STUDIES ON THE MECHANISM OF ACTION OF FILIPIN AND ITS EFFECTS ON INSECTS

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ABSTRACT

STUDIES ON THE MECHANISM OF ACTION OF
FILIPIN AND
ITS EFFECTS ON INSECTS

By

Friedhelm Schroeder

A study on the mechanism of action of filipin and its effects on insects was undertaken. No conclusive evidence for the interaction of filipin with cholesterol existed at the time this research was initiated. Using a computer-centered spectrofluorimeter, it was demonstrated that the interaction of filipin with cholesterol and other 3-beta-OH sterols could be monitored fluorimetrically. These investigations employed the use of a new fluorescence parameter, partial quantum efficiency, which was directly related to the quantum efficiency of fluorescence and was independent of fluorophore concentration. The results demonstrated that filipin interacted stoichiometrically on a 1:1 basis with cholesterol. The interaction was pH independent from pH 4.5 to pH 8.0. As defined by fluorescence criteria, filipin interacted with sterols containing a free 3-beta-OH group, an intact cyclopentanophenanthrene ring, and a hydrophobic alkyl side chain at carbon number 17. These results correlated well with the biological effects

of filipin reported in the literature; only those sterols capable of preventing the biological activity of filipin were capable of reducing the corrected fluorescence and partial quantum efficiency of filipin. A second new discovery was that both sterol-binding and sterol-nonbinding forms of filipin can occur in aqueous solution. These forms were interconvertible and the conversion of the sterol-nonbinding to the sterol-binding form followed first order kinetics. This conversion could be monitored by the ratio of absorbance of filipin at 338 nm/305 nm. The sterol binding ability of filipin was independent of self-aggregation into large micelles detectable by light scattering. The data were consistent with a time and temperature dependent change, possibly conformational, of the sterol-nonbinding to the sterol-binding form of filipin.

The effects of filipin on insects appear to be dependent on the dietary filipin to cholesterol ratio at nonlethal doses of filipin. At dietary filipin to cholesterol ratios greater than 1:1, the following effects were noted: filipin acted as a larvicide and chemosterilant; cholesterol uptake was reduced by 98%; hemolymph ascorbate and cholesterol levels were lowered by 90%; nonesterified-cholesterol excretion was enhanced 8-fold; $^{32}P_i$ and [^{14}C]methyl labeled choline incorporation into larval phosphlipids was inhibited by 90%; and cholesterol-sulfate excretion (the normal excretory product of sterols in Manduca sexta L.) was reduced by 69%. At dietary filipin to cholesterol ratios of 1:2, these effects were largely prevented except that cholesterol uptake and $^{32}P_i$ incorporation into phospholipids was enhanced 50% or more. In contrast, hemolymph cholesterol levels

and cholesterol-sulfate excretion were lower than control values. These results are consistent with filipin causing an increased turnover of cholesterol in the insect. Filipin can act either as a hypo- or hypercholesterolemic agent in insects, depending on the dietary filipin to cholesterol ratio. However, the above data indicated that filipin had more complicated effects on insects than simply sequestering dietary sterols. The enhancement of cholesterol incorporation from the diet noted in Galleria mellonella L. and Manduca sexta L. could be due to indirect effects of filipin; filipin nearly doubled the concentration of hemolymph cholesterol-carrying lipoproteins in M.sexta, but the actual concentration of cholesterol per ml hemolymph was less than control values. If these lipoproteins are involved in the uptake of cholesterol from the diet and its transport to the tissues, then the enhanced cholesterol uptake could be due to the increased level of these proteins in the hemolymph. It is important to note that filipin acted solely in the gut. [^{14}C]labeled filipin was not absorbed into the larval tissues of M. sexta or G. mellonella nor was it metabolized to CO_2. The data are generally consistent with the hypothesis that filipin sequesters dietary cholesterol and affects at least one or more other intestinal processes in insects.

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STUDIES ON THE MECHANISM OF ACTION OF FILIPIN AND ITS EFFECTS ON INSECTS

BY

Friedhelm Schroeder

A DISSERTATION

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DEDICATION

This thesis is dedicated to my loving wife, Mary, and to my parents, Helmut and Irma Schroeder, whose courage and sacrifice brought our family out of war torn Europe. Their wisdom and determination led them to choose this land of freedom as a home and resting place. May those years of sacrifice be rewarded and flulfilled in their grateful son.

ACKNOWLEDGEMENTS

My sincere appreciation is expressed to Professor Loran L. Bieber for his guidance and continual support given during all phases of my graduate training. Thanks are also extended to Professor John F. Holland for his assistance in the evaluation of experiments and in providing the use of a computer-centered spectrofluorimeter used in my research.

Sincere appreciation is also expressed to the other members of my Ph. D. guidance committee: Dr. Charles C. Sweeley, Dr. William W. Wells, Dr. Steve D. Aust, and Dr. Matthew Zabik. I am deeply indebted to my wife, Mary, and to my brother, Dr. Hartmut R. Schroeder, for their continued moral support and helpful discussions. The encouragement of these individuals as well as my parents has allowed this venture to be undertaken.

I would like to thank the Department of Biochemistry and especially the National Science Foundation for financial support.

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ABBREVIATIONS

A	Absorbance
CO	Corrected fluorescence
PQ	Partial quantum efficiency
A_{338}/A_{305}	Ratio of absorbance at 338nm to absorbance at 305nm
A_{310}/A_{292}	Ratio of absorbance at 310nm to absorbance at 292nm
R_{90}	Tyndall light scattering measured at an angle of 90°
F:C	Filipin to cholesterol ratio (mole/mole)
DNA	Deoxyribonucleic acid
BSA	Bovine serum albumin
P_i	Inorganic phosphate
PPO	2,5-diphenyloxazole
POPOP	1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene
Tris	Tris(hydroxymethyl)aminomethane
DMF	Dimethylformamide
CMC	Critical micelle concentration
DPM	Disintegrations per minute
CPM	Counts per minute
Ci	Curie of radioactivity

Chapter I

INTRODUCTION

Organization of the Thesis

The body of this thesis has beenorganized in chapter form. A detailed Introduction comprises Chapter I. Chapter II includes the Experimental Procedures, while Chapter III contains the Results section presented as a reprint of a published article and a rough draft of another article. Each reprinted article includes an abstract, introduction, methods, results, and discussion section. Chapter IV is a comprehensive Discussion of the results detailed in Chapter III. A compilation of all the References is found in Chapter V. The remaining portion of this research appears at the end of the thesis as two reprints and one preprint, Appendices I, II and III, respectively.

Research Objectives and Rationale of Experimental Approach

In order to investigate the mode of action of polyene antibiotics, especially filipin, in insects, it was first necessary to demonstrate the ability of filipin to interact with cholesterol and other sterols. Early investigations presenting spectrophotometric evidence for filipin binding to cholesterol proved to be inconclusive because the data could also be interpreted by precipitation or micellization of filipin (Gottlieb, et al., 1961; Lampen, et al., 1960).

Filipin-Cholesterol Interaction: Since conclusive direct evidence for filipin-cholesterol interaction was unavailable at the time that this research was initiated, Schroeder, et al. (1971, 1972; see Appendices I and II) decided to investigate the possibility of using fluorescence

to monitor the interaction of filipin with cholesterol in aqueous solution. Using a computer-centered instrument system for the simultaneous measurement of absorbance, fluorescence, and partial quantum efficiency (Holland, et al., 1973a,b), it was shown that addition of cholesterol to an aqueous solution of filipin decreases the absorbance, the corrected fluorescence (CO), and the partial quantum efficiency (PQ) of filipin. The latter parameter, PQ, is directly proportional to the quantum efficiency of fluorescence of a fluorophore. The reduction in partial quantum efficiency is independent of concentration of filipin and is definitive evidence that the filipin complex interacts with sterols in aqueous solution. These results correlated well with the biological data of others (Gottlieb, et al., 1960,1961). Sterols that reduce the PQ of filipin also prevent the toxicity of filipin to fungi. These investigations also provided evidence for the existence of active (sterol binding) and inactive (sterol-nonbinding) forms of filipin (Schroeder, et al., 1972,1973; see Appendices II and III).

Sterol Requirement of Insects: In most insects, the sterol requirement is satisfied by cholesterol (Lipke and Fraenkel, 1956). Some insect species, such as tobacco hronworm, Manduca sexta (L.), can replace cholesterol with other dietary sterols such as beta-sitosterol, stigmasterol, campesterol, fucosterol, and brassicasterol (Robbins, et al., 1971) and can convert these sterols to cholesterol. These sterols all have a 3-beta-OH group and a long alkyl side chain at carbon-17. As demonstrated by Schroeder, et al., (1971,1972; see Appendices I and II), such sterols interact with filipin. The required sterols serve as structural components of the insect cells (Clark and Bloch, 1956b) and as precursors of essential steroid metabolites and hormones (Berkoff, 1969;

Robbins, et al., 1971). In addition to absorbing sterols from their diets, insects are able to excrete sterols as polar metabolites (Clayton, 1964; Gilbert, 1967; Ritter and Wientjens, 1967; Hutchins and Kaplanis, 1969). Sulfates of cholesterol, beta-sitosterol, and campesterol were isolated and identified from the meconium of the tobacco hornworm (Hutchins and Kaplanis, 1969).

Effect of Filipin on Insects: The initial investigations of Sweeley, O'Connor, and Bieber (1970) were prompted by the rationale that, since insects are unable to synthesize their own sterols (Clark and Bloch, 1959a) and require a dietary or exogenous source of sterol for normal growth and development (Hobson, 1935), polyenes could be used to kill insects. The polyene should bind dietary or membrane-bound sterols and prevent their utilization by the insect. Sweeley, et al. (1970) found that filipin and other polyenes had larvicidal and chemosterilant effects in some insects. Filipin also prevented the uptake of [^{14}C] cholesterol from the diet by wax moth, Galleria mellonella (L.), larvae. Since the mode and site of action of filipin in insects was not known, it could have effects on sterol metabolism, lipid metabolism, membrane permeability, and other essential processes in the insect. Since much circumstantial evidence had already accumulated in the literature on the probability of filipin interacting wtih sterols (Kinsky, 1967), the author decided to determine if the effects of filipin on insects noted by Sweeley, O'Connor and Bieber (1970) could be prevented or reversed by cholesterol added to the diet in molar excess. Subsequent experiments were designed to determine the effects of filipin on insect cholesterol levels, cholesterol incorporation into insect tissues, cholesterol transport in the hemolymph, cholesterol excretion, and lipid metabolism.

Choice of Insects: Rapidly growing insects such as housefly, wax moth, and tobacco hornworm were chosen for these investigations since they would be expected to have a greater daily cholesterol requirement for membranes than would slowly growing insects. Initially, small insects such as housefly were used, but limitations in their size precluded their convenient use in experiments requiring much dissection. Instead, the much larger insect, tobacco hornworm, was chosen for experiments in which individual tissues were needed such as hemolymph, intestine, fat body, malpighian tubules or body wall. The tobacco hornworm, Manduca sexta L., proved to be an ideal organism in these studies. For instance the larvae increased 7000-fold in mass (from a 1 mg. newly hatched larvae) when reared on a laboratory diet during a normal 15 day larval period (Robbins, et al., 1971); as much as 1 ml. of hemolymph could be obtained from each 5th instar larva; and M. sexta was essentially non-cannibalistic.

Literature Review

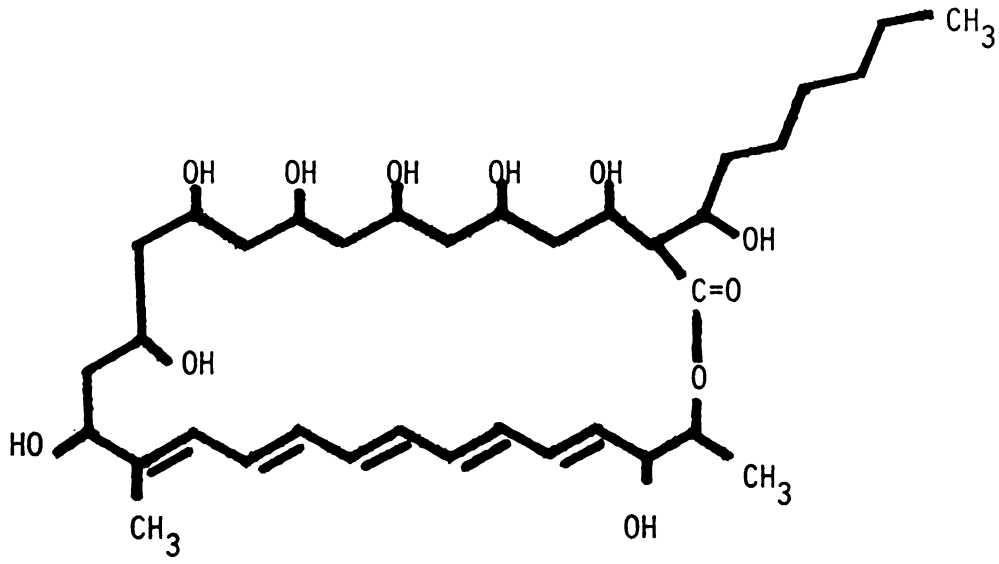
Importance of Filipin and Other Polyene

Over eighty polyenes have been discovered since 1950. Probably the greatest importance of polyene antibiotics has been their clinical usefulness in treating a number of systemic mycoses: histoplasmosis, coccidioidomycosis, blastomycosis, cryptococcal meningitis, and disseminated candidosis (Hamilton-Miller, 1973). Polyenes are also used as food preservatives, for selection of nutritional mutants of yeasts, and for suppression of microbial growth in tissue culture media (Hamilton-Miller, 1973). Recently, polyenes have been used to treat heart valves during homograft aortic valve insertion (Gonzalez-Lavin, et al., 1973). Another

exciting new development has been that polyenes potentiate antitumor agents (Kuwano, et al., 1972; Medoff, et al., 1973). Apparently the polyenes preferentially interact with the membranes of tumor cells rather than those of non-neoplastic cells.

Chemistry of Filipin

Filipin is a polyene, a member of the macrolide class of antibiotics. A macrocyclic lactone ring and five conjugated double bonds characterize filipin as a pentaene. The double bonds determine much of the antibiotic's biological activity (Kinsky, 1967; Hamilton-Miller, 1973). Filipin was originally isolated from Streptomyces filipinensis (L.), a Philippine soil organism, by Whitfield, et al., (1955). Until 1968 the antibiotic was considered to be a pure compound. However Bergy and Eble (1968) found that filipin was actually a complex of 8 or more similar components. Components I, II, III, IV comprise 96% of the complex and differ structurally by one or two hydroxyl groups (Pandey and Rinehart, 1970). The structure of the major component, filipin III (56% of the complex), is shown below (Pandey and Rinehart, 1970):



Filipin III

Two types of degradation of filipin have been discovered. When exposed to light or heat in the presence of oxygen, filipin loses it biological activity (Whitfield, et al., 1955; Tingstad and Garrett, 1960). The loss of biological activity was correlated with a reduction in the absorbance of the pentaene group at 335 nanometers. An oxidative degradation of filipin also occurs during prolonged standing in concentrated methanolic solution (Whitfield, et al., 1955; Rickards and Smith, 1970). During methanolic decomposition, filipin loses one double bond by expoxide formation. The epoxide is also biologically inactive and has reduced absorbance at 338 and 355 nanometers, two of the absorbance maxima of filipin's pentaene group. As shown by Schroeder, et al. (1972; see Appendix II), the epoxides of the filipin complex do not interact with cholesterol as determined by fluorescence criteria.

Interaction of Filipin with Sterols

The mechanism of polyene action suggested by previous studies is the binding to sterols, free or membrane-bound, by polyene macrolides. This evidence is, however, largely indirect (Kinsky, 1967; Hamilton-Miller, 1973). The first attempt to show complex formation between polyenes and cholesterol by spectrophotometric means indicated that cholesterol almost uniformly reduced the absorption of the polyene chromophores, with the possible exception of filipin (Lampen, et al., 1960). Since polyenes such as filipin are very insoluble in water (Kinsky, 1967), the reduction in filipins' absorbance observed with cholesterol could also be interpreted by cholesterol causing a lowered solubility or an aggregation of the antibiotic in solution. The findings that these antibiotics do not follow Beer's Law in aqueous solution (Norman, et al., 1972) and that

filipin forms large aggregates or micelles (Schroeder, et al., 1973; see Appendix III) are consistent with this interpretation. However, it was observed by Lampen, et al. (1960) and by Gottleib, et al. (1961) that cholesterol also altered the ratio of two absorbance maxima of filipin (absorbance at 338 nanometers/absorbance at 305 nanometers). This finding was not consistent with just lowered solubility of filipin in solution. In addition, methanol and other alcohols prevented this alteration in absorbance ratio and returned the value of the absorbance ratio of filipin-cholesterol solutions to values of filipin in the absence of cholesterol. Thus, at least qualitative spectrophotometric evidence gave evidence that filipin and cholesterol might interact in aqueous solution. Norman, et al. (1972) later utilized the findings of these early investigators to determine the interaction of a variety of free and membrane-bound sterols with filipin. But, the reduction of absorbance maxima of filipin by sterols did not correlate well with the biological results of others (Gottlieb, et al., 1960,1961) and did not take into account the insolubility problems of the polyene. Norman, et al. (1972) also found that the absorbance ratio of filipin and its alterations by cholesterol were dependent on a number of other parameters in addition to the presence of cholesterol: the method of adding filipin to the solution, the time of mixing, the age of the filipin stock solution, and the temperature. Small differences in experimental conditions also affect the absorbance ratio (Schroeder, et al., 1972; see Appendix II). Using the absorbance ratio, these workers also determined that 2 or 3 cholesterol molecules interact with each filipin molecule. Interpretation of these data did not provide conclusive evidence that filipin did interact with cholesterol.

However, changes in specific fluorescent properties, partial quantum efficiency and corrected fluorescence, can be used to monitor the interaction of filipin and other polyenes with sterols (Schroeder, et al., 1971, 1972; see Appendices I and II). The parameter PQ, partial quantum efficiency, is particularly useful since it does not vary with the concentration of the fluorophore. Cholesterol decreases the PQ and CO, corrected fluorescence, of filipin complex 36% and 62%, respectively. Thus, "incontrovertible direct evidence for binding between sterols and filipin by use of a fluorimetric technique involving the measurement of partial quantum efficiencies" has been provided (Hamilton-Miller, 1973). The filipin complex binds sterols that contain both a 3-beta-OH group and a long alkyl side chain that is attached to the D-ring of sterols (Schroeder, et al., 1972; see Appendix II; Norman, et al., 1972). Fluorescence properties indicate that the filipin complex interacts weakly or not at all with cholesterol-palmitate and 3-keto or 3-alpha-OH sterols. The specificity of the antibiotic for sterol is important when the action of filipin on membranes is considered. The 3-beta-OH group of sterols is essential for limiting the permeability properties and membrane phase transitions of liposomes, erythrocytes, and Mycoplasma laidlawii B cells (Bruckdorfer, et al., 1968a,b,1969; De Kruyff, et al., 1972; Demel, et al., 1972). For instance, membrane incorporated 3-beta-OH-cholesterol reduces the permeability of erythrocytes and of mycoplasma for a number of solutes: glucose, glycerol, and $^{86}Rb^{+}$. The 3-alpha-OH isomer of cholesterol does not have this effect. If the 3-beta-OH-cholesterol is removed from the erythrocyte membrane by exchange with plasma 3-alpha-OH-cholesterol, the permeability of the cell greatly increases. Therefore, it seems logical that at least some membrane properties are determined by the structure

of the sterol. LUzzatti and Husson (1962) pointed out that phase transitions of a membrane, such as from a lamellar to a micellar configuration, would produce marked changes in selective permeability. Filipin and other polyenes alter the phase transitions of phospholipid-cholesterol mixtures as determined by differential scanning calorimetry (Norman, et al., 1972a,b).

