two halves of the lipid bilayer.

INTRODUCTION

One problem of great biological significance concerns the mechanism by which cells control the amount of exogenous sterol incorporated into their plasma membrane. While it is generally accepted that cholesterol plays an important role as a regulator of membrane fluidity (Jain, 1975; Chapman, 1975) and as a structural component of the membranes (Nes and McKeen, 1977), the relationships between sterol structure and its uptake, movement, and distribution is not yet understood.

Being the only prokaryote that requires an external sterol source for growth (Edwards and Fitzgerald, 1951; Razin, 1978) and in the same time being capable of autonomous growth, mycoplasma is a unique model system for studying such a relationship. It was shown that sterols that support the growth of mycoplasma must possess a β-hydroxyl group at C_3, a planar steroid ring system, and a side chain at C_{17} (De Kruyff et al., 1973). The necessity for such a molecular configuration for growth was demonstrated in different mycoplasma (Butler and Knight, 1960; Rodwell, 1963; Smith, 1964; Razin and Tully, 1970; Rottem et al., 1971).

Other cells which have been studied in vivo in relation to their sterol needs include Pythiaceous fungi (Hendrix, 1970; Elliot, 1977), yeast (Nes et al., 1978), and yeast mutants (Silbert, 1975). The main sterol in Saccharomyces cerevisiae

is ergosterol,which supports growth without being metabolically altered (Proudlock et al., 1968). There is a significant difference in the response of S. cerevisiae to different sterols: methyl groups at C_4 and C_{14} were inconsistent with growth, a double bond between C_{17} and C_{20} had no deleterious effect on the growth,and the Δ5 system was less effective than the Δ5, 7 diene system (Nes et al., 1978).

In previous studies on cholesterol distribution and movement, two mycoplasma species, M. gallisepticum and M. capricolum were used (Bittman and Rottem, 1976; Rottem et al., 1978; Clejan et al., 1978). In the present study the ability of other sterols to be taken up by these mycoplasmas and to be translocated from the outer leaflet to the inner leaflet of the membrane have been examined. The present chapter describes the growth of these two mycoplasmas with different sterols and the uptake,distribution, and movement of two classes of sterols that differ from cholesterol: one having modification in the cyclopentanophenanthrene ring and the other having a double bond and/or additional substituents in the side chain.

Materials and Methods

Cholesterol and 5α - cholestan - 3β - ol were from Sigma; stigmasterol, β - sitosterol, ergosterol, desmosterol,and

fucosterol were from Steraloids; 4, 6 - cholestadien - 3β-ol, lathosterol, 7-dehydrocholesterol, campesterol, 25-hydroxycholesterol, allocholesterol, and brassicasterol were from Research Plus Steroid Laboratories. β-Norcholesterol was a gift of Dr. W. G. Dauben. All were recrystallized three times from ethanol. The purity was checked by (1) melting points, (2) thin-layer chromatography on silica gel in ether-petroleum ether 90:30 (v/v) and (3) gas chromatography (as trimethyl silyl diethers) at 230°C with N_2 as a carrier gas, and 5α-cholestane as an internal standard on SE-54 glass columns (analysis made by Dr. T. Parker). Based on this method, all the sterols used were at least 94% pure. They do not contain cholesterol. An impurity of low R_f value was detected in fucosterol, brassicasterol, and 4, 6 - cholestadien-3β-ol on charring with sulfuric acid, and may represent an oxygenated sterol.

(4-^{14}C) β-Sitosterol (53.6 mCi/mmol) and (^{3}H) - cholesterol (43 Ci/mmol) were obtained from New England Nuclear.

Bovine serum albumin fraction V from Sigma was found to be cholesterol-free by gas-liquid chromatography.

Growth Conditions and Isolation of Membranes

The conditions for cell growth and membrane isolation were described previously (Clejan et al., 1978). To minimize cell lysis of M. capricolum grown with various sterols, the concentration of BSA in the medium was increased from 1% to 2%. Sterols were added to the growth medium together with the fatty acids in absolute ethanol, so that the total concentration of ethanol never exceeded

1%. The order of the addition was first the sterol-fatty acid solution, then cholesterol oleate, and finally BSA. In this way crystals of lipids were not formed after incubation with the medium containing 10 μg/ml of sterol or less.