The stoichiometry of the filipin-cholesterol interaction, determined by fluorometric means, is, within experimental error, 1:1. The interaction in aqueous solution is independent of pH over the range 4.5 to 8.0. Above or below this pH range, the lactone ring is hydrolyzed and the antibiotic cannot interact with cholesterol. Schroeder, et al. (1972; see Appendix II) found that the interaction of filipin with cholesterol is dependent on the method of isolation and preparation of the filipin complex. The filipin complex isolated by conventional procedures using organic solvents does not interact with sterols, but will interact with sterols after prolonged standing in aqueous solution or heating at 50° C for 2 hours. These studies, as well as data using Tyndall light-scattering, strongly indicate that filipins can exist in aggregates that do not bind sterols (Schroeder, et al., 1973; see Appendix III). Such inactive aggregates slowly undergo disruption in solution, as indicated by decreased light-scattering, to a form or forms that initially do not bind sterols. After a slow, apparently conformational, change this form of filipin becomes active and will interact with cholesterol and other 3-beta-OH sterols. The active, sterol-binding, form of filipin can also aggregate, but retains its ability to immediately interact with cholesterol. The absorbance peak ratio of filipin (A at 338nm/A at 305nm) can be used to monitor the formation of the active, sterol-binding, form of this polyene. In addition, work done simultaneously

by Norman, et al. (1972a) shows that interaction of filipin with cholesterol appears to be specific for certain sterols since BSA, sucrose, galactose, egg lecithin, low concentrations of urea, sodium chloride, and cetyl alcohol had no effect on the absorbance properties of the filipin-cholesterol complex. The results obtained by Schroeder, Holland, and Bieber (1972; see Appendix II) and by Norman, et al. (1972a) demonstrated a stoichiometric interaction of filipin with cholesterol in aqueous solution. Recently the above method for determining the presence of active or inactive forms of filipin has apparently been adopted by others (Bittmann and Fischkoff, 1972). Since the discovery of using fluorescence parameters to monitor the interaction of filipin with sterols (Schroeder, et al., 1971; see Appendix I), numerous other investigators have used fluorescence parameters to monitor the interaction of polyenes with free sterols (Schroeder, et al., 1972; see Appendix II; Bittmann and Fischkoff, 1972) and with membrane bound sterol of liposomes (Bittmann and Fischkoff, 1972), erythrocyte membranes (Strom, et al., 1972), and sarcoplasmic reticulum (Drabikowski, et al., 1973).

Effects of Filipin on Model Membrane Systems

Polyene antibiotics produce a number of effects related to their ability to interact with sterols. Filipin can alter the structure and permeability of lipid monolayers (Demel, et al., 1965,1968; Demel, 1968), lipid bilayers (Van Zutphen, et al., 1966, 1971), and phospholipid spherules or liposomes (Kinsky, 1967; Sessa and Weissman, 1967,1968; Weissmann and Sessa, 1967; Kleinschmidt, et al., 1972). Freeze-etch microscopy demonstrated that filipin induced the formation of aggregates in the membranes of egg lecithin-cholesterol liposomes (Verkleij, et al., 1973). Negative staining of such spherules showed that filipin caused the formation of "pits" in the membranes (Kinsky, et al., 1967). Presumably these

aggregates or pits are due to filipin interacting with sterol in the liposome membrane. Similar effects were found with erythrocyte membranes by these investigators.

Lytic Action of Filipin on Microorganisms and Cells

Filipin can cause lethal permeability alterations in microorganisms that have sterol containing membranes (Amman, et al., 1955; Gottlieb, et al., 1960,1961; Weber and Kinsky, 1965; Zygmunt, 1966; Zygmunt and Tavormina, 1966; Defago, et al., 1969; Gallo and Zygmunt, 1970). This antibiotic also lyses erythrocytes (Kinsky, 1967) and ruptures beef spermatozoa (Morton and Lardy, 1967). Other effects, such as stimulation of glucose utilization in mammalian fat cells (Kuo, 1968), inhibition of photochemical activity in chloroplasts (Bishop, 1973), and decreasing DNA synthesis and lowering cellular respiration in Ehrlich ascites and Novikoff hepatoma tumor cells (Mondovi, et al., 1971) have been regarded as secondary since the metabolic activities of the cell free systems are unaffected by polyenes. Polyenes bind to the cellular membranes of sensitive organisms and cells (Hamilton-Miller, 1973). Filipin has even been used to precipitate and isolate the membrane of an amoeba (Riedel and Gerisch, 1968).

Activity of Filipin in Experimental Animals and Animal Tissues

Some polyenes, especially filipin, increase phospholipid turnover in beef thyroid slices (Larsen and Wolff, 1967), mimic vitamin D-mediated calcium transport in chicks (Adams, et al., 1970; Wong, et al., 1970), disrupt intestinal microvillar membranes of chick ileum (Adams, et al., 1970), decrease serum cholesterol levels in dogs and chicks (Schaffner

and Gordon, 1968; Fisher, et al., 1969), and reduce canine prostatic hyperplasia (an effect that requires mobilization of cholesterol deposits) (Gordon and Schaffner, 1968). Gordon and Schaffner (1968) assumed that prevention of cholesterol uptake was due to sequestration of free cholesterol by filipin molecules in the gut. These investigaitons indicated that filipin does not cross the digestive tract of dogs when administered orally; less than 1% of filipin fed to dogs appeared in the blood stream. Similar results were obtained with other polyenes in human subjects. Little, if any, nystatin or amphotericin B is absorbed fromthe gut of human patients (Hamilton-Miller, 1973; Drouhet, 1962,1968).

Toxicity of Filipin and Other Polyenes to Insects

Filipin had larvicidal activity when added to the diet of housefly, Musca domestica L., wax moth, Galleria mellonella L., and cockroach, Nauphoeta cinerea L. (Sweeley, et al., 1970). This polyene also had chemosterilant effects on adult housefly, but did not affect the viability of housefly eggs. Gemrich (1972) confirmed the larvicidal and chemoserilant activity of filipin on housefly. Filipin also prevented the uptake of [4-^{14}C] cholesterol into hemolymph of wax moth larvae. In contrast to filipin and amphotericin, flavofungin possessed larvicidal activity towards M. domestica, but did not reduce the absorption of [4-^{14}C] cholesterol into the hemolymph of wax moth larvae. Flavofungin actually enhanced cholesterol uptake by 18%. This may be similar to enhancement of cholesterol uptake by filipin at filipin to cholesterol ratios in the diet less than one.

Chapter II

EXPERIMENTAL PROCEDURES

General Methodology

The results of this thesis have been compiled in manuscript form. Each manuscript or reprint of published work contains a detailed Materials and Methods section. The reader is referred to these sections for the experimental procedures. In addition, the following article is included in this Chapter since it deals with the methodology used in rearing and maintaining a colony of *Manduca sexta* L.

Prolongation of the Life Stages of the Tobacco Hornworm, *Manduca sexta* L., for Experimental Uses

by

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The tobacco hornworm, Manduca sexta (L.), is very useful for studying the biochemistry and physiology of insects. However, a major difficulty encountered in laboratory investigations using hornworm larvae is the availability of eggs, larvae, and pupae over an extended time period without mass rearing. At 28°C, eggs normally hatch 3 (± 1) days after oviposition, while pupae are obtained 18 (± 2) days later (Yamamoto, 1969). M. sexta do not remain viable when refrigerated at 4°C. Herein we report rearing conditions that will increase the availability of M. sexta eggs, larvae, and pupae from 7 to 50 days per cycle.

MATERIALS AND METHODS.-The hornworms were reared on diets as reported by Yamamoto (1968, 1969) and as modified by Bell (1972) with minor alterations. Four ml of raw soy oil, instead of linseed oil, were added per 1000 g larval diet. In this laboratory, larvae were reared in clear plastic shoe boxes (30 x 16 x 8 cm) with green lids. Five 0.5 cm holes were drilled in each lid. A sheet of paper toweling was placed in the bottom of each box to absorb condensed water. Ten larvae with diet were placed on wire mesh (1.2 x 1.2 cm) raised 2 cm above the bottom of each box. Diet and toweling were replaced every 2 days. Prepupae were maintained in water-saturated redwood blocks (Bell, 1972). Prepupae were placed in holes drilled in the redwood blocks (2 cm diam., 15 cm deep). The holes were sealed with corks (2 cm diameter) and the pupae were removed after 7 days. Adult M. sexta were reared in a Sherer Mobile Greenhouse, Model No. MG 8, Sherer-Gillet Co., Marshall, Mich. Eggs were collected daily for 10 days from 50 adults/Sherer Model Greenhouse. Oviposition sites were tobacco plants and tomato plants. The rearing or storage temperature was varied from 15 to 36°C, as described in each experiment.

Viability is defined as follows for each stage of metamorphosis: egg to larvae formation; larvae to prepupal formation; prepupae to pupal

formation; pupae to adult emergence; adult ovipositon. The degree of viability is measured as high (+++), medium (++), low (+), trace (±), and non (-).

RESULTS AND DISCUSSION.-Lowering the rearing temperature increased the duration of metamorphosis of the tobacco hornworm (Table 1). TKe photoperiod used, 16 h, produces nondiapause pupae (Yamamoto, 1969). With these conditions, lowering the storage temperature from 28 to 15°C extended teh development time of eggs from 4 to 14 days (3.5-fold increase) and of pupae from 18 to 36 days (2.0-fold increase). Decreasing the rearing temperature from 28 to 24°C increases the duration of the larval, prepupal, and pupal stages by 33, 100, and 33%, respectively, but decreases adult survival by 1 to 2 days.

Table 2 shows the effect of storage at 15°C on viability of diapausing pupae. Pupae were stored by placing them on dry toweling and refrigerating at 15°C. Viability remained high up to 9 months at 15°C. Diapause was broken by increasing the temperature in small increments consecutively as follows: from 15 to 24°C for 2 days, 24 to 28°C for 3 days, 28 to 32°C for days, and 28°C until emergence.

Storage of eggs, larvae, prepupae, pupae, and adults from either diapause or nondiapause conditions at 15°C for times shorter than indicated in Tables 1 and 2 increased the viability for each developmental stage (data not shown).

The data show that lowering the rearing temperature increased the duration of each developmental stage of M. sexta except the adult. This increased the viability of eggs and pupae without mass rearing or elaborate rearing facilities; therefore, the usefulness of this insect as a convenient laboratory animal can be extended.

TABLE 1.-Effect of Temperature on the Development of Nondiapausing (16 h Photoperiod) Tobacco Hornworms

Stage of development[a]	Maximum storage time tested (days)	Temperature (°C)	Viability (28°C)[b]
Egg (4)	4	28	+++
	14	15	++
	> 14	15	--
Larvae (18)	18	28	+++
	24	24	++
	2	36	--
Prepupae (8)	7	28	+++
	14	24	++
	2	36	--
Pupae (18)	18	28	+++
	24	24	++
	30	15	+
	> 30	15	±
Adult (10)	10	28	+++
	10	24	+
	2	36	--

[a]The numbers in parentheses are controls and indicate the normal development times (days) using standard rearing conditions.

[b]Viability is measured by transferring M. sexta to a 28°C chamber and standard rearing conditions.

TABLE 2.-Effect of Temperature on the Development of Diapausing (12 h Photoperiod) Tabacco Hornworms

Stage of development[a]	Maximum storage time tested (days)	Temperature (°C)	Viability (28°C)[b]
Egg (4)	4	28	+++
Larvae (20)	20	28	++
Prepupae (7)	7	28	++
Pupae (270)	120	15	+++
	270	15	++
Adult (10)	10	28	++

[a]The numbers in parentheses are controls and indicate the normal development times (days) using standard rearing conditions.

[b]Viability is measured as in Table 1.

Chapter III

RESULTS

The Results are presented in the form of a reprint and a preliminary draft of a manuscript to be submitted for publication. The initial investigations on the prevention by cholesterol of the effects of filipin in insects are detailed in the reprint entitled "Effects of Filipin and Cholesterol on Housefly, Musca domestica L., and Wax Moth, Galleria mellonella, L.". Cholesterol prevented both the larvicidal and chemosterilant effects of filipin on housefly and prevented the inhibition of [4-^{14}C] cholesterol uptake in G. mellonella. However, other experiments indicate that filipin has more complex modes of action than simply sequestering cholesterol. The data from this investigation provided the basis for the experiments detailed in the manuscript entitled "Studies on Hypo- and Hypercholesterolemia in Insects Induced by Filipin".

Effects of Filipin and Cholesterol on Housefly, *Musca domestica* L., and Wax Moth, *Galleria mellonella* L.

by

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EFFECTS OF FILIPIN AND CHOLESTEROL ON HOUSEFLY, *MUSCA DOMESTICA* L., AND WAX MOTH, *GALLERIA MELLONELLA* L. *

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SUMMARY

The effects of filipin on insects are dependent on the molar ratio of cholesterol to filipin. The larvicidal effects of the polyene antibiotic, filipin, can be prevented by excess cholesterol ("excess" herein is defined as a molar ratio of cholesterol to filipin of greater than 2 : 1) in housefly, *Musca domestica* L., and wax moth, *Galleria mellonella* L., larvae. Excess cholesterol also prevents the chemosterilant effect of filipin in housefly adults. The filipin-induced inhibition of [^{14}C]cholesterol uptake by wax moth larvae is prevented by excess cholesterol; cholesterol uptake is increased severalfold. Dietary filipin, in the absence of added cholesterol, caused loss of ^{32}P from housefly tissues and decreased the incorporation of ^{32}P- and [^{14}C]methyl-labeled choline into phospholipids of wax moth tissues. Addition of excess cholesterol to filipin-containing diets enhanced incorporation of ^{32}P into the different classes of phospholipids, and phospholipid synthesis was nearly doubled.

The filipin complex***, a mixture of polyene macrolides, is a larvicide and chemosterilant for the housefly, *Musca domestica* L. The complex also inhibits uptake of ^{14}C-labeled cholesterol into the hemolymph of wax moth, *Galleria mellonella* L., larvae[1]. The larvicidal and chemosterilant effects were attributed to the interaction of the polyene with sterols because polyene antibiotics, including the filipin complex, have antifungal activity which is apparently due to interaction with membrane-bound sterols[2-5]. These antibiotics have also been used as probes for studying properties and functions of sterols in natural and artificial membranes[6-15]. Herein we show that cholesterol can prevent the larvicidal and chemosterilant effects of filipins on the

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** NSF Predoctoral Fellow.
*** Unless otherwise indicated, filipin herein refers to the filipin complex.

housefly. Filipin in the presence of excess cholesterol enhances cholesterol uptake and phospholipid synthesis in housefly and wax moth larvae.

METHODS

Rearing of insects

Musca domestica larvae were reared on 1-g casein-containing diets as described by BIEBER *et al.*[16] with some modifications. No cholesterol was added to the basic diet except as stated in the legends of the experiments; sterility was not maintained.

Adult housefly and wax moth larvae were reared as described by SWEELEY *et al.*[1]. Wax moth larvae (average weight, 110 mg/larvae) were reared for 2 days at 37° on 1.8-g diets. 10 wax moth larvae were placed on each diet. The diets were prepared by combining the filipin complex, dissolved in methanol, with cholesterol dissolved in chloroform and vitamins dissolved in water. Solid medium was then added and thoroughly mixed with a magnetic stirrer for 10 min to ensure uniform distribution of antibiotic. Subsequently, solvents were removed with a rotary evaporator. Residual methanol, which is toxic to the larvae, was removed by placing each diet on a lyophilizer for 3 h.

Analysis of insect tissue

Wax moth larvae hemolymph was collected as described previously[1] with the following modifications. The chloroform–methanol extracts were washed with 0.2–0.4 vol. of distilled water, and the organic and aqueous fractions were transferred to separate tared glass scintillation vials, evaporated, weighed and assayed radiometrically. Less than 1% of the total ^{14}C remained in hemolymph protein after chloroform–methanol extraction of the hemolymph. The remaining insect tissue was homogenized with 10 vols. of chloroform–methanol (1 : 1) for 5 min in a Waring blender. The homogenate was then centrifuged at 3000 rev./min on an IEC Model-HN trunnion-head centrifuge for 20 min. The residue was reextracted with 10 vols. of chloroform–methanol (2 : 1) and again centrifuged. Combined supernatant fluids were washed with 0.2 vol. of an 0.8% NaCl–0.1% $CaCl_2$ solution and centrifuged. The aqueous as well as organic layers were retained for drying, weighing, isotope counting and phosphate analysis. Phosphate was determined by the method of BARTLETT[17].

Assays

The 3β-OH sterol content of the diets was determined as previously described[18].

In the ^{32}P loading studies, care was taken to ensure removal of external ^{32}P during transfers. The larvae and diet were placed on a cloth screen with a dark background and then placed under a light source. The larvae crawled through the screen to avoid the light, thus separating them from the diet. Rinsing with warm water removed any remaining solid material clinging to the larvae.

Housefly eggs were collected on moist pads placed over alfalfa in plastic containers. The eggs were quantitated (ml) after centrifugation in water for 4 min at

2000 rev./min on an IEC Model-HN trunnion-head centrifuge. Identical aliquots of eggs were dispensed onto the diets by packing the eggs wet into a glass-wool-plugged serological pipette and dispensing as separate aliquots (each 0.1-ml aliquot is approximately equivalent to 50 mg wet eggs).

Percent survival and body length of larvae were used as parameters for larvicidal effects. In the latter, the housefly larvae were immobilized by placing them on a black metal plate lying on crushed ice. After placing a ruler next to the larvae, a photograph was taken with a Polaroid Land camera. The lengths of the larvae were measured from the photograph and averaged for larvae in each sample.

All ^{14}C counts were determined in a toluene scintillator (4 g PPO, 100 mg POPOP, in 1 l toluene), except for protein-bound ^{14}C which was counted in a hyamine hydroxide + toluene scintillator[19]. ^{32}P was determined by measuring Cerenkov radiation[20] in polyethylene vials; otherwise glass vials were used throughout. ^{14}C on thin-layer chromatographic plates was counted by suspending the silica gel in Cabosil + toluene scintillator. A Packard 3310 liquid-scintillation spectrometer and a Beckman CPM-100 spectrophotometer were used for scintillation counting. ^{32}P incorporation into the phospholipid classes was quantitated as described by HILDENBRANDT *et al.*[21].

MATERIALS

Radioisotopes, [4-^{14}C]cholesterol and [^{14}C]methyl-labeled choline were purchased from New England Nuclear. ^{32}P was in the form of the inorganic isotope. Filipin was kindly provided by the Upjohn Company, Kalamazoo, Mich. Precoated thin-layer-chromatographic, silica gel F-254 plates (Brinkman Instruments, Westbury, N.Y.) were used in thin-layer analysis of lipids. A molecular weight of 650.14 was used in calculating filipin concentration on a molar basis[22]. This molecular weight is based on the percent composition of the filipin complex and the molecular weight of the individual components.

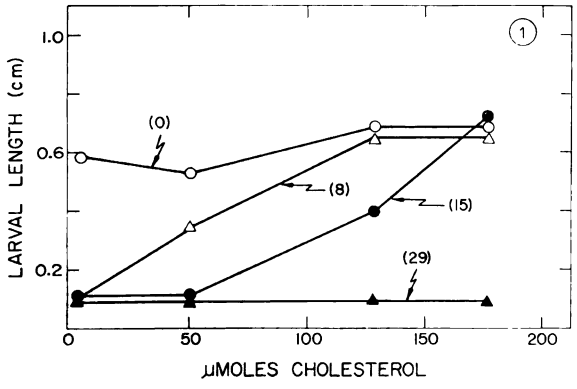

Fig. 1. Reversal by cholesterol of filipin-induced larvicidal effects on housefly larvae. 0.1 ml of *Musca domestica* eggs was placed on diets containing 1 g solids and 12 ml water per flask at 37° for 3 days. Larval size was determined as described in METHODS. Larvae less than 0.15 cm long were dead. The numbers in parentheses indicate the μmoles of filipin complex added to the diet. O——O, no filipin; △——△, 8 μmoles filipin; ●——●, 15 μmoles filipin; ▲——▲, 29 μmoles filipin.

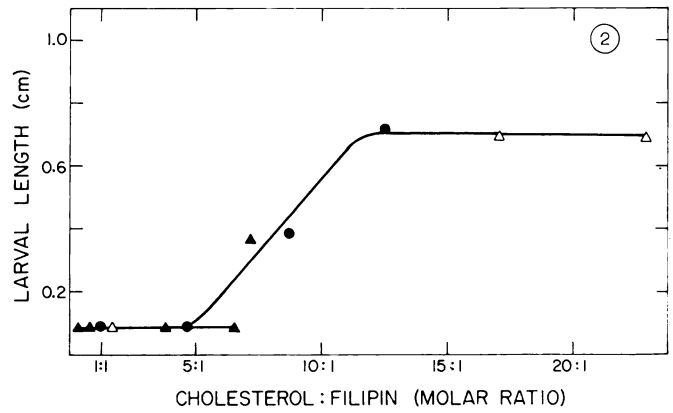

Fig. 2. Effect of the dietary cholesterol–filipin ratio on housefly larval growth. *Musca domestica* larvae were reared as described in the legend of Fig. 1. Control larvae averaged 0.6–0.7 cm body length. The molar ratio of cholesterol to filipin is based on a molecular weight of 650.14 for filipin. △, 8 μmoles filipin; ●, 15 μmoles filipin; ▲, 29 μmoles filipin.