Lipid Analysis

Lipids were extracted from wet cells according to the method of Bligh and Dyer (1959). When the cells were harvested from growth medium containing ^{14}C-cholesterol and the lipids were extracted, negligible counts of ^{14}C were present in the upper methanol-water layer after extraction. The separation of neutral lipids was as described in Chapter V. Lipid spots were visualized by iodine vapor. Iodine was removed by heating the plates. For determination of the radioactivity in the lipid spots, the spots were scrapped from the plates into scintillation vials, and 5 ml of Bray's solution was added. The radioactivity was measured in a Beckman liquid scintillation spectrometer. In order to study the competition between two sterols, [^{14}C] β-sitosterol and [^{3}H] cholesterol were added together to the medium. All of the ^{14}C counts were made on channel B and normalized to the amount of overlapping ^{3}H, and all counts for ^{3}H were made on channel A and normalized for ^{14}C that interferes with ^{3}H counts. Standards with

0 to 20,000 counts per minute were used. The actual amount of ^{14}C and ^{3}H after normalization differed in each sample by less than 20%.

Kinetic Measurements.

Rates of filipin-cholesterol association were as described previously (Clejan et al., 1978). The initial rate of filipin-sterol association in cells and membranes containing different sterols remained first order in each reactant. To determine whether these cells remained intact, the percent efflux of [^{3}H] thymidine-labeled-components and of NADH oxidase were determined. Lysis was also checked at 450 nm in the stopped-flow apparatus when cells were mixed with filipin (Bittman and Rottem, 1976).

Analytical Methods.

Protein and phospholipid analyses were made as described previously (Clejan et al., 1978). Total sterol was measured colorimetrically (Zlatkis and Zak, 1969). The sterols were analyzed at the following wavelengths: cholesterol, 550 nm; β-cholestanol 530 nm; stigmasterol and β-sitosterol, 542 nm; ergosterol, 560 nm.

RESULTS

Effects of Sterols on Growth.

Figure 1 shows the concentration dependence of sterols to support growth of young M. capricolum cultures (20 hr). Growth was monitored by absorbance at 640 nm. At concentrations of 1.25 or 2.5 μg / ml of sterol in the medium, growth on β-sitosterol, stigmasterol, ergosterol, cholestanol, and lathosterol is inhibited compared with growth on cholesterol. At concentrations of 10 and 20μg/ml β-sitosterol gave the best growth of the cholesterol analogs examined (Figures 1 and 2). At β-sitosterol concentrations of 20 μg / ml or higher, cell growth, as judged by turbidity at 640 nm, approached that of cultures grown on 10 μg / ml of cholesterol. The growth response of 25-hydroxycholesterol was very poor, while β-norcholesterol inhibited growth. Cells grown on these cholesterol analogs are more prone to lysis than cholesterol-grown cells; NADH oxidase efflux was 10-20% higher than in cholesterol-grown cells. The lower absorbance of β-sitosterol-grown cells at the stationary phase may arise from partial lysis (Figure 2). Indeed, NADH oxidase release from these cells was 40% after 40 hr of growth.

Attempts to grow M. gallisepticum on stigmasterol, campesterol, desmosterol, ergosterol, 4, 6-cholestadien-3β-ol, cholestanol, and lathosterol were not successful. Only with β-sitosterol was some very poor growth seen. When cells grown on β-sitosterol (20 μg ml) were transferred to a cholesterol-rich medium (10 μg / ml), the growth rate was markedly enhanced after a lag period of 2 hr, but still the cells reached the stationary phase of growth at a lower turbidity than cultures of M. gallisepticum grown from the beginning on cholesterol.

M. capricolum was adapted to grown on 1.25 μg / ml of β-sitosterol or stigmasterol by serial passage of cultures, initially grown on 20 μg / ml, into media containing one-half of the sterol concentration of the previous medium. A minimum of three passages were made at each concentration. This method failed for cholestanol, lathosterol, ergosterol, and desmosterol.

Sterol Uptake, Distribution, and Movement.

The amount of free sterol taken up from the medium was between 120 - 150 μg / mg of membrane protein in cells grown on cholesterol, cholestanol, and 4, 6-cholestadien-3β-ol (young cultures). The uptake decreased to 80 - 110 μg /mg membrane protein in cells grown on β-sitosterol, stigmasterol, and ergosterol (Table I). The distribution of the free sterol in

the two halves of the lipid bilayer of the membrane of native M. capricolum cells was probed with filipin (Table I). Approximately two-thirds of the sterol remained in the outer half of the bilayer where cells were grown on cholestanol and 4, 6-cholestadien-3β-ol, as was observed in the membranes of cholesterol-grown cells (Bittman and Rottem, 1976). With cells grown on β-sitosterol,stigmasterol, or ergosterol, about 90% of the sterol was localized in the outer half of the bilayer (mid-exponential-phase culture). Therefore, in these cells, sterol translocation from the outer half to the inner half is much slower than in cells grown on cholesterol. Thus, sterol accumulates in the outer half of the bilayer.