RESULTS

Prevention of the larvicidal effect of filipin in Musca domestica by cholesterol

Cholesterol can reverse the larvicidal activity of dietary filipin as shown in Fig. 1. Cholesterol prevents the inhibition of larval growth at low concentrations of filipin (< 15 μmoles/g diet), but at higher filipin concentrations (29 μmoles/g diet), cholesterol does not overcome the toxicity. In Figs. 1 and 2, larvae less than 0.15 cm long were dead. Fig. 2 shows that larvicidal effects are overcome at a molar cholesterol–filipin ratio between 1 : 1 and 4 : 1; however, the growth is severely retarded until the cholesterol–filipin ratio is greater than 5. Thus, at low filipin concentrations but not at high filipin concentrations inhibition of larval growth is prevented, but the ratio of cholesterol to filipin must be greater than 1 : 1 before cholesterol overcomes filipin's larvicidal activity.

Inhibition by cholesterol of filipin-induced chemosterilancy in M. domestica

Dietary cholesterol can prevent the chemosterilant activity of filipin in houseflies as shown in Table I. Total egg production in filipin-treated flies was reduced to less than 1% that of the controls (diets 1 and 3). Addition of 50 mg cholesterol per g diet increased the egg production 140-fold and restored egg production to that of the controls.

Effect of filipin on loss of ^{32}P from housefly larvae

Filipin causes yeast membranes to become leaky[23]. Therefore, experiments were designed to determine if similar effects occur in housefly larvae. Housefly larvae were reared on a diet containing $^{32}P_i$ for 1.5 days, then transferred to filipin-containing diets (1 ± 0.05 g larvae/diet) without $^{32}P_i$. The ^{32}P content of the larvae and of the diets was determined. Fig. 3A shows that more ^{32}P was lost from larvae that were transferred to filipin-containing diets than from larvae that were transferred to control diets. When larvae containing ^{32}P were transferred to diets that were free of

TABLE I

CHOLESTEROL INHIBITION OF CHEMOSTERILANCY CAUSED BY FILIPIN IN HOUSEFLY

500 *Musca domestica* pupae were placed in each of 5 cages at 32°. The emerged houseflies were reared on the 10-g diets, replaced after 11 and 16 days, for 3 weeks. Eggs were collected every 3–4 days for 2 weeks, beginning 6 days post emergence of the adult houseflies.

Diet No.	*Diet*	*Filipin*	*Cholesterol*	*Total egg production*
1	5 g sucrose + 5 g powdered milk	0	0	11.0 ml
2	5 g sucrose + 5 g powdered milk	0	50 mg/g diet	4.4 ± 0.7 ml
3	5 g sucrose + 5 g powdered milk	3.3 mg/g diet	0	0.03 ml
4	5 g sucrose + 5 g powdered milk	3.3 mg/g diet	50 mg/g diet	4.4 ml

added cholesterol, ^{32}P loss was greater than that shown in Fig. 3A. Although filipin caused loss of ^{32}P to the diet, the total phospholipid content of the tissues increased (see Fig. 3B). The filipin-induced increase in tissue phospholipid occurred only when excess cholesterol was added to the diet. When cholesterol was not added to the diet, the phospholipid content of the larvae was reduced by as much as 50%.

Cholesterol reversal of the inhibition of [^{14}C]cholesterol uptake by filipin in G. mellonella hemolymph and tissue

Nonlethal amounts of dietary filipin retard the growth of wax moth larvae. When wax moth larvae were reared on filipin-containing diets in which cholesterol was added in increasing concentration, growth was nearly normal. Cholesterol increased weight gains for control larvae as well as overcoming weight loss due to filipin until the weight gain was 70% of the control value. The inhibition by filipin of [^{14}C]cholesterol uptake into the hemolymph of wax moth larvae is prevented by added cholesterol, as shown in Fig. 4A. At the two filipin concentrations shown, the absolute amount of [^{14}C]cholesterol uptake into hemolymph is increased by increasing dietary cholesterol until the levels are much higher than controls. Similar results were obtained when the incorporation of cholesterol into wax moth tissue was determined, as shown in Fig. 4B. When carrier cholesterol was omitted from the diet, more than 80% of the total ^{14}C was cholesterol, as determined by the criteria described previously[1]. When both cholesterol and filipin were added to the diet, increasing the filipin concentration caused less ^{14}C to appear in the cholesterol esters of hemolymph; at 8 μmoles filipin, 95% of the total ^{14}C (from [^{14}C]cholesterol) in the hemolymph was found in nonesterified cholesterol. Increased esterification of [^{14}C]cholesterol did not occur in tissue lipids since 95% of the [^{14}C]cholesterol was nonesterified. It may be significant that some (as much as 4% in wax moth larvae reared on diets containing cholesterol–filipin ratios greater than 1) of the ^{14}C of the dietary cholesterol does not occur in hemolymph as cholesterol or cholesterol esters.

Effect of filipin and filipin plus cholesterol on the uptake of ^{32}P- and [^{14}C]methyl-labeled choline in wax moth larvae

Dietary filipin decreases the amount of [^{14}C]methyl-labeled choline and ^{32}P incorporated into the hemolymph of wax moth larvae by more than 90% (see Fig. 5A).

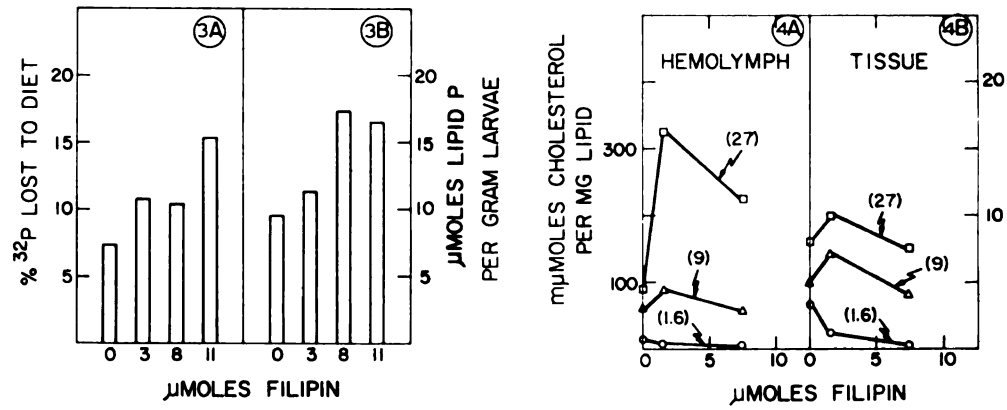

Fig. 3. The effect of filipin on loss of ^{32}P from *Musca domestica* larvae. Housefly larvae were reared at 32° for 1.5 days on diets containing 1 g solids, 12 ml water, 52 μmoles cholesterol and 1.6 μCi $^{32}P_i$. The larvae were then transferred to filipin-containing diets without $^{32}P_i$ and reared for 1 day at 32° as described in METHODS. The ^{32}P content of the larvae and of the second diet was determined as the sum of d.p.m. ^{32}P appearing in the chloroform–methanol extract, in the aqueous extract and in a 5 *N* KOH digest (12 h at 100°) of remaining solids from larvae or diet. A represents % ^{32}P lost to the diet, and B indicates the phospholipid content of the larvae.

Fig. 4. Effect of filipin and cholesterol on cholesterol uptake in *Galleria mellonella* larvae. Wax moth larvae were reared as described in METHODS. The values in parentheses indicate the μmoles of cholesterol in the diet. O——O, 1.6 μmoles cholesterol; △——△, 9 μmoles cholesterol; and □——□, 27 μmoles cholesterol. Each point on the graph represents the average value for 9–10 larvae. Cholesterol uptake was determined from d.p.m. ^{14}C appearing in the hemolymph (A) and in the organic layer of the chloroform–methanol extract of the remaining tissues (B) and from the specific activity of [^{14}C]cholesterol in the diet (0.28 μCi per/g diet).

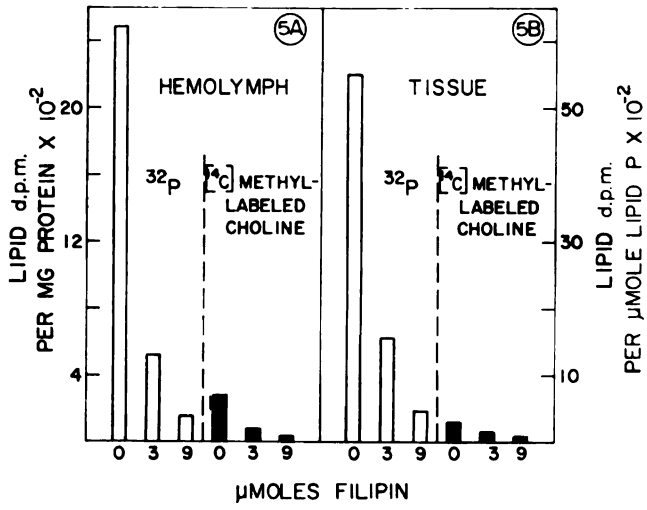

Fig. 5. Effect of filipin on ^{32}P- and [^{14}C]methyl-labeled choline incorporation into *Galleria mellonella* larval hemolymph and tissue. Wax moth larvae were reared as described in METHODS. $^{32}P_i$ (5 μCi per g diet) or [^{14}C]methyl-labeled choline (0.28 μCi per g diet) was added to the diet. The total cholesterol content of each diet was 1.6 μmoles. Lipid d.p.m. refers to radioactivity appearing in the chloroform layer of the chloroform–methanol extract, and protein refers to hemolymph protein as described in METHODS. Each point represents the average value for 9–10 larvae. A, wax moth larval hemolymph; B, wax moth larval tissue.

Similar results were obtained for the whole tissue as shown in Fig. 5B. Thus, as with housefly larvae, filipin causes a reduction of intracellular ^{32}P content. If 6.6 μmoles cholesterol/diet is added, an increase rather than a decrease is obtained, as described below.

Thin-layer chromatography of the lipids from a chloroform–methanol extract of the hemolymph and of the tissue demonstrated that the [^{14}C]methyl-labeled choline appeared in phosphatidylcholine. Less than 1 % of the ^{14}C was found in neutral lipids, phosphatidylethanolamine and phosphatidylserine. This indicates that little, if any, [^{14}C]methyl-labeled choline enters the C_1 pool of *G. mellonella* larvae.

Effect of the ratio of filipin to cholesterol on the incorporation of ^{32}P into phospholipids of wax moth and housefly larvae

When filipin and carrier cholesterol were added to the wax moth diet, the lipid content of the tissue increased. The increase in lipid was dependent on the amount of cholesterol as well as filipin in the diet. Similar increases in phospholipid content were obtained. For example, when wax moth larvae were reared for 2 days on $^{32}P_i$-containing diets without added cholesterol, filipin greatly reduced the incorporation

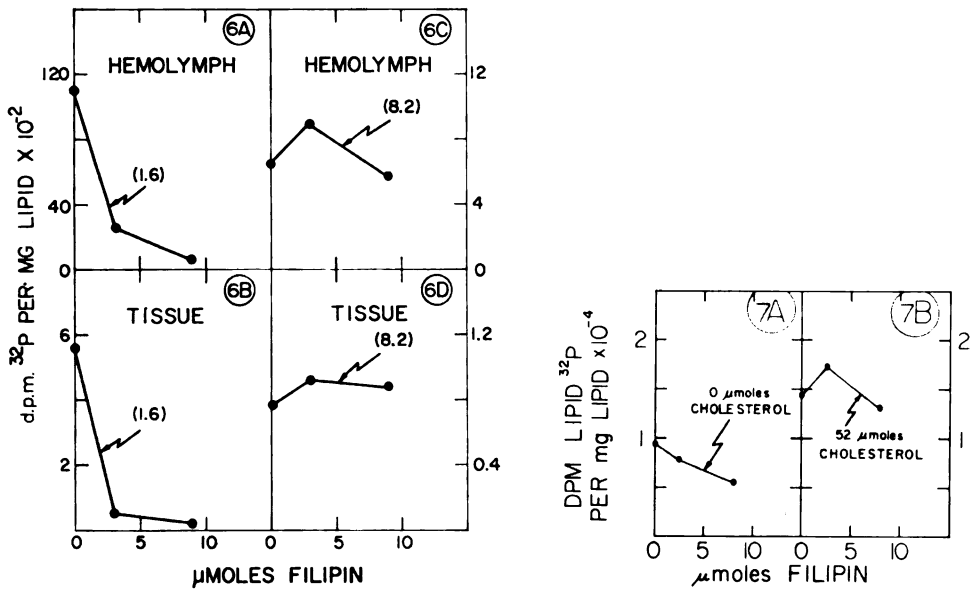

Fig. 6. Effect of filipin *plus* cholesterol on ^{32}P appearing in *Galleria mellonella* larvae hemolymph and tissue. Wax moth larvae were reared as described in METHODS. Diets for A and B contained no added cholesterol; C and D contained 6.6 μmoles added cholesterol. The $^{32}P_i$ content of diets A and B differed by a factor of 10 from diets C and D. The numbers in parentheses indicate the μmoles of cholesterol in the diet. Each point represents the average value for 9–10 larvae. A and B, wax moth larval hemolymph; C and D, wax moth larval tissue.

Fig. 7. Effect of filipin on ^{32}P uptake in *Musca domestica* larvae. Housefly larvae were reared on diets as described in the legend of Fig. 3, except that the larvae were reared for 2 days at 32° without $^{32}P_i$ and then transferred for 1 day at 32° to a ^{32}P-containing diet (6 μCi/g solids in the diet). Diet A contained no added cholesterol; B contained 52 μmoles added cholesterol.

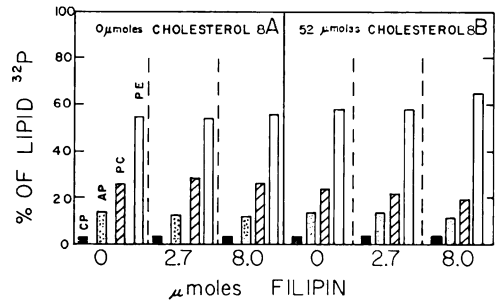

Fig. 8. Effect of filipin on the distribution of phospholipid classes in *Musca domestica* larval lipids. Housefly larvae were reared as described in the legend of Fig. 7. In A, no cholesterol was added to the diet; in B, 52 μmoles cholesterol was added to the diet. The distribution of ^{32}P lipids was determined as described by HILDENBRANDT *et al.*[21]. CP, ceramide phosphorylethanolamine; AP, acid phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, and cardiolipin); PC, phosphatidylcholine; and PE, phosphatidylethanolamine.

of ^{32}P into hemolymph and tissue phospholipids, as shown in Fig. 6A and B. Incorporation of ^{32}P into phospholipids was restored by adding cholesterol to the diets. At low filipin concentrations, excess cholesterol increased the incorporation of ^{32}P into hemolymph and tissue lipids to levels above the control (see Fig. 6C and D). The elevated incorporation of ^{32}P into lipids was dependent on the ratio of filipin to cholesterol and on the absolute amount of filipin in the diet. When filipin was added in amounts that approached toxic levels, excess cholesterol was less effective in promoting ^{32}P incorporation into lipids; compare the 3- and 9-μmole filipin values in Fig. 6C and D.

Similar results were obtained when ^{32}P incorporation was plotted against mg protein or per larvae. The increased ^{32}P incorporation was probably not due to increased lipid turnover because other experiments demonstrated that the total phospholipid content per mg protein or lipid increased.

Housefly larvae were reared for 2 days on normal diets and then transferred to diets containing $^{32}P_i$ and varying amounts of filipin and cholesterol. As shown in Fig. 7A, ^{32}P incorporation into phospholipids was inhibited by filipin; however, when excess cholesterol was added to the same diet, ^{32}P incorporation was greater than in controls (see Fig. 7B).

The filipin *plus* cholesterol stimulation of ^{32}P incorporation and the filipin-induced decrease of ^{32}P incorporation into phospholipids was similar for the principal larval phospholipids. The relative distribution of ^{32}P in phosphatidylethanolamine, phosphatidylcholine, ceramide phosphorylethanolamine and acidic phospholipids was the same, as shown in Fig. 8A and B. Thus, the overall phospholipid synthesis mechanism was affected.

DISCUSSION

The results show that many of the deleterious effects of filipin on housefly and wax moth larvae can be prevented by excess dietary cholesterol. Cholesterol prevents larvicidal and chemosterilant effects of low concentrations of filipin. Cholesterol

(18 μmoles/g diet) restores growth in *Galleria mellonella* which is retarded by non-lethal concentrations of filipin. Cholesterol also completely reverses the inhibition by filipin of the uptake of cholesterol into the hemolymph and tissues of wax moth larvae. Such results are consistent with the conclusion that one of the primary modes of action of filipin is to sequester dietary sterols or prevent normal use of the sterols.

Other data indicate that the filipin complex has more complicated modes of action or multiple modes of action. The reduction of ^{32}P- and [^{14}C]methyl-labeled choline in hemolymph and tissues of wax moth larvae placed on diets containing no added sterol indicates that the filipin complex affected the permeability of the insect's gut. Cholesterol did not prevent toxic effects of filipin when the filipin complex was present in large amounts (Figs. 1 and 2). The large increase in total cholesterol content of the hemolymph and of total wax moth tissue and hemolymph lipids indicates a different mode of action. The increase in hemolymph cholesterol and tissue lipids in housefly and wax moth larvae were obtained when both cholesterol and filipin were added to the diet, but the molar ratio of cholesterol–filipin exceeded 1. Such results indicate that filipin also affects the cholesterol (sterol) uptake mechanism or affects the control of the uptake mechanism, thereby permitting an increase in hemolymph cholesterol. Filipin in the presence of excess cholesterol appears to turn on the cholesterol uptake mechanism.

Alternatively, a nonfunctional cholesterol–filipin complex could be present in hemolymph which sequesters cholesterol. Additional cholesterol uptake would be required to maintain the concentration of unbound cholesterol and thus compensate for that bound in a cholesterol–filipin complex. This explanation may be untenable because: *(1)* the filipin complex prevents uptake of cholesterol in these insects when the molar ratio of cholesterol-to-filipin is less than 1. This indicates that a sterol–filipin complex is not absorbed; and *(2)* data obtained with another system (dogs) indicates that little, if any, filipin complex is absorbed into the bloodstream[24].

The elevated phospholipid levels in tissue and hemolymph could be an indirect effect of filipin. In these experiments, the amount of cholesterol in hemolymph was more than double the control value. Thus, the increase in phospholipids and neutral lipids would be a consequence of elevated cholesterol levels or increased levels of cholesterol metabolites. Similar type results have been obtained with isolated fat cells. The filipin complex induces lipogenesis and glucose uptake similar to insulin in fat cells[25]. The filipin complex also stimulates phospholipid synthesis in thyroid slices[26]. If filipin damages the insect's gut membranes as it does yeast membranes[23], chick ileal tissue[27] and bovine sperm[28], then the increased phospholipid synthesis could be an attempt by the organism to repair the damaged membranes which contain phospholipids. It is apparent that the total phospholipid synthesis system has been affected in housefly larvae. The relative distribution of the major phospholipid classes and the phosphosphingolipid, ceramide phosphorylethanolamine, were not altered in any of the experiments. Thus, filipin in the presence of cholesterol has changed the total concentration of phospholipids but not their relative concentration.

It appears that the hypercholesteremia and elevated phospholipids we have reported herein may be similar to experimentally induced atherosclerosis (*cf.* ref. 29,

p. 46). If so, the insect could be an excellent system for studying the cholesterol uptake mechanism because pools of *in vivo* synthesized cholesterol would not be present.