In order to examine the movement of sterols in growing cells, cells were adapted to grow on 1.25 μg / ml of β-sitosterol or stigmasterol as described above and then transferred to a sterol-rich medium (10 μg / ml) (Table II). Immediately after the transfer, 85-90% of the sterol was found in the outer half of the bilayer, and this localization did not change within 1 or 2 hr after the transfer to sterol-rich medium. After 4 and 6 hr of incubation in β-sitosterol-or stigmasterol-rich medium, the distribution of sterol was only slightly changed. With β-sitosterol-grown cells, the initial disturbance in the 100 to 150 msec following mixing seen with cholesterol was not detected.

When different ratios of β-sitosterol to cholesterol were present in the medium and taken up by the adapted strain, stopped-flow analysis of filipin binding to cells and membranes revealed a very fast and a very slow rate process, together with one or two processes of intermediate time constants. The rapid process may involve filipin - β - sitosterol interaction and the slower process may involve filipin - cholesterol association. Rate constants and transmittance changes for each step were not calculated because the final transmittance (T_∞) did not reach a stable value. A number of factors may be responsible for this continuous increase in T_∞. For example, filipin may progressively disrupt the intact membrane at times longer than 400 msec.

Permeability of Sterol-Grown Cells

The change from cholesterol to other sterols in the growth medium had a marked effect on the initial rate of erythritol permeation into the cells. The effect on permeability toward erythritol was measured (Table III). Modifications in the sterol side chain made the cells much more permeable compared with cholesterol-grown cells. Membranes from cholesterol-grown cells harvested at mid-exponential phase of growth showed a low fluidity, as indicated by the decreased freedom of motion of the spin-label 5-doxyl stearate (Rottem, personal communication).

The initial rate of filipin-sterol binding was much more rapid than with cholesterol (k_{cells} = 15 - 20 x 10^5 M^{-1} s^{-1} for M. capricolum cells containing stigmasterol or β-sitosterol compared with k_{cells} = 14.3 x 10^3 for those containing cholesterol).

β-Sitosterol-Cholesterol Exchange Studies.

Figure 3 suggests a lack of competition between β-sitosterol and cholesterol. When adapted M. capricolum cells (grown on only 1.25 μg / ml of ^{14}C - β-sitosterol) were transferred to a medium containing various ratios of ^{3}H-cholesterol to ^{14}C-β-sitosterol (up to 10 μg / ml of total free sterol), the amount of β-sitosterol remained almost constant at the initial level (20 μg / mg protein), and cholesterol was taken up preferentially into the cell membrane. The time course for transfer of labeled cholesterol is shown in Figure 3, A - D. The amount of cholesterol incorporated is higher and the movement of cholesterol more rapid when more cholesterol (7 μg / ml) and less β-sitosterol (3 μg / ml) (Figure 3B) were added to the medium than when more β-sitosterol (7 μg/ml) and less cholesterol (3 μg / ml) were added to the medium (Figure 3D). The rate of cholesterol transbilayer movement may, therefore, depend on cholesterol concentration gradient.

DISCUSSION

Data presented here show that structural changes in the cholesterol molecule have marked effects on the growth of mycoplasmas. No correlation was obtained between active maximum cell growth and the localization of sterols. Sterols that are found to be localized predominantly in the outer half of the M. capricolum membrane bilayer are poor supporters of growth (e.g., stigmasterol and ergosterol); however, the corrollary is not true, since some sterols that supported only poor growth were found to have the same distribution as did cholesterol (e.g., cholestanol and 4, 6 - cholestadien - 3β - ol). Therefore, the degree of unsaturation in the cyclopentanophenanthrene ring appears to play a minor role at least for the localization and movement of sterols, while the nature of the side chain is a crucial feature. The addition of methyl, ethyl, or hydroxyl groups, or of double bonds in the side chain lowered the growth rate and increased the extent of lysis and fragmentation.

We have previously speculated (Clejan et al., 1978) that growing M. capricolum possesses a mechanism catalyzing cholesterol incorporation into the inner half of the bialyer. The present data indicate that this mechanism, if it exists, is specific, at least in relation to the side chain. Steric factors appear to be of primary importance. But the differences in sterol uptake

capacity could be the result of many other factors, e.g., a large gradient of fluidity across the bilayer, or differences in membrane phospholipid content or organization.

The finding that the ratio of membrane lipid to protein decreases markedly on aging of mycoplasma cultures (Amar et al., 1974; Rottem and Greenberg, 1975) and increases with the addition of chloramphenicol (Razin, 1974) was used to show that the amount of cholesterol incorporated into the cell membrane of any specific mycoplasma depends on the phospholipid content of the membrane and is not influenced by variation in membrane protein content (Razin, 1974). Therefore, membranes with a high specific polar lipid content should be able to incorporate large quantities of exogenous sterol. However, the ratio of polar lipid to membrane protein is not significantly higher in the cholesterol-rich Mycoplasma species than in Acholeplasma species (Razin et al., 1974) and addition of exogenous phospholipids did not change the incorporation and distribution of sterols (Chapter V).

Specific interactions of cholesterol with membrane proteins (Klappauf and Schubert, 1977) and membrane enzymes (Warren et al., 1975) have been reported. Recently, the importance of the length of the cholesterol side chain in interaction with phosphatidylcholine has been studied (Suckling et al., 1979). Cholesterol was proposed to have the exact length required to

maximize interactions between neighboring molecules without disturbing the bilayer structure. The possibility that a specific protein is capable of catalyzing cholesterol movement in M. capricolum warrants further investigation.

TABLE I: Sterol Incorporation and Transbilayer Distribution in the Membrane of *M. capricolum* Cells[a]

Sterol in Growth Medium	Free Sterol (μg/mg membrane protein)	$k_{cells}/k_{membranes}$[b]
Cholesterol	153	0.66 ± 0.04(18)
β-Cholestanol	142	0.67 ± 0.02(2)
4,6-Cholestadiene-3β-ol	126	0.65 ± 0.03(3)
β-Sitosterol	109	0.82 ± 0.04(3)
Stigmasterol	91	0.84 ± 0.02(2)
Ergosterol	82	0.92 ± 0.04(2)

[a]Sterol was added to the medium (10 μg/ml) as ethanolic solutions. Cells were harvested at the mid-exponential phase of growth (A_{640} = 0.2).

[b]The initial rates for filipin-sterol association were measured at various sterol concentrations in intact cells and isolated membranes. The number of cell cultures investigated is indicated in parantheses in the last comumn. The results are expressed as average ratio of rate constants ± standard error of the mean.

TABLE II. Transbilayer Distributions of β-sitosterol and Stigmasterol in Adapted *M. capricolum* upon Transfer to a Sterol-Rich Medium[a]

Time of Incubation with 10 µg/ml Sterol (hours)	$k_{cells}/k_{membranes}$[b]	
	β-Sitosterol adapted cells	Stigmasterol adapted cells
0	0.84	0.89
1	0.82	
2	0.74	0.85
4	0.75	0.79
6	0.70	0.78

[a] *M. capricolum* was adapted to grow on low sterol concentration (1.25 µg/ml β-sitosterol or stigmasterol) and transferred at A_{640} = 0.1 to a sterol rich medium (10µg/ml β-sitosterol or stigmasterol) for the indicated period of time.

[b] Average ratio of the second-order rate constants obtained from two different cultures.

TABLE III. Effect of Different Sterols on the Initial Rates of Swelling of Adapted and Native M. capricolum Cells upon Erythritol Permeation[a]

	$d1/A/dt$ (sec^{-1})
Normal strain grown on Cholesterol	0.49 ± 0.09
" " " " β-Sitosterol	1.06 ± 0.12
" " " " Desmosterol	1.22 ± 0.10
" " " " Stigmasterol	1.25 ± 0.12
" " " " Ergosterol	1.17 ± 0.14
Adapted strain grown on Cholesterol	0.78 ± 0.08
" " " " β-Sitosterol	1.65 ± 0.14

[a] Cells were harvested at mid-exponential phase and suspended in 200 mM sucrose. Cell solutions were added to the isotonic solution (200 mM) of erythritol. 1/A at 450 nm was measured at 37°C.

For the normal strain, the concentrations of sterols were: cholesterol 174 μM, β-sitosterol 142 μM, desmosterol 166 μM, stigmasterol 159 μM, ergosterol 150 μM. For the adapted strain, the concentrations of sterols were: cholesterol 21 μM, β-sitosterol 14 μM.