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STUDIES ON HYPO- AND HYPERCHOLESTEROLEMIA INDUCED IN INSECTS BY FILIPIN[†]

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SUMMARY

1. The polyene antibiotic, filipin, can produce both hypocholesterolemic and hypercholesterolemic effects in insects. The type of cholesterolemia induced is dependent on the dietary ratio of filipin to cholesterol.
2. When present in the diet at a filipin to cholesterol ratio of 38:1, filipin decreases cholesterol uptake by *Manduca sexta* (L.) larvae by 99% and lowers the concentration of hemolymph cholesterol by 90%. At a dietary filipin to cholesterol ratio of 1:2, filipin causes an increase in cholesterol incorporation from the diet into larval tissues. Cholesterol incorporation by tobacco hornworm larvae (*M. sexta*) is increased by 50 $\pm$ 4%, but the hemolymph cholesterol concentration is lower than control values.
3. Dietary filipin alters the concentration of sterol-carrying lipoproteins in hemolymph and appears to change the cholesterol-binding ability of these lipoproteins. Filipin causes a two-fold increase in the concentration of sterol-carrying lipoproteins in the hemolymph. However, at high dietary filipin to cholesterol ratios (38:1), the cholesterol binding ability of the lipoprotein fraction is reduced. At filipin to cholesterol ratios of 1:2, the cholesterol binding ability of the cholesterol carrying lipoproteins is approximately the same as control values.

4. At filipin to cholesterol ratios of 38:1, filipin increases excretion of unesterified cholesterol 8-fold. No effect on cholesterol esterification is noted with filipin, but cholesterol-sulfate excretion is reduced by 60%. Excess dietary cholesterol (filipin to cholesterol ratio of 1:2) prevents these alterations in cholesterol excretion, but cholesterol-sulfate excretion is below control levels.
5. Filipin is not absorbed in the digestive tract of *Galleria mellonella* (L.) or *M. sexta* larvae. More than 99% of ingested dietary [^{14}C]filipin appears in the feces. Most of the remaining 1% is found bound to the intestine. Injected filipin is rapidly excreted; 95% of the injected [^{14}C]filipin appears in feces in less than 2 hours. Filipin is not metabolized to CO_2.

INTRODUCTION

The data of Sweeley *et al.* (1970)[1], as well as other investigators[2, 3], are consistent with the hypothesis that the filipin complex and other polyenes are toxic to insects because filipin prevents the uptake of dietary cholesterol. Recent investigations have provided conclusive evidence that polyenes, especially filipin, interact with those sterols needed for insect growth and differentiation[4-7]. Filipin interacts with cholesterol in an aqueous system with a 1:1 stoichiometry[5]. It now appears that, at nonlethal doses, the effects of filipin in insects are dependent upon the dietary filipin to cholesterol ratio. At excess dietary filipin concentrations, [4-^{14}C]cholesterol uptake[1] and [^{14}C]methyl labeled choline or ^{32}P incorporation into phospholipids[3] are reduced by 90% or more in *Musca domestica* (L.) and *Galleria mellonella* (L.) larvae. When dietary cholesterol is present in concentrations much greater than filipin, the above effects are prevented. However, at dietary filipin to cholesterol ratios between 1:1 and 1:2, [4-^{14}C]-cholesterol uptake and $^{32}P_i$ incorporation into phospholipids of wax moth larvae are enhanced above control values[3]. Sweeley *et al.* (1970)[1] noted that flavofungin, a polyene similar to filipin, also increased [4-^{14}C]cholesterol uptake by *G. mellonella*. As previously suggested[1, 3], filipin may therefore have a more complex mode(s) of action in insects, other than simply sequestering dietary sterols.

Since the housefly and wax moth are small insects and are difficult to dissect, the investigations outlined herein were conducted with the tobacco hornworm, *Manduca sexta* (L.). *M. sexta* proved to be an ideal larger organism for these studies since the insect increased 7,000-fold in weight during its normal larval period (final weight ~ 7 g/larvae), ingested over 90% of experimental diets provided, and was essentially non-cannibalistic[8]. The experiments reported herein were designed to determine some of filipin's more complex mode(s) of action, especially to find if filipin (1) similarly affects all insect tissues, (2) alters the levels of hemolymph cholesterol transport proteins, and (3) influences cholesterol excretion in *M. sexta*. Lastly, some evidence from mammals has indicated that polyenes are unable to cross the digestive tract of dogs and humans[9, 10]. Using [^{14}C]-labeled filipin produced in this laboratory, the uptake and possible metabolism of filipin by insects are reported herein.

MATERIALS AND METHODS

Sterols

Cholest-5-en-3β-ol (cholesterol) and cholest-5-en-3β-ol-3-palmitate (cholesterol-palmitate) were purchased from Sigma Chemical Co., St. Louis, Mo. Cholest-5-en-3β-ol-3-sulfate was kindly provided by W. E. Robbins, ARS, USDA, Beltsville, Md. Purity of the sterols was tested by gas chromatography and thin

layer chromatography.

Other chemicals

Filipin (approximately 86% pure) was a generous gift of the Upjohn Co., Kalamazoo, Mich. The antibiotic was further purified as described previously[11,12]. All other materials were obtained as follows: L-ascorbic acid from Sigma Chemical Co., St. Louis, Mo.; 1-phenyl-2-thiourea from Eastman Kodak Co., Rochester, N.Y.; sodium dodecyl sulfate from Pierce Chemical Co.; bovine serum albumin (fraction V, fatty acid poor) from Miles Laboratories, Kankakee, Ill.; silicic acid (Bio-Sil A, 100-200 mesh) from Bio Rad Laboratories, Richmond, Calif.; Celite-535 from Johns-Manville Co., Detroit, Mich.; acrylamide, N,N'-methylenebisacrylamide, ammonium persulfate, and N,N,N',N'-tetramethylethylendiamine from Canal Industrial Co., Rockville, Md.; xylene brilliant cyanin G from K. and K. Laboratories, Plainview, N.Y.; sudan black B from Fisher Scientific Co., Fair Lawn, N.J.; bromophenol blue from Matheson, Coleman, and Bell, Norwood, Ohio; and Triton X-100 from Rohm & Haas, Philadelphia, Pa.

Radioisotopes

[4-^{14}C]Cholest-5-en-3β-ol ([4-^{14}C]cholesterol), 58 mCi/mM, was obtained from New England Nuclear, Boston, Mass. Sodium [1-^{14}C]acetate, 61 mCi/mM, was purchased from International Chemical and Nuclear Corporation, Waltham, Mass. [^{32}P] was in the form of the inorganic isotope.

Rearing of insects

Wax moth larvae, *Galleria mellonella* (L.), and tobacco horn-

worm larvae, *Manduca sexta* (L.), were reared as previously described[1,13]. Eggs for the original colony of *M. sexta* were kindly provided by R. A. Bell, ARS, USDA, Fargo, N.D. The diet used herein is a modification of that provided by Dr. Bell[13]. Diets containing cholesterol, [4-^{14}C]cholesterol, filipin, or [^{14}C]-filipin were prepared by the method stated previously[1,3]. *M. sexta* larvae were injected with radioactive materials, [4-^{14}C]cholesterol or [^{14}C]filipin, dissolved in ethanol. Four microliter aliquots of such ethanol solutions were injected into the hemolymph of each larvae with a Biotronics automated micro-injection device. The site at which the cuticle was punctured was sealed with paraffin. The injection procedure did not cause detrimental effects to *M. sexta*; injected and control larvae gained weight at the same rate and contained similar amounts of lipid and phospholipid. Unless otherwise stated, *M. sexta* larvae were fifth instar, phase one, classified as per Greene and Dahlman (1973)[14].

Isolation of insect tissues

Hemolymph was obtained from *G. mellonella* and *M. sexta* larvae as described previously[1,14]. The hemolymph was collected in ice-cooled glass centrifuge tubes. Each tube contained a few crystals of phenylthiourea to inhibit the phenoloxidase system. The hemolymph was centrifuged for 15 min. at 3000 revolutions/min on an IEC Model-HN trunnion head centrifuge to remove hemocytes. The supernatants were retained and stored at -20°C until used for

gel electrophoresis. Freezing at -20°C did not affect the electrophoretic patterns of *M. sexta* hemolymph proteins. Fat body, malpighian tubules, and intestine were removed and washed with an insect-Ringer solution (1.1% NaCl, 0.14% KCl, 0.11% $CaCl_2$). The excreta and intestinal contents were combined and are collectively referred to as feces.

Lipid extractions

Hemolymph and tissue lipids were extracted by a modification of the method of Folch *et al.*[15] as previously described[3] except that a Potter-Elvehjem homogenizer was used.

Lipid fractionation

Lipids were fractionated on silicic acid by using the modification of the system of Goodman and Shiratori (1964)[16] described by Svoboda *et al.* (1967)[17]. Cholesterol, cholesterol esters, and cholesterol-sulfate were also separated by thin-layer chromatography on Silica gel F-254 plates (Brinkman Instruments, Westbury, N.Y.) in three solvent systems: hexanes-diethylether-glacial acetic acid (80:20:2); dichloromethane-methanol (85:15); and chloroform-methanol-concentrated NH_4OH (65:35:4). Cholesterol, cholesterol-palmitate, and cholesterol-sulfate standards were used. Radioactive sterols were separated as above. Thin layer chromatography plates were scanned to locate radioactive areas with a Packard Radiochromatogram Scanner, Model 7201, Packard Instrument Co., Inc., Downers Grove, Ill. Radioactive areas were scraped off and counted as previously described[3].

Assays

Lipid was determined both gravimetrically and colorimetrically using cholesterol as a standard[18]. Phosphate and lipid-phosphate were determined by the method of Bartlett[19]. The method of Lowry *et al.*[20] was used to assay for protein content. Cholesterol was determined on a 1% SE-30 column[17]. Ascorbic acid was extracted and quantitated as the trimethylsilyl derivative[21]. [^{14}C] CO_2 evolution was measured by adjusting the flow rate of air through a 250-ml florence flask such that 90% of CO_2 released by the insects was removed every 16 min. The released [^{14}C] CO_2 was trapped in ethanolamine and counted, [^{14}C] and [^{32}P] were determined by dissolving aliquots of each sample in a 1:1 mixture of Triton X-100 with toluene scintillator (4 g PPO, 100 mg POPOP in 1 ℓ toluene). Samples were counted in a refrigerated Packard 3310 liquid-scintillation spectrometer.

The cholesterol-binding ability of the hemolymph proteins was determined by incubating 0.1 ml of hemolymph, centrifuged as above, with 10 μCi [4-^{14}C]cholesterol (> 4 μg) adsorbed on the surface of 1.0 mg celite-535. [4-^{14}C]cholesterol adsorbed on Celite-535 readily exchanges with cholesterol of serum lipoproteins[22] and also exchanged with the hemolymph lipoproteins of *M. sexta* larvae. Only negligible amounts of [4-^{14}C]cholesterol were found in solution in the absence of protein[22]. After incubation with [4-^{14}C]cholesterol, Celite-535, and centrifugation as above, radioactivity was associated only with cholesterol carrying lipoproteins when the

incubated hemolymph was electrophoresed and the gels were sliced and counted as described in the following section. Avigan (1959)[22] found that the cholesterol content of human serum lipoproteins was 11.6 ± 1.8% after incubation with [4-^{14}C]cholesterol-treated Celite. Reproducibility with [4-^{14}C]cholesterol incorporation into hemolymph lipoproteins of *M. sexta* was ± 10% in these experiments.

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out with 4.5% acrylamide as described by Davis (1964)[23] with the following modifications: sample and spacer gels were omitted, pH was adjusted to 8.6, and 10-20 µl of centrifuged hemolymph were layered directly on the gel. Samples were electrophoresed from cathode to anode at constant current of 1 mA/gel for 10 min followed by 2.5 mA/gel for 60 min. Samples were run in triplicate. 1) One gel of each triplicate set was stained for protein with xylene brilliant cyanin G[24]. This stain was prepared by a modification of the Malik-Berrie procedure[25] as described by Blakesly (1974)[24]. The xylene brilliant cyanin G stain is approximately as sensitive as Coomassie Blue R-250, but does not require destaining. Xylene brilliant cyanin G stain is prepared as follows[24]: mix equal parts of 0.2% xylene brilliant cyanin G (dissolved in distilled H_2O) and 2 N H_2SO_4; then add 10 N KOH until the solution changes color from brown to purple; and finally add trichloroacetic acid (TCA) to make 12% TCA in the final solution. The staining solution is stable for several

months. Gels are stained for 5-8 hours, but protein bands become visible in less than 1/2 hour. Stained gels are rinsed two times with distilled H_2O and stored in distilled H_2O at 4°C in the dark. The absorbance of the gel stained with xylene brilliant cyanin G was determined at 550 nm on a Gilford Model 220 spectrophotometer equipped with a Gilford Linear Transport. 2) The second gel of each sample set was stained with Sudan black B by the method of Swahn[26]. Absorbance was determined at 600 nm as above. 3) The third gel of each sample was finely divided with a Savant Autogel-divider, Savant Instruments, Hicksville, N.Y. Fractions of the finely divided gel particles were collected in distilled water. Each fraction was placed in a scintillation vial containing 1 ml gel solubilizer (1% sodium dodecyl sulfate in 0.1 N NaOH) and incubated at 40°C for 24 hours[24]. Ten ml of the aforementioned toluene:Triton X-100 (1:1) scintillation cocktail were then added. The vials were capped, vortexed for 20 sec, and counted.

[^{14}C]*Filipin production*

A lyophilized culture of *Streptomyces filipinensis* was kindly provided by T. G. Pridham, ARS, U.S.D.A., Peoria, Ill. The organism was maintained at 28°C on a medium containing 0.4 g yeast extract (Difco), 1.0 g malt extract (Difco), and 0.4 g glucose per 100 ml distilled H_2O[27]. Maximum filipin production occurs when palmitate is used as the carbon source instead of glucose[28]. [^{14}C]Filipin production was maximized when the organism was grown on 5 ml of the palmitate (0.4 g/100 ml) medium for three days,

followed by addition of 1 mCi Na[1-^{14}C]acetate. The culture was harvested 10 days later. [^{14}C]Filipin was extracted and purified by the procedure used for preparation of unlabeled filipin[11,12]. The [^{14}C]filipin (10 μCi/mg) had the same ultraviolet absorption spectrum, thin layer chromatographic behavior, and distribution of [^{14}C] among the four major components of the filipin complex as unlabeled filipin complex[11, 12]. The [^{14}C]filipin was approximately 96+% pure as judged by the criteria used previously[11, 12]. In order to determine if [^{14}C]filipin had a random labeling pattern, the [^{14}C]filipin was subjected to alkaline followed by acidic $KMnO_4$ oxidation[29]. In randomly labeled [^{14}C]filipin, 22-28% of the [^{14}C] should have theoretically been released as [^{14}C] CO_2. Our results showed that 24% of the radioactivity was released as [^{14}C] CO_2, consistent with a random [^{14}C] labeling of the carbon atoms of the [^{14}C]filipin molecule.

RESULTS

Cholesterol uptake in Manduca sexta

Figure 1 shows the effects of filipin and cholesterol on the incorporation of cholesterol from the diet into the larval tissues of *M. sexta*. Cholesterol incorporation was calculated from the known specific activity of [4-^{14}C]cholesterol in the diet and from the amount of [4-^{14}C]cholesterol found in the larval tissues. As shown in Fig. 1, fifth instar tobacco hornworm larvae take up increasing amounts of dietary cholesterol as the concentration of

cholesterol is increased in the diet. At dietary cholesterol levels greater than 5 μmole/g diet, the insects were capable of absorbing a minimum of 0.3 μmole cholesterol/g larvae/day as calculated from the data shown in Fig. 1. When the dietary cholesterol concentration was low (0.13 μmole/g diet), at least 20% of the daily ingested cholesterol was absorbed. Filipin added to the diet at a filipin to cholesterol ratio (herein referred to as the F/C ratio) of 38:1 reduced the incorporation of dietary cholesterol into larval tissues by 98%. In contrast, as also indicated previously for wax moth[3], at a filipin to cholesterol ratio of 1:2, tissue cholesterol levels were increased by 50 ± 4%.

Effect of filipin on incorporation of cholesterol into M. sexta larval tissues

The effect of filipin on dietary cholesterol incorporation into individual tissues of tobacco hornworm larvae was investigated. The effect varied depending on the ratio of filipin to cholesterol and the concentration of cholesterol in the diet. At F:C = 38:1, cholesterol incorporation was reduced by 98% in hemolymph, fat body, intestine, and remaining larval tissue; see Table I. In the feces, more [4-^{14}C]cholesterol appeared in the filipin treated larvae. As shown in the Table, at F:C = 1:2, cholesterol levels were increased in all larval tissues, especially the fat body (175% increase). In three experiments using 10-20 larvae per determination, hemolymph cholesterol uptake was stimulated 49 ± 3%

at F:C = 1:2. When cholesterol uptake was stimulated, a concomitant decrease in [4-^{14}C]cholesterol per g feces was noted. Similar data were obtained if cholesterol incorporation was expressed on a per mg lipid or per mg dry weight basis. Therefore, filipin can either prevent or enhance cholesterol uptake in *M. sexta* larvae, depending on the ratio of filipin to cholesterol in the diet.

Effect of filipin on cholesterol esterification in tissues of tobacco hornworm larvae

M. sexta larvae were injected with [4-^{14}C]cholesterol and then allowed to feed on cholesterol containing diets. At 5 μmole cholesterol/g diet, the appearance of [4-^{14}C]cholesterol esters in the fat body was increased 10-fold (Fig. 2A) above that found at low cholesterol. Filipin appeared to have little effect on the percentage of injected [4-^{14}C]cholesterol converted to [4-^{14}C]-cholesterol esters found in the fat body (Fig. 2B). Only small amounts of [4-^{14}C]cholesterol esters were found in the other tissues of the insect. At low dietary cholesterol (0.13 μmole/g diet), approximately 1.5% (0.05 μmole/g larvae/day) of the cholesterol is esterified; while at dietary cholesterol levels above 4 μmole/g diet, 11.2% (0.9 μmole/g larvae/day) of the incorporated cholesterol is esterified by *M. sexta* tissues, mainly the fat body.

Effect of filipin on cholesterol carrying lipoproteins of hemolymph

Since filipin increased cholesterol incorporation into tobacco hornworm larvae at F:C = 1:2, it was decided to investigate the effects of filipin on the proteins that transport cholesterol in

M. sexta hemolymph. Previously, Chino *et al.* (1969)[30] demonstrated that insect hemolymph proteins carry cholesterol. Cholesterol had little effect on hemolymph protein concentration of *M. sexta*, but as shown in Fig. 3, dietary filipin caused an increase in the total protein content of hemolymph (50-200% increase). Because part of this increase in protein content could be due to the presence of hemocytes in the hemolymph, the hemolymph hemocytes were removed by centrifugation and the supernatants were subjected to electrophoresis. These results are shown in Fig. 4. At least 19 major protein bands in hemolymph from larvae fed on diets without filipin are indicated by xylene brilliant cyanin G (Fig. 4A). Six of these protein bands also contained lipid, as shown by the Sudan black B stain (bands 1, 5, 7, 8, 11, and 12). [4-^{14}C]Cholesterol was found in three of the lipid containing protein bands (bands 5, 7, and 8). A comparison of Fig. 4A with Fig. 4B indicated that at F:C = 38:1, little [4-^{14}C]cholesterol was found in the hemolymph cholesterol-carrying lipoproteins. As shown in Table II, the cholesterol content of the centrifuged hemolymph, undoubtedly the lipoproteins, is decreased by 90% at F:C = 38:1. Free cholesterol migrates electrophoretically in the region of band 19; but as shown in Fig. 4, almost all [4-^{14}C]cholesterol is associated with bands 5, 7, and 8. The area under the protein stained bands 5, 7, and 8 was increased by 69% at F:C = 38:1, indicating that filipin had affected the concentration of hemolymph cholesterol carrying lipoproteins. Since the Coomassie dyes (including xylene brilliant cyanin G)

interact with protein and, to an extent, with lipid[31], two other methods were employed to determine the protein content of the cholesterol-carrying lipoproteins resolved by these gels. 1) Measurement of absorbance at 280 nm indicated that the amount of cholesterol-carrying lipoproteins increased 2-fold above control values at F:C = 38:1. 2) The cholesterol-carrying lipoproteins were also sliced out of the gels; the proteins were extracted[32], and the protein content was determined by the method of Lowry[20]. The concentration of cholesterol-carrying lipoproteins per ml hemolymph increased 1.6-fold, and per mg hemolymph protein increased 2-fold from 0.11 mg/mg to 0.21 mg/mg. Therefore, filipin caused a doubling of cholesterol-carrying lipoprotein/mg hemolymph protein at dietary F:C = 38:1.

A comparison of Figs. 4C (10.23 μmoles cholesterol/g diet) and 4D (10.23 μmoles cholesterol/g diet and 5.0 μmoles filipin/g diet) shows no major differences in protein, lipid, or [4-^{14}C]cholesterol due to filipin at an F:C ratio = 1:2. The protein, lipid, and [4-^{14}C]cholesterol containing bands on the gels were relatively unchanged. However, the absorbance at 280 nm and the protein/ml hemolymph of the cholesterol-carrying lipoproteins increased 1.5 and 1.8-fold, respectively. On the basis of hemolymph protein, as measured by the method of Lowry[20], the cholesterol-carrying lipoprotein concentration increased 1.8-fold (from 0.13 mg/mg to 0.24 mg/mg hemolymph protein). Feeding high cholesterol diets alone had little effect on the concentration of hemolymph cholesterol-carrying

lipoproteins (Fig. 4C). Thus, filipin elevated the hemolymph cholesterol-carrying lipoprotein levels at both high (F:C = 38:1) and low (F:C = 1:2) dietary filipin to cholesterol ratios.

Effect of filipin on the cholesterol binding ability of hemolymph cholesterol carrying lipoproteins

The above experiments have shown that the increased incorporation of cholesterol at F:C = 1:2 by *M. sexta* may be due to the increase in cholesterol-carrying lipoprotein in hemolymph. However, at F:C = 38:1, the cholesterol-carrying lipoprotein concentration of hemolymph was also increased. It would appear that filipin had simply sequestered dietary cholesterol at F:C = 38:1 and prevented its uptake by the insect. But, it is not known if filipin altered the sterol-binding capacity of the hemolymph lipoproteins at F:C = 38:1.

Experiments were designed to test the cholesterol-binding ability of hemolymph cholesterol-carrying lipoproteins. Lipoprotein-bound cholesterol can exchange with [4-^{14}C]cholesterol adsorbed on Celite-535[22]. Avigan (1959)[22] showed that he could reproducibly increase the cholesterol content of lipoproteins, as well as effect maximal exchange of [4-^{14}C]cholesterol between lipoprotein cholesterol and Celite adsorbed cholesterol. It is known that cholesterol of lipoproteins exchanges with other sterols of red blood cell membrane such that, if lipoproteins had been prelabeled *in vivo* or *in vitro* with [4-^{14}C]cholesterol, the specific activity of choles-

terol would be the same in both the lipoproteins and the red blood cells[44,45]. Avigan (1959)[22] showed that the [4-^{14}C]cholesterol labeled lipoproteins prepared *in vitro* by exchange with [4-^{14}C] cholesterol were capable of exchanging [4-^{14}C]cholesterol with rat red blood cells *in vivo* in a manner similar to that with reinjected [4-^{14}C]cholesterol lipoproteins initially prepared by feeding rats [4-^{14}C]cholesterol.

It is not known if all the cholesterol of lipoproteins from mammalian blood or insect hemolymph is free to exchange with cholesterol-Celite. As indicated in Table II, at F:C = 38:1, the hemolymph cholesterol concentration is reduced over 90% despite a doubling in hemolymph cholesterol-carrying lipoprotein concentration. Thus, *in vivo* at least, almost all (> 95%) of the hemolymph cholesterol can be removed or prevented from binding to the lipoproteins. With the above considerations in mind, it was found that [4-^{14}C] cholesterol on Celite exchanged with cholesterol bound to hemolymph lipoproteins. Electrophoresis of the incubated hemolymph indicated that all of the [4-^{14}C]cholesterol was associated with hemolymph cholesterol-carrying lipoproteins 5, 7, and 8 (see Fig. 4 for explanation of numbering). In three experiments, the exchange was reproducible $\pm$ 5-10%. Table II indicates that the cholesterol-carrying lipoproteins from hemolymph of larvae fed on F:C = 38:1 were able to bind 25.5 $\pm$ 1.3 µg cholesterol/ml, or over 10 times as much cholesterol as was present prior to incubation with excess

[4-^{14}C]cholesterol-Celite. However, the hemolymph of the control larvae was capable of carrying at least 81 ± 3 µg cholesterol/ml. This hemolymph bound 3 times as much cholesterol as the hemolymph from filipin-fed larvae. Since the filipin treated larvae had 2 times as much hemolymph protein in the electrophoretic region where bound cholesterol is found, it appears that filipin affected the availability of cholesterol and the cholesterol-carrying ability of these lipoproteins. It is important to note, however, that even at F:C = 38:1, the hemolymph lipoproteins are able to bind much more cholesterol than is present in the hemolymph. Such data are consistent with the ability of filipin to bind sterols, especially in the diet, and preventing net uptake of cholesterol at F:C = 38:1.

At dietary F:C = 1:2, the apparent cholesterol-carrying ability of the hemolymph was 2.3 times greater than control values (180 ± 5 µg/ml versus 76.8 ± 2.8 µg/ml). Since the hemolymph cholesterol-carrying lipoproteins per ml had also increased 1.7-fold (see previous section), the cholesterol-carrying ability of the cholesterol-carrying lipoproteins increased only approximately 34% above control values. Thus, filipin causes an increase in the hemolymph cholesterol-carrying lipoproteins, and may also affect their cholesterol binding ability.

Sterol excretion influenced by dietary filipin

Earlier investigators found that filipin and other polyenes lowered serum cholesterol in chickens[33] and dogs[9]. The sterol

content of the feces of polyene treated chickens was approximately 2-fold greater than that of controls[33]. However, the origin of this sterol was not determined. Since filipin and other polyenes bind sterols, these data could be interpreted by the polyene simply sequestering dietary sterol and preventing its uptake. The following experiments with *M. sexta* were designed to determine if filipin could also increase fecal sterol content by removing cholesterol from the insect's tissues. Table III shows that at dietary F:C = 38:1, filipin increased fecal cholesterol content 8-fold. At the same time, the specific activity of the ingested fecal [4-^{14}C]-cholesterol was diluted 8-fold. Since filipin prevented cholesterol uptake, it is apparent that cholesterol excretion was increased 8-fold.

Filipin also increased the appearance of injected [4-^{14}C]-cholesterol in the feces at F:C = 38:1 (see Total Fecal Sterols in Fig. 5). Free cholesterol excretion was increased, cholesterol-ester excretion was unchanged, and cholesterol-sulfate excretion was decreased. Cholesterol-sulfate was found primarily in the feces and intestine (70% of total [4-^{14}C]cholesterol-sulfate and 25% of total [4-^{14}C]cholesterol-sulfate, respectively, data not shown). Insects do not excrete cholesterol as bile acids, but cholesterol-sulfate appears to be the normal excretory product[8, 34]. Therefore, filipin increased free cholesterol excretion in both [4-^{14}C]cholesterol-fed and [4-^{14}C]cholesterol-injected larvae. Increasing dietary cholesterol reduced the excretion of injected [4-^{14}C]cholesterol and increased the appearance of [4-^{14}C]choles-

terol in the larval tissues. Only 7% of the injected [4-^{14}C]-cholesterol appeared in the fat body at F:C = 38:1, while 42% appeared in fat body at F:C = 1:2. However, as shown in Fig. 5, the excretion of injected [4-^{14}C]cholesterol was still greater in filipin fed larvae at F:C = 1:2 than in controls. Thus it appears that filipin increased the excretion of cholesterol independent of the F:C ratio, but prevented uptake only at high F:C.

Effects of filipin on hemolymph ascorbic acid and cholesterol sulfate excretion

In the previous section, it was shown that filipin decreased cholesterol-sulfate excretion at F:C = 38:1. The mechanism by which filipin might affect cholesterol-sulfate excretion in insects remains unknown. Filipin apparently will not interact with sterols that do not contain a free 3-β-OH group. It has been demonstrated that ascorbic acid is converted to ascorbate-sulfate in mammals[35]. The ascorbate-sulfate serves as the sulfating agent of cholesterol[35]. Both ascorbic acid and ascorbate sulfate have been shown to be hypocholesterolemic agents in mammals[35, 36]. Since insects excrete cholesterol-sulfate, the levels of hemolymph ascorbate were determined. As shown in Table IV, high dietary cholesterol (10.23 μmole/g diet) doubled the hemolymph cholesterol concentration as compared with low dietary cholesterol (0.13 μmole/g diet), but both the hemolymph ascorbate concentration and excretion of injected [4-^{14}C]cholesterol as cholesterol-sulfate were lowered 48% and 49%,

respectively at high dietary cholesterol. Filipin decreased hemolymph ascorbic acid concentrations by 88% at F:C = 38:1. A 69% decrease in [4-^{14}C]cholesterol excretion was also noted. In the presence of filipin as well as high levels of cholesterol (F:C = 1:2), hemolymph cholesterol levels were not at control values. Hemolymph cholesterol, hemolymph ascorbic acid, and [4-^{14}C]cholesterol-sulfate excretion were 33%, 23%, and 21%, respectively, below the control levels (10.23 μmole cholesterol/g diet). Therefore, ascorbate levels in the hemolymph may affect excretion of injected [4-^{14}C]cholesterol as [4-^{14}C]cholesterol-sulfate. Filipin does not interact with ascorbic acid as determined by the criteria and methods previously used[5].

Studies on site of action of filipin in M. sexta and G. mellonella

Since the data show that at F:C = 38:1, filipin prevents the uptake of cholesterol, affects the hemolymph cholesterol carrying lipoproteins, increases the excretion of free cholesterol; and decreases both the hemolymph ascorbate levels and fecal cholesterol-sulfate, studies on the fate of filipin in *M. sexta* were undertaken. [^{14}-C]Filipin was produced as described in the Methods from [1-^{14}C]-acetate by a culture of *S. filipinensis*. When the [^{14}C]filipin was included in the diet of *G. mellonella* and *M. sexta* larvae, less than 1% of radioactivity appeared in the insect tissues (see Table V). Both *G. mellonella* and *M. sexta* ingested more than 90% of the diet. Most of the [^{14}C]filipin appearing in the larvae was found

in the intestine; less than 0.1% appeared in the hemolymph. Addition of cholesterol in large molar excess reduced the binding of [^{14}C]filipin to the intestine and lowered [^{14}C]filipin in the larval tissues.

In order to determine if the low activity of [^{14}C]filipin in insects might be due to the metabolism of [^{14}C]filipin, [^{14}C] CO_2 evolution was measured. In three days, less than 0.0008% of the ingested [^{14}C]filipin appeared as [^{14}C] CO_2. When [^{14}C]filipin was injected into the hemolymph of *M. sexta* larvae, very little [^{14}C] CO_2 was released as shown in Fig. 6. Less than 0.04% of the injected [^{14}C]filipin appeared as [^{14}C] CO_2 in two hours. Acidification of the feces did not release additional [^{14}C] CO_2. The low amounts of [^{14}C] CO_2 released may be due to small impurities in the [^{14}C]filipin (96+% pure as determined by the criteria established by others[22, 23]. In two hours, 95% of injected [^{14}C]filipin was found as [^{14}C]-containing material in the feces (Fig. 7). The fecal [^{14}C]-containing material separated into two major radioactive areas on thin-layer chromatography plates developed in either of two solvent systems -- CH_2Cl_2:MeOH (85:15); $CHCl_3$:MeOH: Ammonia (65:35:4). The r_f values of the two [^{14}C]-containing areas were identical with r_f values of intact [^{14}C]filipin and base hydrolyzed [^{14}C]filipin (filipin incubated at pH 10 for 2 hrs at 50°C has the lactone ring hydrolyzed[10]) in the same solvent systems. The fecal pH of *M. sexta* larvae was measured at pH 9.0.

It was previously shown that filipin is inactivated at pH 9.0[5]. Therefore, base hydrolysis of the lactone ring of filipin could have occurred in the feces. [^{14}C]-labeled material isolated from hemolymph of *M. sexta* injected with [^{14}C]filipin had the same chromatographic behavior as [^{14}C]filipin. The [^{14}C]filipin did not appear to be hydrolyzed in the hemolymph. Although the [^{14}C]-labeled material isolated from the feces was not further characterized, it is apparent that insects cannot metabolize [^{14}C]filipin to [^{14}C] CO_2 and do not absorb [^{14}C]filipin.

DISCUSSION

Two effects of filipin were apparent with fifth instar *M. sexta* larvae and both were dependent on the dietary ratio of filipin to cholesterol (F:C). The first effect noted was on cholesterol uptake and excretion. (1) At F:C = 38:1, filipin prevented the uptake of [4-^{14}C]cholesterol from the diet and decreased hemolymph cholesterol. This effect was initially described for *Galleria mellonella* larvae[1]. At high dietary filipin, ascorbic acid levels were reduced by nearly 90%, and cholesterol-sulfate excretion was decreased by 69%. Cholesterol-ester formation and excretion were unaffected, but free cholesterol excretion was increased 8-fold. (2) At F:C = 1:2, filipin increased larval cholesterol incorporation from the diet by 50 $\pm$ 4%. However, the hemolymph cholesterol concentration at F:C = 1:2 was 33% lower than the control (10.23 μmole cholesterol/g diet). These levels are the

result of both cholesterol uptake from the diet and cholesterol excretion into feces. Thus, filipin can produce both hypo- and hypercholesterolemic effects in insects. The following explanation may help to clarify these results. If the assumption is made that the larval cholesterol system is composed of three major pools (dietary cholesterol in the gut; hemolymph cholesterol bound to transport proteins; and cholesterol accepted or needed by the tissues), the action of filipin may be explained. Reversible exchange of cholesterol is assumed to occur among these pools of cholesterol. At high dietary filipin (F:C = 38:1), filipin binds dietary cholesterol. This would prevent the incorporation of dietary cholesterol into the hemolymph and tissue pools. The data indicate that incorporation of dietary [4-^{14}C]cholesterol into the hemolymph and tissues is decreased by 98% at F:C = 38:1. Since filipin is present in large molar excess in the diet, it would be expected to remove additional cholesterol from the insect and lower the hemolymph cholesterol levels. The results show that cholesterol excretion is enhanced 8-fold and hemolymph cholesterol levels are decreased by 90% at F:C = 38:1. The insect appears to attempt to compensate for this loss of cholesterol by decreasing the excretion of cholesterol-sulfate by 69%. Cholesterol-sulfate seems to be the normal excretory product of cholesterol in tobacco hornworm[34]. In contrast, at dietary filipin to cholesterol ratios of 1:2, the net excretion of free cholesterol is prevented, but, as

indicated in Figure 5, the excretion of injected [4-^{14}C]cholesterol is higher than control levels. Hemolymph cholesterol levels are also lower (33%) at F:C = 1:2 than control levels. Despite this lowering of hemolymph cholesterol level, the incorporation of dietary cholesterol is enhanced 50 $\pm$ 4% and 49 $\pm$ 3% into tissues and hemolymph, respectively. These data seem to be consistent with the hypothesis that at F:C = 1:2, filipin causes an increased turnover of cholesterol between the ingested dietary cholesterol and the hemolymph and tissue cholesterol pools. This may be due to an increased demand for cholesterol in the tissues. Since cholesterol turnover seems to be increased at F:C = 1:2, loss of cholesterol as cholesterol-sulfate would be expected to be lower than control values. Cholesterol-sulfate excretion at F:C = 1:2 was indeed lower than control values.

The data are consistent with the conclusion that filipin affected both the cholesterol absorption mechanism and the cholesterol excretory mechanism of insects. At high dietary F:C ratio, filipin increased excretion of free cholesterol. Possibly the antibiotic bound to intestinal wall cholesterol and removed it from the intestinal membranes. Since cholesterol absorption appears to occur by dietary cholesterol displacing membrane cholesterol in mammalian intestines, such an explanation may be feasible[37]. Cholesterol-sulfate seems to be the normal excretory product of cholesterol in *M. sexta*[8, 34]. Filipin reduced the excretion of

cholesterol-sulfate in *M. sexta* larvae. Since filipin only interacts with sterols containing a free 3-β-OH group[5], a direct effect of filipin on cholesterol excretion is not likely. However, it is known that in rats, cholesterol-sulfate is formed from ascorbic acid via ascorbate sulfate[35]. In *M. sexta* larvae, hemolymph ascorbic acid and hemolymph cholesterol levels were lowered whenever cholesterol-sulfate excretion was decreased. If cholesterol-sulfate is synthesized similarly in insects and in mammals, then lowered hemolymph cholesterol and ascorbate levels would effectively decrease the amount of cholesterol available for sulfation and decrease the amount of sulfating agent precursor. It is also possible that sulfate may be the limiting factor in cholesterol-sulfation in insects. Filipin is known to affect the permeability of membranes and cause loss of P_i from housefly larvae[3]. It could also increase loss of sulfate. Similar correlations between serum cholesterol and urinary cholesterol-sulfate excretion were observed in normal and hypercholesterolemic human subjects[38]. However, as demonstrated by the fluorometric assays previously developed[4, 5], filipin apparently did not interact directly with ascorbic acid. Thus, the mechanism whereby filipin lowered hemolymph ascorbic acid levels and decreased cholesterol-sulfate excretion is not known. Since cholesterol is necessary for formation of membranes and hormones as well as in growth and differentiation of insects[8], it is possible that the insect responded to filipin's binding of cholesterol by a homeostatic mechanism designed to prevent efflux

of cholesterol. Cholesterol-sulfate in insects may be involved in cholesterol excretion[8, 34], reproduction[39], and hormone formation[8, 39-41].

The second major effect of filipin on *M. sexta* larvae was to elevate the concentration of hemolymph cholesterol-carrying lipoproteins. In mammals, the ability of lipoproteins to bind cholesterol is greatly influenced by their lipid composition[37]. At high dietary filipin, filipin is known to decrease $^{32}P_i$ incorporation into phospholipids and alter phospholipid metabolism in *G. mellonella* and *M. domestica* larvae[3]. In *M. sexta*, filipin altered the lipid distribution in the lipoproteins, decreased the hemolymph lipid content slightly (10 $\pm$ 2%), and lowered the incorporation of [$^{32}P_i$] into hemolymph phospholipids over 90% (data not shown). The apparent impaired cholesterol-binding ability of the sterol-carrying lipoproteins may be due to these changes in hemolymph lipid. An increase in the amount of cholesterol-binding lipoproteins as well as their ability to bind cholesterol may account for the enhanced uptake of cholesterol at F:C = 1:2. If so, this implies that the circulating levels of lipoproteins have a major effect on the amounts of cholesterol in the hemolymph. Lastly, [^{14}C]filipin was neither taken up by tissues nor metabolized to [^{14}C] CO_2 by either *G. mellonella* or *M. sexta* larvae. Therefore, filipin apparently acts by sequestering dietary cholesterol and also affects one or more intestinal processes.

The phenomena described herein may be generally applicable to other insects as well as mammals. Earlier results from our laboratory indicated that cholesterol uptake was also blocked by filipin in two other insects, *G. mellonella* and *Musca domestica*[1]. Cholesterol in dietary molar excess of filipin prevented the inhibition of [4-^{14}C]cholesterol incorporation by *G. mellonella*[3] and *M. sexta*. The hypocholesterolemic effects of filipin have therefore been established in three insect species. Filipin has also been tested in mammals. It was shown to lower blood cholesterol levels in chicks[33] and dogs[9]. Since filipin is known to interact stoichiometrically with cholesterol[5], this polyene could simply sequester dietary cholesterol of affected animals. However, our results indicate that filipin has a more complicated mode(s) of action. Filipin caused an increase in the concentration of cholesterol-carrying lipoproteins in *M. sexta* hemolymph. This may provide an increased ability to transport cholesterol. Filipin is known to mobilize cholesterol deposits in prostatic hyperplasia of dogs[42]. However, the mechanism whereby cholesterol mobilization was achieved in dogs was not investigated. With *M. sexta*, it has been shown that filipin can increase free cholesterol excretion from the larval tissues. An alternative mode of action of filipin may therefore be its ability to influence cholesterol transport and remove cholesterol from the tissues by sequestration in the intestinal lumen. Enhanced cholesterol incorporation was also noted in *M. sexta* at F:C = 1:2. Sweeley *et al.* (1970)[1] found that flavofun-

gin, a polyene similar to filipin, had larvicidal effects on *M. domestica*, but actually enhanced cholesterol uptake in *G. mellonella*. The flavofungin to cholesterol ratio was not determined in these experiments. In a previous investigation[3], filipin also enhanced cholesterol incorporation by *G. mellonella* at F:C between 1:1 and 1:2. Filipin caused an increase in cholesterol-carrying lipoproteins in hemolymph, thereby providing more cholesterol acceptor on the serosal side of the insect gut. If so, this could account for the enhancement of cholesterol incorporation. The results showing that filipin is not taken up by the insect are consistent with similar observations noted in dogs[9, 42] and with other polyenes in humans[10, 43]. Filipin acts as a chemosterilant in *M. domestica*, but the viability of the eggs was not affected[1, 2]. These data are also consistent with filipin preventing cholesterol incorporation into the eggs without actually entering the housefly ovaries or eggs.