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FIGURE CAPTIONS

Figure 1: Growth of young cells of M. capricolum with various sterols

●–● Cholesterol; ○–○ β-Sitosterol; □–□ Desmosterol; △–△ Ergosterol;
▲–▲ Stigmasterol; ■–■ Campesterol; ⬡–⬡ 25-hydroxycholesterol;
●–● β-norcholesterol; ◐–◐ β-cholestanol; ◧–◧ Lathosterol;
▲–▲ 4,6-cholestadien-3β-ol

Figure 2: Effect of the age of the culture on the growth of M. capricolum cells adapted to grow on cholesterol, β-sitosterol, or cholestanol.

●–● Cholesterol; ○–○ β-Sitosterol; ◐–◐ Cholestanol

Figure 3: Time course of increase in membrane sterol upon transfer of adapted M. capricolum cells to sterol-rich medium. ○(^{14}C)-β-sitosterol; ●(^{3}H)-cholesterol.

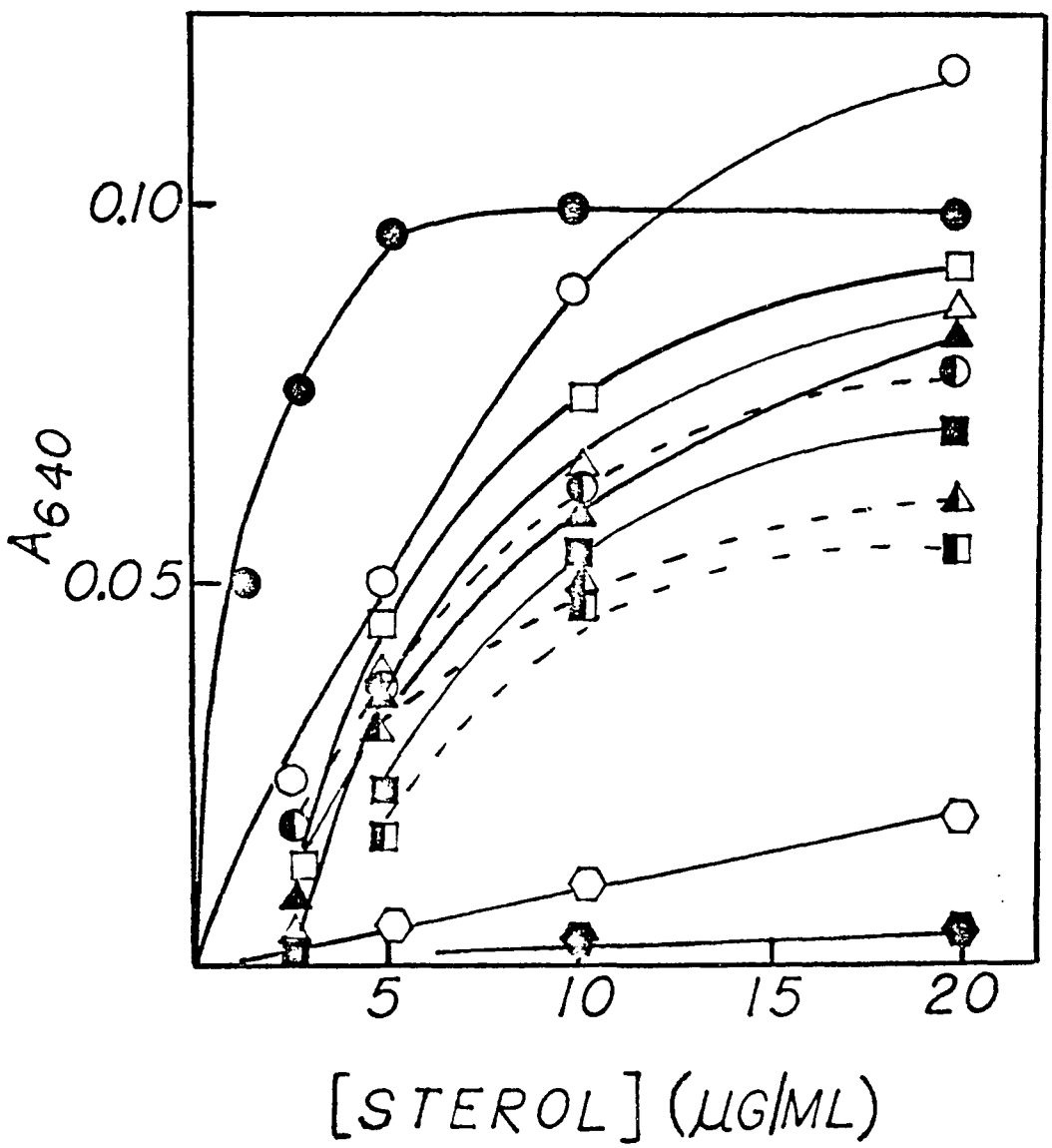
A. Transfer to 10 µg/ml cholesterol

B. 7 µg/ml cholesterol, 3 µg/ml sitosterol

C. 5 µg/ml cholesterol, 5 µg/ml sitosterol

D. 3 µg/ml cholesterol, 7 µg/ml sitosterol

FIG. 1



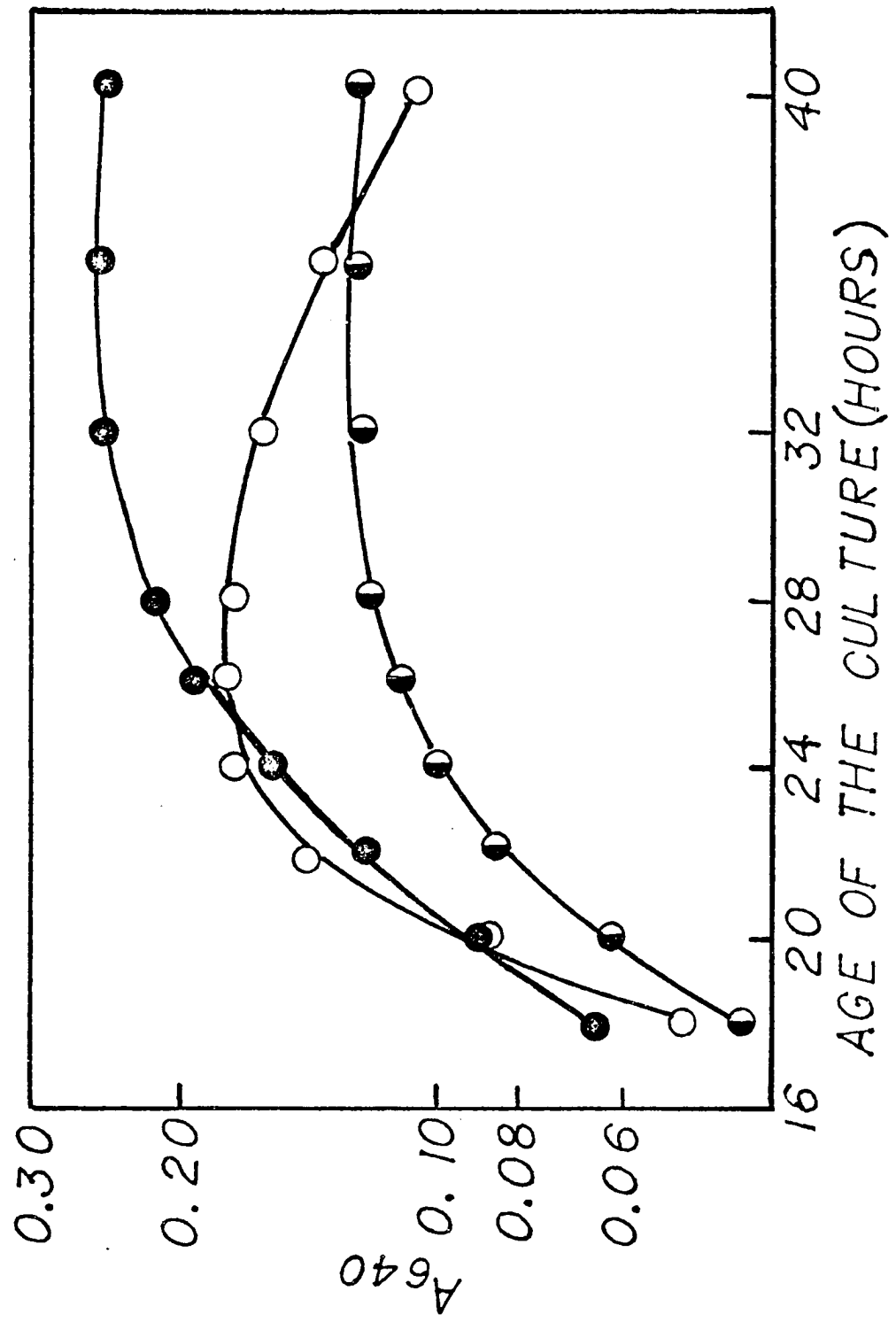


FIG. 2

FIG.3A

FIG. 3

FIG.3

FIG. 3