In conclusion, the cholesterolemic effects of filipin may be due to several modes of action, but are dependent on the dietary filipin to cholesterol ratio.

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TABLE I - EFFECT OF HIGH CONCENTRATIONS OF FILIPIN ON CHOLESTEROL INCORPORATION INTO LARVAL TISSUES

Manduca sexta larvae were reared and harvested as described in the legend of Fig. 1. Control diets contained 0.5 uCurie [4-C^{14}]cholesterol and cholesterol as described in the table. Experimental diets were identical to control diets except that 30.8 umole filipin was added per g. diet. Lipid-PO_4 was determined as stated in Methods. Cholesterol incorporation was determined from the specific activity in the diet and from the radioactive cholesterol appearing in the insect tissues. Determinations represent the average of 3 experiments with 10-20 insects per sample.

TISSUE	Cholesterol (0.80 umole/g diet)		Cholesterol (26.7 umole/g diet)		Cholesterol (65.4 umole/g diet)		Cholesterol (130.0 umole/g diet)	
	Control	Filipin	Control	Filipin	Control	Filipin	Control	Filipin
	(nmoles Cholesterol/umole Lipid-PO_4)							
Hemolymph	6.9±0.3	0.3±0.2	39.2±1.8	41.0±1.9	82.1±2.8	126.9±4.4	110.8±4.1	111.3±5.2
Fat Body	9.5±0.4	0.1±0.1	63.7±2.6	54.4±3.0	53.0±3.2	162.2±6.8	77.6±5.0	69.5±5.2
Intestine	13.9±1.0	0.3±0.1	135.4±7.0	103.0±6.4	137.0±8.0	144.0±7.6	155.0±8.4	175.0±9.0
Remaining Larval Tissue	2.2±0.2	0.1±0.1	21.5±1.1	26.1±1.2	44.4±2.0	57.4±3.8	47.3±4.0	44.9±3.7
Filipin to Cholesterol Ratio in Diet	38:1		1.2:1		1:2		1:4	

TABLE II - EFFECT OF FILIPIN ON CHOLESTEROL BINDING ABILITY OF M. SEXTA HEMOLYMPH

M. sexta larvae were reared and harvested as described in the legend of Fig. 4. Hemolymph was collected from 20 larvae and centrifuged. The hemolymph supernatant was then incubated with [4-^{14}C]cholesterol adsorbed on Celite-535 as described in Methods. Celite-535 was then removed by centrifugation and [4-^{14}C]cholesterol bound to hemolymph protein was determined after electrophoresis as in Fig. 4. Numbers in parentheses refer to the F:C ratio. The experimental data for hemolymph cholesterol represent the average of two experiments utilizing 20 larvae in each experiment. The specific activity of the hemolymph before incubation was determined from the amount of radioactivity in the hemolymph-Celite mixture divided by the quantity of cholesterol in the mixture. The specific activity of the hemolymph after incubation was determined after removal of [4-^{14}C]cholesterol bound to Celite by centrifugation. Cholesterol was quantitated by gas chromatography before and after incubation with [4-^{14}C]cholesterol-Celite.

Filipin umole/g diet	Cholesterol umole/g diet	Specific Activity of Hemolymph Cholesterol (dpm/mg cholesterol x 10^{-5})		Hemolymph Cholesterol		
		Before Incubation	After Incubation	Before Incubation ug/ml	After Incubation ug/ml	Fold Increase In Bound Cholesterol
0	0.13	2.88	2.34	30.0±1.5	81.0±3.0	2.7
5	0.13 (38:1)	2.99	0.76	2.4±1.0	25.5±1.3	10.6
0	10.23	2.77	2.12	61.8±2.0	76.8±2.8	1.2
5	10.23 (1:2)	2.84	5.20	41.3±2.2	180.0±5.0	4.4

TABLE III - EFFECT OF FILIPIN AND CHOLESTEROL ON CHOLESTEROL EXCRETION

M. sexta larvae were reared, placed on experimental diets, and harvested as stated in the legend of Fig. 1. Cholesterol and [4-^{14}C]cholesterol content of feces were determined as stated in Methods. Numbers in parentheses refer to the F:C ratio. Each determination represents the average of two experiments with pooled samples from 20 larvae per determination. S.A.=specific activity.

Filipin umole/g diet	Cholesterol umole/g diet	Fecal Cholesterol (umole)	Fold Increase in Fecal Cholesterol	Specific Activity of Fecal Cholesterol (dpm/umole x 10^{-4})	Fold Increase in Fecal Cholesterol Calculated from s.a.
0	0.13	0.55±0.05	---	25.66±0.80	---
0	4.13	13.8±0.3	---	5.24±0.60	---
0	10.23	49.4±2.5	---	2.55±0.55	---
0	20.42	122.0±6.4	---	1.01±0.13	---
5.0	0.13 (38:1)	4.5±0.2	8.3	2.90±0.14	8.9
5.0	4.14 (1.2:1)	23.1±1.2	1.7	4.18±0.50	1.3
5.0	10.23 (1:2)	66.6±3.4	1.4	1.84±0.13	1.4
5.0	20.42 (1:4)	120.0±7.6	1.0	1.05±0.09	1.0

TABLE IV - EFFECT OF FILIPIN ON HEMOLYMPH CHOLESTEROL AND ASCORBIC ACID CONTENT OF HEMOLYMPH AND ON [4-^{14}C]CHOLESTEROL-SULFATE EXCRETION

M. sexta larvae were reared and placed on experimental diets as stated in the legend of Fig. 4. Cholesterol and ascorbic acid of centrifuged hemolymph were determined by gas chromatography as described in Methods. [4-^{14}C]cholesterol-sulfate excretion of injected [4-^{14}C]cholesterol was determined as described in Methods. Numbers in parentheses refer to the F:C ratio. The data are the average of two experiments using pooled samples from 20 larvae per determination.

Filipin umole/g diet	Cholesterol umole/g diet	Hemolymph		% of Injected [4-^{14}C]Cholesterol Excreted as [4-^{14}C] Cholesterol-Sulfate
		Cholesterol ug/ml	Ascorbic Acid ug/ml	
0	0.13	30.0±1.5	92.5±2.5	9.1±0.3
5	0.13 (38:1)	2.4±1.0	11.3±0.4	2.8±0.2
0	10.23	61.8±2.0	48.0±1.4	4.6±0.2
5	10.23 (1:2)	41.3±2.2	37.4±1.3	3.6±0.3

TABLE V - DISTRIBUTION OF INGESTED [^{14}C]FILIPIN IN TOBACCO HORNWORM AND WAX MOTH LARVAE

M. sexta larvae and *G. mellonella* larvae were reared as described in Methods. [^{14}C]Filipin was prepared as stated in Methods. Larvae were reared for two days on experimental diets containing 0.5 uCurie [^{14}C]filipin/g diet. The data are the average of two experiments using pooled samples from 20 larvae per determination.

Filipin	Cholesterol	% of Ingested [^{14}C]Filipin				
umole/g diet	umole/g diet	Hemolymph	Fat Body	Malpighian Tubules	Intestine	Excreta
M. sexta Larvae						
0	0.16	0.04±0.01	0.13±0.01	0.09±0.02	0.53±0.04	99.2±0.03
0	1.46	0.02±0.01	0.23±0.03	0.05±0.01	0.09±0.01	99.6±0.02
0.92	0.16	0.08±0.02	0.02±0.01	0.01±0.01	0.13±0.02	99.8±0.02
0.92	1.46	0.08±0.02	0.08±0.02	0.01±0.01	0.26±0.03	99.6±0.02
G. mellonella Larvae						
0	0.59	0.01±0.01	0.03±0.01	0.01±0.01	0.35±0.08	99.6±0.04
0	5.63	0.01±0.01	0.04±0.01	0.00±0.01	0.32±0.20	99.6±0.04
1.21	0.59	0.03±0.02	0.05±0.02	0.00±0.01	1.00±0.60	98.9±0.03
1.21	5.63	0.01±0.01	0.04±0.01	0.01±0.01	0.11±0.09	99.8±0.02

Fig. 1. Effect of filipin on cholesterol incorporation in *Manduca sexta* (L.) larvae. *Manduca sexta* (5th instar, phase 1) larvae were reared as stated in Methods and placed on experimental diets containing 0.5 μCurie [4-^{14}C]cholesterol (0.13 μmole to 21.1 μmole cholesterol/g diet) with and without filipin (5 μmole/g diet) as shown on the Figure. After two days, larvae were collected, dissected, and lipid extracted as described in Methods. Cholesterol uptake was determined from the known specific activity of cholesterol in the diet and from the quantity of [4-^{14}C]cholesterol appearing in the larval tissues, excluding feces. Each point on the figure represents the average of pooled tissues from 20 larvae. ●—● = Control (no filipin); ▲—▲ = 5 mole filipin/g diet.

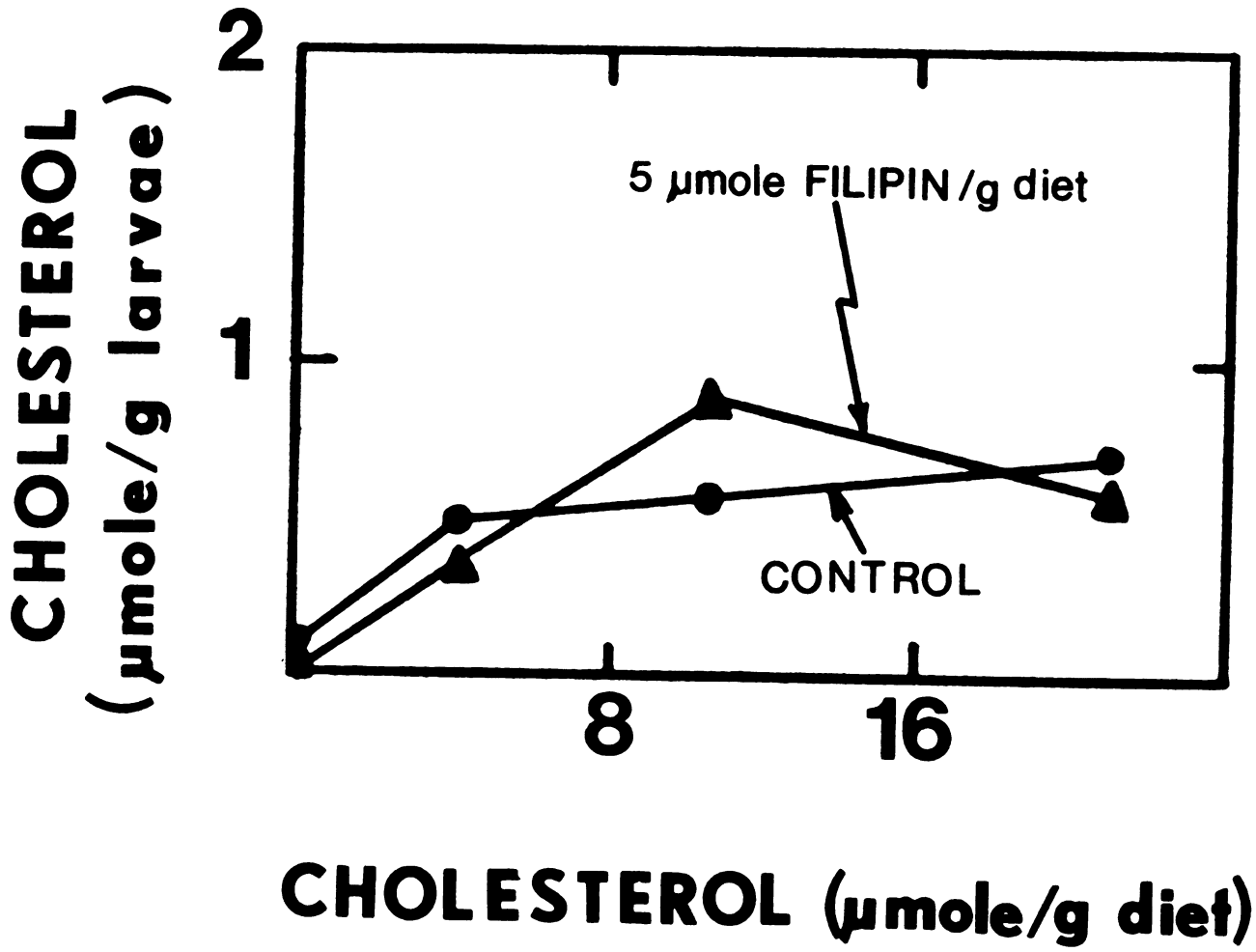

CHOLESTEROL
(µmole/g larvae)
2
1
5 µmole FILIPIN/g diet
CONTROL
8
16
CHOLESTEROL (µmole/g diet)

Fig. 2. Effects of filipin on cholesterol esterification in tobacco hornworm larvae. *M. sexta* larvae were reared as described in Methods. Fifth instar, phase 1 larvae were injected with 0.1 μCurie [4-^{14}C]cholesterol/ larvae and placed on experimental diets containing cholesterol (0.13 to 21.1 μmole/g diet). After two days, [4-^{14}C]cholesterol and [4-^{14}C]cholesterol-esters of larval tissue and feces were isolated and quantitated as described in Methods. Each point on the figure represents the average of pooled tissues from 20 larvae. ●—● = hemolymph; ▲—▲ = fat body; ■—■ = intestine; O—O = remaining larval tissue; Δ—Δ = feces.

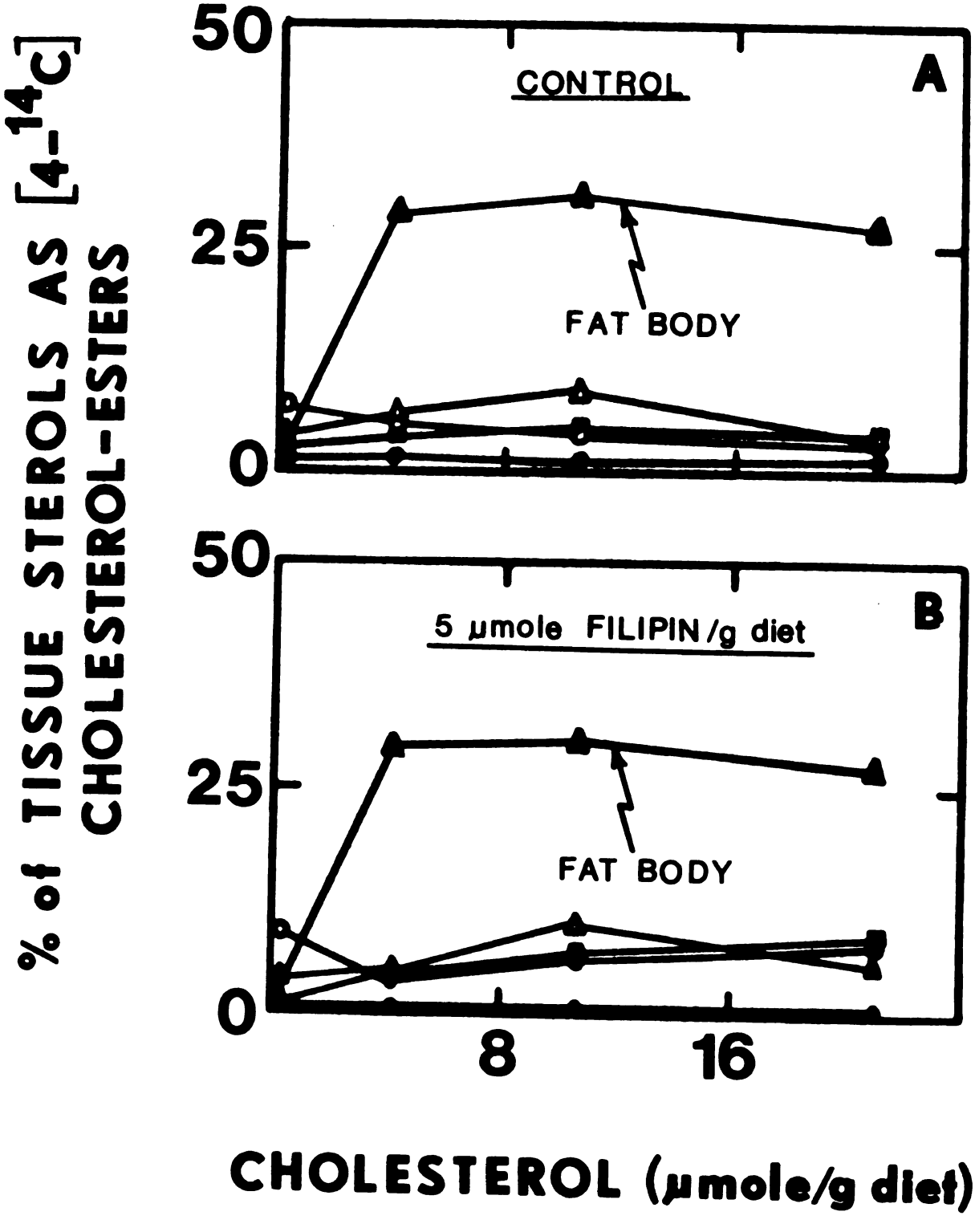

% of TISSUE STEROLS AS [4-14C] CHOLESTEROL-ESTERS
50
25
0
CONTROL
A
FAT BODY
50
25
0
5 µmole FILIPIN/g diet
B
FAT BODY
8
16
CHOLESTEROL (µmole/g diet)

Fig. 3. Effect of dietary filipin on hemolymph proteins. *M. sexta* larvae were reared and placed on experimental diets as in Fig. 1. Hemolymph was collected as described in Methods, but not centrifuged. Protein was determined by the method of Lowry[20].

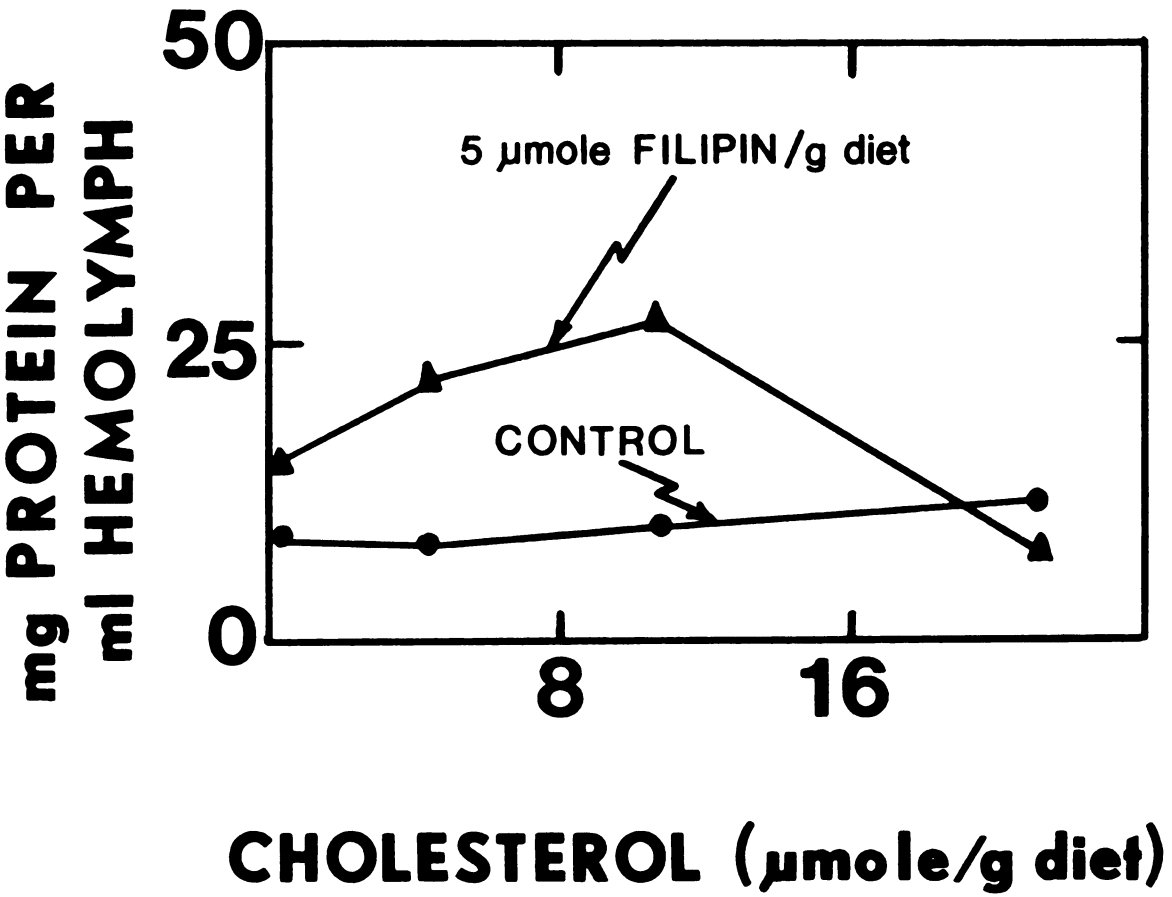

50
25
0
8
16
5 µmole FILIPIN/g diet
CONTROL
mg PROTEIN PER
ml HEMOLYMPH
CHOLESTEROL (µmole/g diet)

Fig. 4. Effects of filipin on the distribution of hemolymph proteins. *M. sexta* larvae were reared as described in Methods. The 5th instar, phase 1 larvae were placed on experimental diets for two days as follows: diet of 4A contained 0.13 µmole cholesterol/g diet, 0.16 µCurie [4-^{14}C]cholesterol/g diet, and no filipin; diet of 4B was the same as diet of 4A except that 5 µmole filipin/g diet was added; diet of 4C contained 10.23 µmole cholesterol/g diet, 0.92 µCurie [4-^{14}C]cholesterol/g diet, and no filipin; diet of 4D was the same as diet of 4C except that 5 mole filipin/g diet was added. Hemolymph from 10-20 larvae on each diet was collected, pooled, and centrifuged to remove hemocytes, as described in Methods. Three 10 µl samples of hemolymph supernatant taken from larvae fed on diets of 4A, 4B, 4C, and 4D were subjected to electronphoresis. Gels were stained for lipid or protein. [4-^{14}C]Cholesterol was determined as described in Methods. —— = protein (xylene brilliant cyanin G); --- = lipid (Sudan black B); ●—●—●—● = [4-^{14}C]cholesterol.

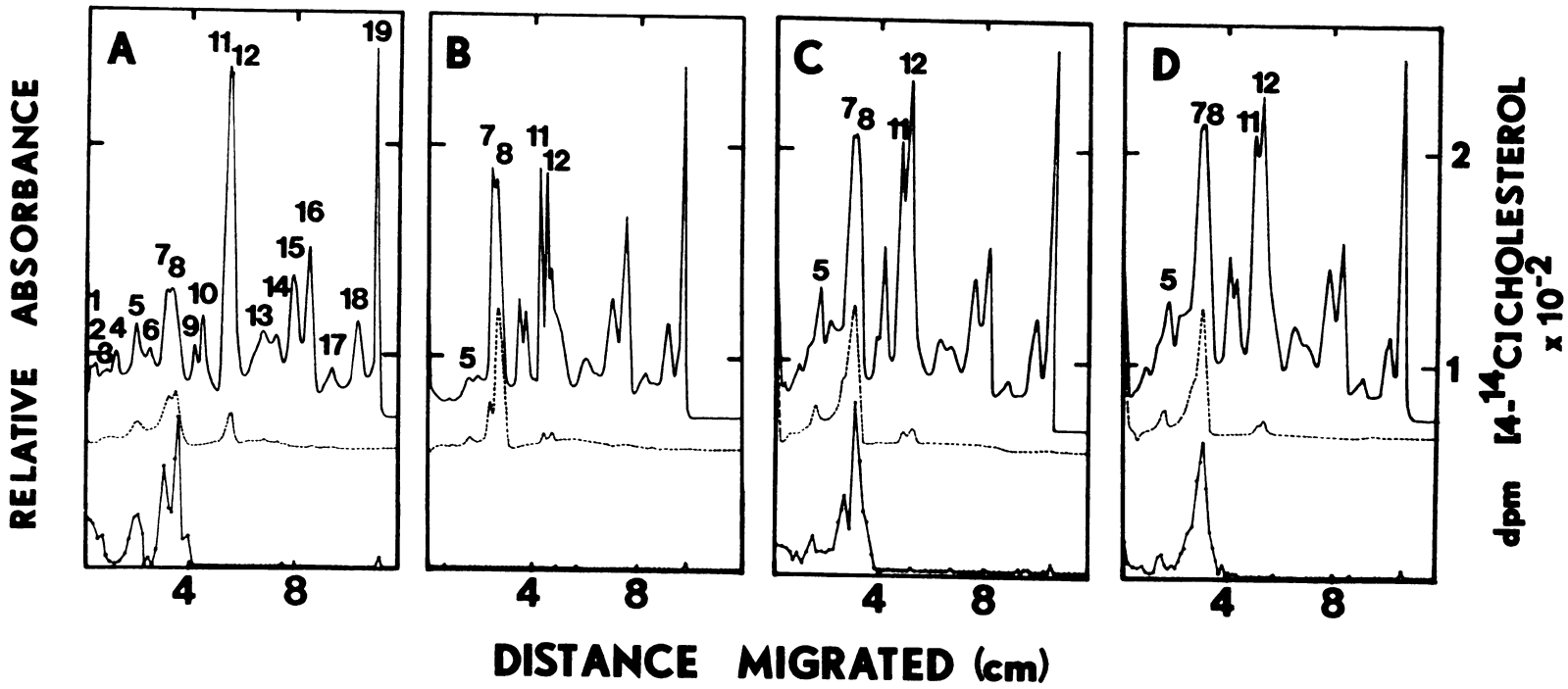

A
B
C
D
RELATIVE ABSORBANCE
dpm 14-14C CHOLESTEROL x 10-2
DISTANCE MIGRATED (cm)
4
8
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Fig. 5. Effect of filipin on excretion of injected [4-^{14}C] cholesterol. *M. sexta* larvae were treated as in Fig. 2. [4-^{14}C]Cholesterol, [4-^{14}C]cholesterol-esters, and [4-^{14}C]cholesterol-sulfate of feces were determined as described in Methods. Each point represents the average from pooled feces from 20 larvae. The F:C ratio of the experimentally determined points were 38:1, 1.2:1, and 1:2 from left to right. ●——● = 0 μmole filipin/g diet; ▲——▲ = 5 μmole filipin/g diet.

% of INJECTED [4-14C] CHOLESTEROL IN FECES
20
10
0
TOTAL FECAL STEROLS
FREE CHOLESTEROL
CHOLESTEROL ESTERS
CHOLESTEROL SULFATE
4 8
CHOLESTEROL (μmole/g diet)

Fig. 6. [^{14}C] CO_2 evolution by *M. sexta* larvae injected with [^{14}C]filipin. Tobacco hornworm larvae were reared and injected with 0.024 μCurie [^{14}C]filipin as described in Methods. [^{14}C] CO_2 evolution was measured as stated in Methods. Each determination represents the average [^{14}C] CO_2 evolution by 20 larvae.

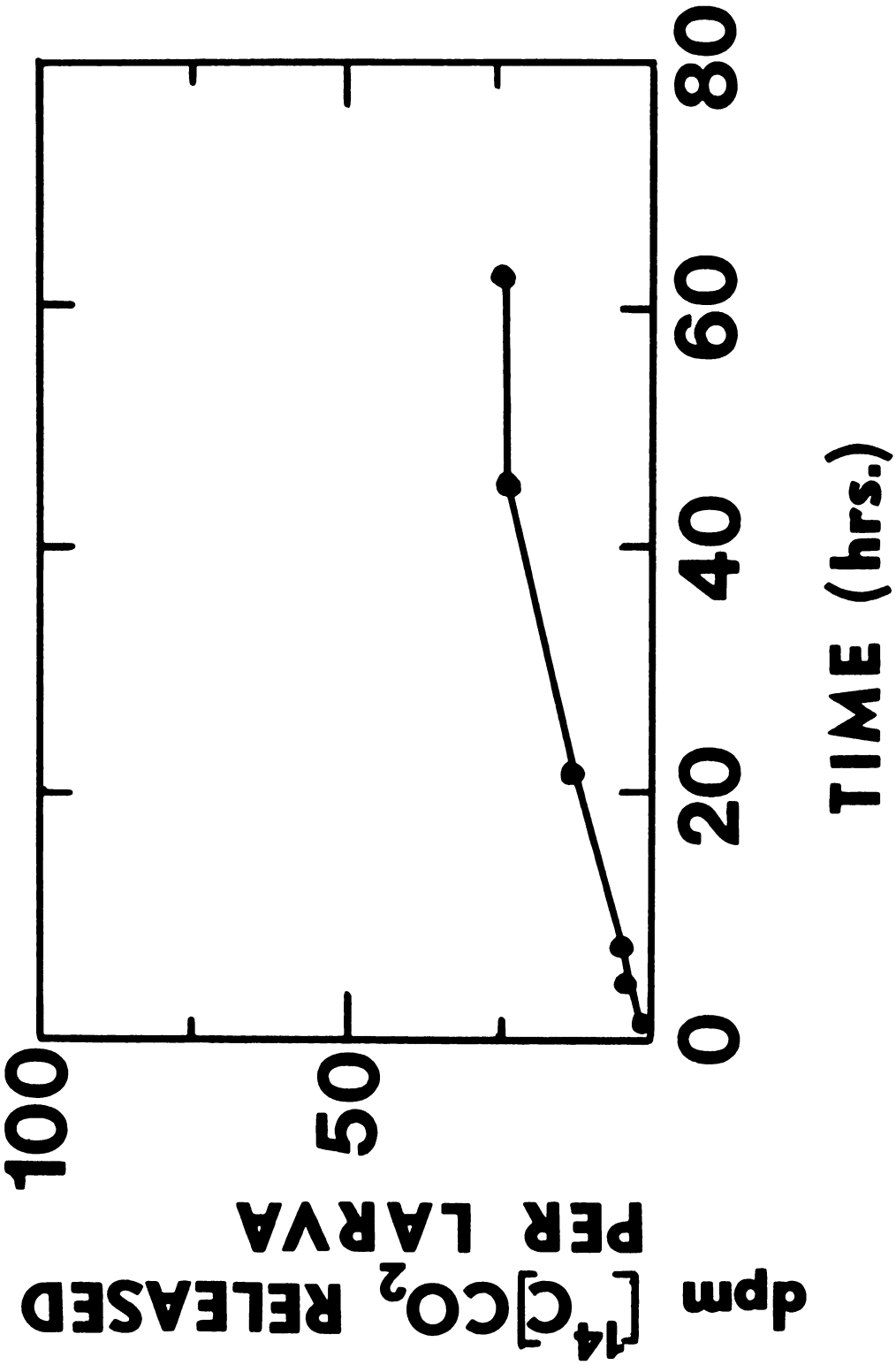
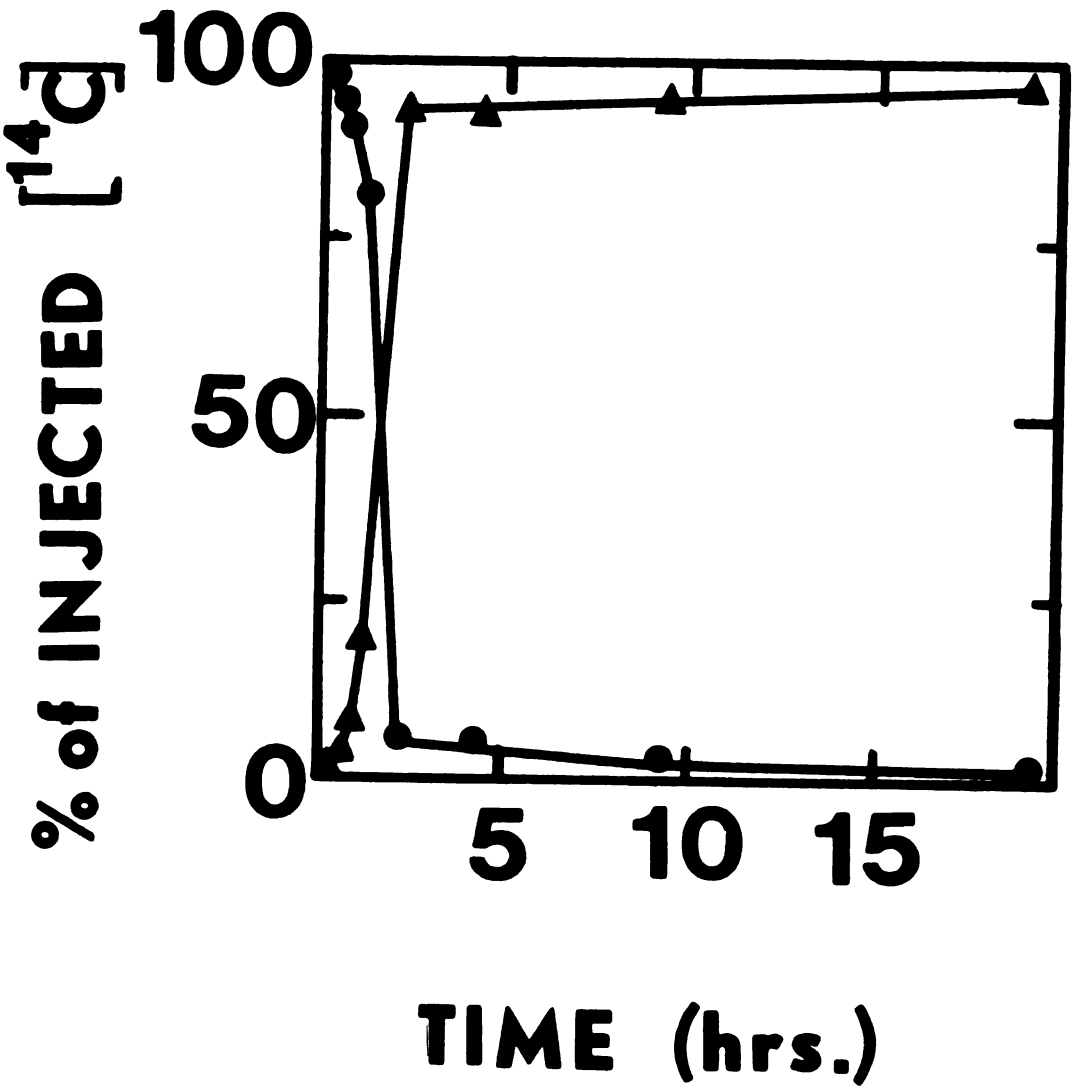

dpm [14C]CO2 RELEASED PER LARVA
100
50
0
0
20
40
60
80
TIME (hrs.)

Fig. 7. Fate of [^{14}C]filipin by *M. sexta* larvae injected with [^{14}C] filipin. *M. sexta* larvae were treated as in Fig. 6. [^{14}C] material was extracted from feces and tissue as described in Methods. ●—● = larvae tissues; ▲—▲ = feces.


100
50
0
% of INJECTED [14C]
5
10
15
TIME (hrs.)

Chapter IV

DISCUSSION

The results detailed in this thesis were obtained from two major investigations on the mechanism of action of filipin: 1) one study provided direct evidence for and characterization of the filipin-cholesterol interaction, which aided in the interpretation of 2) the effects of filipin on insects, organisms that require a dietary source of cholesterol.

Fluorometric Evidence for the Interaction of Filipin and Cholesterol

The initial data of Sweeley, O'Connor and Bieber (1970) were consistent with the hypothesis that filipin prevents the uptake of dietary cholesterol in insects. A general 'sterol-hypothesis' had existed for some time and much indirect evidence indicated that filipin interacts directly with free and membrane bound cholesterol. Support for this hypothesis came from two sources. First, filipin affected only organisms or cells whose membranes contained cholesterol. Second, cholesterol and other 3-beta-hydroxy sterols prevented the lytic effects of filipin on sensitive microorganisms and cells. In view of the circumstantial nature of this evidence, it seemed imperative to show conclusively that filipin interacts with cholesterol. Simple spectrophotometric methods were of questionable value since any process that changed the concentration of filipin in solution, such as preicpitation or adsorption to a solid surface or particle will alter the intensity of absorption, thereby producing apparent, but not necessarily real changes in absorption. Polyenes such as filipin are quite insoluble in water and have amphipathic properties. Therefore, the absorption measurements were undesirable since they would not have been particularly useful for studying molecular interactions of

filipin and cholesterol unless the exact nature of the concentration changes were known. However, certain fluorescence parameters proved to be ideally suited for studying the filipin-cholesterol interaction.

The computer-centered spectrofluorimeter of Holland, et al. (1972) simultaneously provides absorbance, corrected fluorescence (CO), and partial quantum efficiency (PQ) measurements. It is of importance to note that the latter parameter, PQ, is dependent upon the quantum efficiency of the fluorophore and independent of its concentration. Three major conclusions may be drawn from the fluorometric investigations detailed in Appendices I, II, and III.

1). Fluorescence can be used to monitor interactions of filipin with sterols. Addition of cholesterol decreases the PQ and CO of filipin in aqueous solution 36% and 62%, respectively. TCe higher the affinity of the sterol for filipin, apparently the greater was the decrease in PQ. Sterols containing a free 3-beta-OH group interacted with filipin: cholesterol, cholestanol, beta-sitosterol, and stigmasterol (listed in order of increasing ability to reduce teh PQ of filipin). Norman, et al. (1972) also found that stigmasterol was most effective in reducing the absorbance of filipin. These results are also in agreement with biological studies of others (Gottlieb, et al., 1960, 1961). Sterols such as cortisone, cholesterol-esters, and androstane-3-beta-ol-17-one did not reduce the PQ of filipin, nor did they prevent fungicidal effects of filipin (Gottlieb, et al., 1960, 1961). These data strongly indicate that the 3-beta-OH configuration of sterols is of prime importance for interacting with filipin. The 3-beta-OH configuration of sterols is

also important in limiting the permeability properties of membranes (Bruckdorfer, et al., 1968a,b; De Kruyff, et al., 1972; Demel, et al., 1972). If the 3-hydroxyl group is in the alpha configuration, smaller changes in PQ occur. The 3-alpha-OH isomers of cholesterol and other sterols are also much less effective in limiting the permeability of membranes to small ions and molecules. Filipin alters the phase transitions of cholesterol-phospholipid dispersions (Norman, et al., 1972). Such phase changes are important in controlling the permeability of membranes (Luzzatti, 1962).

2). The stoichiometry of the filipin-cholesterol interaction: The filipin complex interacts with cholesterol in aqueous solution with a 1:1 stoichiometry. The interaction is independent of pH 4.5 to 8.0. At pH's above or below this range, the lactone ring of filipin is cleaved (Kinsky, 1967; Amman, et al., 1955). Conditions which sould cleave the lactone, high and low pH's, prevented PQ and fluorescence changes of filipin by cholesterol. Methanol and other alcohols prevent the filipin-cholesterol interaction and disrupt the filipin-cholesterol complex. These results indicate that specific hydrogen and hydrophobic bonds are major forces involved in the interaction of filipin with cholesterol. The fact that the absorption maxima are decreased but not shifted on interaction with cholesterol indicates that no bond changes are occurring in the vicinity of the fluorophore; rather, the concentration of the fluorophore is reduced or the absorptivity of the molecule has been diminished. The change in the raito of the absorption peaks indicates that a simple reduction in concentration is not an adequate explanation; however, analysis of these changes does not clearly delineate

any interactions that may be involved. Since PQ is independent of concentraiton, it can provide a measure of interactions between the molecules of filipin and the various sterols occurring in the solution itself.

3). Existence of sterol-binding and sterol-nonbinding forms of filipin: The specific interaction of filipin with cholesterol that is detected fluorometrically depends on how filipin was treated prior to exposure to sterols. Freshly prepared aqueous solutions of the filipin complex that were isolated using organic solvents or dissolved in solvents such as methanol do not show changes in PQ immediately. Thus these solutions of filipin apparently do not interact with sterols. If such solutions are allowed to stand for long periods of time or are heated for 2 hours at 50°C, the ratio of the absorption maxima at 338nm/305nm changes from 1.96 to 1.55. Such solutions readily interact with sterols as shown by changes in PQ. Thus, the absorbance ratio is a sensitive measure of the capability of filipin to bind sterols in a specific manner that affects fluorescence properties. Interactions between filipin and sterols, specific or nonspecific, that do not alter the fluorescence properties of the system would not be detected. A likely explanation of these results is that filipin, isolated using organic solvents or dissolved in alcohols acquires a conformation or aggregated condition that is unfavorable for complexing with sterols in a specific manner. However, the sterol-binding activity of filipin, as measured by its capacity to immediately alter PQ when exposed to cholesterol, appears to be independent of the presence or absence of large micelles or aggregates of filipin. Initially filipin exists as inactive micelles in solution. These micelles are quickly disrupted by incubation at 50°C into small aggregates or

possibly monomers that also do not bind cholesterol. The decrease in absorbance ratio during further incubation or long periods of standing suggests that filipin undergoes a time- and temperature-dependent change, most likely conformational, to an active, sterol-binding form(s) of filipin which can also form micelles. The active micelles are immediately reactive with cholesterol. The finding that the converison of the sterol-nonbinding form(s) of filipin to the sterol-binding form(s) follows first-order kinetics is consistent with a conformational change.

4). Proposed model for the interaction of filipin with cholesterol: This proposed model is based on our studies, biological data, and structural investigations reported in the literature. The major components (92%) of the filipin-complex are capable of interacting with cholesterol (Schroeder, et al., 1972; see Appendix II). Herein, primarily the interaction of filipin III (see p. 6 for structure) with cholesterol will be discussed. The absolute configuration of the filipin is not known. However, the absolute structure of amphotericin B, a polyene similar to filipin, has been determined by X-ray diffraction crystallography (Mechlinsky, et al., 1970). It will be assumed in this model that the configuration of filipin is similar to that of amphotericin B. In the proposed model, the R and S configuration of the methyl group, the hydroxyl groups, and the hydrocarbon tail of filipin are arbitrarily assigned such that they resemble the configurations of similar groups in the amphotericin B moleule. Filipin has a hydrocarbon tail, a lactone ring, and a conjugated pentaene group which we conclude to be the fluorophore. The macrocyclic ring is planar. In the model, almost all the hydroxyl groups in the lactone ring face toward one side, leaving the other side of the ring

relatively hydrophobic. The cholesterol molecule fits into this hydrophobic 'groove' of filipin such that the hydrophobic tails of filipin and cholesterol interact, and the hydroxyl group of cholesterol hydrogen bonds to filipin. The bulk of the cholesterol lies over the double bonds of filipin. It appears in this model, that the R or S configuration of the nine hydroxyl groups of filipin would not sterically prevent interaction of the antibiotic with cholesterol. The following data appear to be consistent with this model:

a). The stoichiometry of the filipin-cholesterol interaction is 1:1.

b). The filipin-cholesterol interaction in water appears to be largely hydrophobic with some hydrogen bonding since alcohols such as methanol or ethanol easily disrupt the complex (Gottlieb, et al., 1960, 1961; Norman, et al., 1972a; Schroeder, et al., 1972; see Appendix II). The presence of high salt concentraitons has little effect on the filipin-cholesterol complex. Heating to 80°C disrupts the complex; while cooling from 80°C allows it to reform. Failure to observe any shift in the energy of the excitation or emission spectra indicates that any strong interactions between filipin and cholesterol are not located in the vicinity of the chromophores or fluorophores.

c). The observed decrease in PQ (Schroeder, et al., 1971, 1972; see Appendices I and II) strongly suggests pi*-pi interaction between the excited states of the fluorophore and the orbitals of cholesterol. Stigmasterol and beta-sitosterol are sterols that contain one more double bond than cholesterol; both these sterols are able to reduce the CO and PQ of filipin more than cholesterol does. This may indicate a closer approximation of stigmasterol or beta-sitosterol's double bonds to the pentaene of filipin such that the charge distribution of the excited state orbitals

of the filipin could be altered to produce a greater loss in CO and PQ.

d). Only sterols with a 3-beta-OH configuration are able to prevent the biological effects of filipin (Gottlieb, et al., 1960, 1961). Filipin does not interact as well with the 3-alpha-OH forms of cholesterol and cholestanol (Schroeder, et al., 1972; see Appendix II; Norman, et al., 1972a). In a space filling model of the filipin molecule complexed with cholesterol, the 3-alpha-OH form of cholesterol or cholestanol is not capable of hydrogen bonding with any hydroxyl of filipin.

e). Hydrogen bonding between filipin and cholesterol seems to be an important factor in the interaction since the confiugraiton of the sterol's hydroxyl group at the number-3 position is critical for interaction and prevention of biological activity of filipin. Esterification of this group with palmitate or changing this group to a ketone also results in inactivation of the antibiotic and prevents interaction with filipin (Gottlieb, et al., 1960, 1961; Norman, et al., 1972a; Schroeder, et al., 1971, 1972; see Appendix I and II).

f). Hydrophobic interactions between the tails of filipin and of cholesterol are essential for the interaction since sterols that contain keto or hydroxyl groups instead of the hydrocarbon tail do not interact with filipin or prevent its biological activity.

g). Lastly, the presence of an intact planar macrocyclic ring with a conjugated pentaene group is needed for interaction. Epoxidation of the double bond nearest the lactone group results in inactivation of the filipin molecule (Gottlieb, et al., 1960, 1961; Rickards and Smith, 1970). The epoxide does not interact with cholesterol (Schroeder, et al., 1972; see Appendix II). Epoxidation of this double bond alters the configuration

of a space filling model of filipin sufficiently such that the hydrophobic interactions discussed above cannot occur. Cleavage of the lactone ring at high or low pH abolishes the biological activity of filipin (Kinsky, et al., 1967) and prevents its interaction with cholesterol (Schroeder, et al., 1972; see Appendix II).

This model may provide insight into the interaction of filipin with cholesterol. Hopefully it will help to indicate how filipin might affect the sterol uptake mechanism of insects as well as provide a useful tool in elucidating similar parameters in other systems.

Effects of Filipin on Insects

Sweeley, O'Connor, and Bieber (1970) found that polyenes such as filipin act as larvicides and chemosterilants in rapidly growing insects such as housefly (Musca domestica L.) and wax moth (Galleria mellonella L.). Less toxic effects, decreased weight gain for example, were obtained with the slow growing cockroach (Nauphoeta cinera L.) and southern field cricket (Acheta domestica L.). The larvicidal and chemosterilant effects of filipin on housefly were later confirmed by Gemrich (1972). Secondly, Sweeley, et al. (1970) found that filipin prevented uptake of [4-^{14}C]-cholesterol into G. mellonella larvae. Digitonin, a plant substance that complexes 3-beta-OH sterols with a 1:1 stoichiometry, had similar larvicidal effects on M. domestica and reduced [4-^{14}C]cholesterol uptake in G. mellonella. These data are consistent with the hypothesis that filipin prevents the uptake of dietary cholesterol in insects. As discussed in the previous section, filipin interacts with cholesterol in an aqueous system with a 1:1 stoichiometry. Thus the initial hypothesis of Sweeley, et al., (1970)

appears sound. However, some indications existed that filipin may have more complex modes of action. In some experiments filipin was effective at molar concentrations less than the concentration of added cholesterol. In addition, flavofungin, a polyene similar to filipin, had larvicidal effects on M. domestica but, surprisingly, flavorungin increased [4-^{14}C]-cholesterol uptake in G. mellonella. These results posed several interesting problems on the mechanism of action of filipin in insects. Further research indicated that the effects of filipin were primarily dependent upon the dietary filipin to cholesterol ratio. The following sections discuss the results obtained from these investigations.

1). Effects of filipin at high filipin to cholesterol ratios: At high dietary filipin to cholesterol ratios of approximately 38:1, the antibiotic acts primarily as a hypocholesterolemic agent in Galleria mellonella (Sweeley, et al., 1970) and in Manduca sexta larvae. Hemolymph cholesterol levels were lowered nearly 90% in M. sexta, tobacco hornworm. Filipin inhibited [4-^{14}C]cholesterol incorporation into hemolymph and tissues of G. mellonella (Sweeley, et al., 1970) and M. sexta. The prevention of [4-^{14}C]cholesterol incorporation was similar in all tissues: hemolymph, fat body, intestine, and remaining tissue of tobacco hornworm.

At filipin to cholesterol ratios of 38:1, filipin increased free cholesterol excretion from M. sexta over 8-fold. This is consistent with the hypothesis that filipin sequesters dietary cholesterol and removed cholesterol from the insect tissues. But, cholesterol-sulfate excretion was reduced. Cholesterol-sulfate seems to be the normal excretory product of cholesterol in M. sexta (Hutchins and Kaplanis, 1969). Cholesterol-ester excretion or formation was relatively unaffected by filipin in the tobacco hornworm. Since filipin removes free cholesterol from the insect

tissues, it seems logical that the insect might respond by decreasing the excretion of cholesterol as cholesterol-sulfate. Filipin does not interact with sterols such as cholesterol-sulfate which do not contain a free 3-beta-OH group (Schroeder, et al., 1972; see Appendix II). Therefore, a direct effect of filipin by sequestration of cholesterol-sulfate does not appear likely. It seems more logical to presume that filipin affected cholesterol-sulfate excretion indirectly, perhaps by affecting the insects hormones or simply by decreasing the tissue levels of cholesterol-sulfate precursors. Filipin may, for instance, reduce cholesterol-sulfate formation and excretion because it also reduces hemolymph cholesterol levels and prevents the incporation of dietary cholesterol into the insect tissues. This may effectively lower the amount of cholesterol available for sulfation and excretion. Alternatley, an indirect effect of cholesterol on the sterol sulfation mechanism may occur. In mammals it has been shown that ascoribc acid forms ascorbate-sulfate which in turn is the in vivo sulfating agent of cholesterol (Mumma and Verlangieri, 1971). Ascorbic acid was also a hypocholesterolemic agent in mammals. Fluorometric studies indicated that filipin does not interact with ascorbic acid. However, at filipin to cholesterol raitos of 38:1, the hemolymph ascorbic acid content of M. sexta was greatly reduced. At the same time cholesterol-sulfate excretion was also lowered. If a relationship between ascorbate and cholesterol-sulfate similar to that found in mammals exists in insects, then the decreased cholesterol-sulfate excretion of M. sexta could also be due to filipin causing a lowering of ascorbic acid levels in the insect. These effects may be a homeostatic response by the insect to spare cholesterol in its tissues and prevent additional cholesterol excretion. If ascorbate were also a hypocholesterolemic agent

in insects as it is in mammals, then a lowering of insect ascorbate levels may also spare cholesterol from excretion. Since filipin increases the permeability of membranes to small ions, filipin could also increase the efflux of sulfate and reduce the availability of sulfate for sulfation of cholesterol. Alternately, filipin could affect cholesterol excretion in insects by a completely unknown mechanism. However, either one or both of the above detailed hypotheses could explain why filipin decreases cholesterol-sulfate excretion in insects.

Filipin decreased [$^{32}P_i$] and [^{14}C]methyl labyled choline incorporation into phospholipids of wax moth and hosuefly larvae (Schroeder and Bieber, 1971). Similar effects on [$^{32}P_i$] incorporation into phospholipids were noted with tobacco hornworm larvae. Since cholesterol and phospholipids are important membrane components, inhibition of growth or of cholesterol uptake would be expected to reduce membrane formation. This may explain why [$^{32}P_i$] and [^{14}C]methyl labeled choline were not incorporated into phospholipids to any large extent. Filipin increased the loss of [^{32}P] from [^{32}P]labeled phospholipids in housefly larvae and reduced the phospholipid content of the larvae by almost 50%. These results are also consistent with filipin affecting the permeability of the insect gut membranes to small ions such as $PO_4^{(-3)}$.

Lastly, at filipin to cholesterol ratios of 38:1, filipin caused an increase in the level of hemolymph cholesterol-binding proteins in M. sexta. This may also be an indirect effect of the antibiotic. Cholesterol is necessary for insect growth, development, and reproduction. Since filipin causes an increase in free cholesterol excretion and prevents cholesterol incorporation into larval tissues, it would be reasonable to expect the insect to respond in some manner that would reduce or compensate for these

effects of filipin. One such possible reduction has already been discussed: cholesterol-sulfate excretion was reduced. A second possibility might be to increase the absorption capability of the insect for cholesterol. If the level of cholesterol carrying lipoproteins in the hemolymph is a determining factor in cholesterol absorption or transport, then an increase in the concentration of these proteins in the hemolymph may increase the ability to absorb cholesterol and thereby compensate for the loss in free cholesterol due to filipin.

2). Effects of filipin at dietary filipin to cholesterol ratios less than 1:1 (molar excess of cholesterol): If filipin is toxic to insects because it sequesters dietary cholesterol and prevents its use by the insect, then a molar excess of dietary cholesterol should prevent the larvicidal activity and inhibiton of [4-^{14}C]cholesterol uptake. It was shown by Schroeder and Bieber (1971) that excess cholesterol prevents the larvicidal effects of filipin in housefly larvae. The chemosterilancy observed in housefly adults (Sweeley, et al., 1970; Gemrich, 1972) was also prevented by excess dietary cholesterol. Filipin, fed to G. mellonella larvae in non-toxic doses, retards growth and weight gain. Excess cholesterol in the diet (1:2 ratio of filipin to cholesterol) prevented this growth inhibiton and larval weight gains were the same as controls.

At filipin to cholesterol ratios between 1:1 and 1:2, filipin not only prevented the inhibiton of [4-^{14}C]cholesterol incorporation in wax moth and tobacco hornworm larvae, but enhanced [4-^{14}C]cholesterol incorporation into the insect hemolymph, fat body, intestine, and remaining tissue. Thus, at filipin to cholesterol ratios of 1:2, filipin can also act as a hypercholesterolemic agent in insects.

Excess cholesterol prevents the drastic lowering of hemolymph cholesterol levels caused by filipin. Hemolymph ascorbate levels and cholesterol-sulfate excretion were also increased, but not quite to control levels. The 8-fold enhancement of free cholesterol excretion noted at dietary filipin to cholesterol ratios of 38:1 was also prevented at excess cholesterol levels in the diet.

In the presence of excess cholesterol, filipin increased the total phospholipid content of housefly larvae. If excess cholesterol is present in addition to filipin, incorporation of [$^{32}P_i$] into the different classes of phospholipid was the same. Similar data were obtained with housefly, wax moth, and tobacco hornworm larvae. Therefore, the overall phospholipid biosynthetic mechanism in insects appears to have been affected by filipin. Filipin had similar effects in other systems. It stimulated phospholipid synthesis in beef thyroid slices (Larsen adn Wolff, 1967). In chick ileum filipin disrupted intestinal microvilli membranes (Adams, et al., 1970; Wong, et al., 1970). Larval growth inhibition is prevented at filipin to cholesterol ratios of 1:2 and cholesterol incorporation into the tissues is enhanced (hemolymph cholesterol incorporation is enhanced 49 $\pm$ 3%). It seems likely therefore that since growth resumed, increased membrane formation also took place. This would explain why [4-^{14}C]cholesterol and [$^{32}P_i$] incorporation into phospholipids is uninhibited by filipin at filipin/cholesterol ratios of 1:2.

At dietary filipin to cholesterol ratios of 1:2, the concentration of hemolymph cholesterol-carrying lipoproteins was elevated, similar to the elevation noted at filipin to cholesterol 38:1. Such results are consistent with the increased cholesterol uptake at filipin to cholesterol ratios of

1:2, if one assumes that the level of cholesterol-carrying proteins in the hemolymph is related to the incorporation of cholesterol into M. sexta. It seems that filipin sequesters or binds dietary sterols and prevents the insect from utilizing them at high dietary filipin to cholesterol ratios. If excess sterol is added to the diet, the filipin would bind equal amounts of cholesterol (Schroeder, et al., 1972; see Appendix II) and the remaining cholesterol could be taken up by the insect. This may be a very simplistic interpretation in which all other effects of filipin may be largely indirect.

3). Studies on the incorporation of filipin into insect tissues: Investigations with chicks, dogs and humans (Fischer, et al., 1969; Gordon and Schaffner, 1968; Hamilton-Miller, 1973) indicated that filipin and other polyenes lowered serum cholesterol levels and increased the mobilization of cholesterol deposits in prostatic hyperplasia without penetrating the gut. Using [^{14}C]labeled filipin, prepared in our laboratory, it was shown that insects do not absorb orally administered filipin. Less than 1% of ingested [^{14}C]filipin appeared in G. mellonella or M. sexta larval tissues. Most of this 1% of ingested [^{14}C]filipin was found associated with the intestine. M. sexta did not metabolize ingested or injected [^{14}C]filipin to [^{14}C]CO_2 or [^{14}C]labeled bicarbonate. More than 96% of injected [^{14}C]filipin was rapidly excreted into the feces in less than two hours after injection.

Summary

Filipin interacts stoichiometrically with cholesterol on a 1:1 basis in an aqueous system. Other data are consistent with the hypothesis that similar interactions between filipin and cholesterol of insects or of insect diets occur. Thus it appears that the effects of filipin in insects may be largely attributed to its ability to sequester cholesterol. The

action of filipin on insects is modified by the cholesterol content of the diet. At high dietary filipin to cholesterol ratios, filipin causes increased excretion of free cholesterol from the insect and prevents the incorporation of dietary cholesterol. The insect appears to respond to the toxic effects of high filipin by decreasing cholesterol-sulfate excretion and by increasing its ability to absorb cholesterol by raising hemolymph cholesterol-carrying lipoprotein levels. At dietary filipin to cholesterol ratios of 1:2, the toxic effects of filipin on insects are prevented. Filipin appears to be effectively 'titrated' out of the diet. Because of this sequestration and since the concentration of hemolymph cholesterol and lipid-carrying proteins is elevated, the insect may then incorporate increasing amounts of cholesterol in order to grow and repair damaged or cholesterol depleted tissues. If the concentration of cholesterol-carrying lipoproteins in hemolymph is a determining factor in cholesterol uptake, then the increased concentration of these proteins at filipin to cholesterol ratios of 1:2 may account for the hypercholesterolemic effects of filipin at this filipin to cholesterol ratio. It is not known now filipin induces an increase in the level of hemolymph cholesterol-carrying lipoproteins, possibly a homeostatic mechanism is involved.

Chapter V

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Appendix I

Fluorometric Evidence for the Binding of Cholesterol to the Filipin Complex

by

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FLUOROMETRIC EVIDENCE FOR THE BINDING OF CHOLESTEROL TO THE FILIPIN COMPLEX*

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The data show that the filipin complex in water has a characteristic fluorescence spectrum with a broad emission maxima at 497 nm. Addition of cholesterol to the aqueous solution decreases the absorbance, the corrected fluorescence, and the partial quantum efficiency. The reduction in partial quantum efficiency, which is independent of the concentration of filipin, is definitive evidence that the filipin complex interacts with sterols in aqueous solution. The data indicate that changes in fluorescence may be a sensitive tool for monitoring the interaction of filipins with sterols.

The filipin complex (1) has been used to probe properties and functions of sterols in natural and artificial membranes[1~8]; (2) has antifungal activity[9~13]; (3) has larvicidal and chemosterilant activity towards houseflies[14,15]; (4) reduces serum cholesterol levels in dogs[16,17] chicks[18] and also (5) mimics vitamin D in ileal segments from vitamin D-deficient chicks[19,20]. The above-mentioned effects have been attributed to the apparent affinity of the filipin complex for certain sterols. Herein definitive evidence is presented that filipins interact with cholesterol and that this interaction can be monitored fluorometrically.

Materials and Methods

Filipin, 86 % pure, was kindly provided by the Upjohn Company, Kalamazoo, Michigan. The complex was further purified by washing twice with chloroform and twice with petroleum ether. After drying, the filipin complex was dissolved in tertiary butyl alcohol, lyophilized, and stored in the dark at −20°C. The standard filipin solution was prepared by dissolving 1 mg in 100 ml distilled water. Partial quantum efficiency (PQ), correct fluorescence (CO), and absorption spectra were determined as decribed by HOLLAND and TIMNICK[21].

Results

The fluorescence emission spectrum of the filipin complex in water is shown in Fig. 1. Filipin also has a characteristic absorption spectrum (see Fig. 2A and ref. 9~11) which is decreased by addition of cholesterol. Cholesterol caused a 60 % reduction of the absorption maxima of the filipin complex at 356 and 338 nm and a smaller decrease of the absorption maxima at 305 and 321 nm as shown in Fig. 2B. Cholesterol also decreased the corrected fluroescence (compare Fig. 2D to 2E) and the

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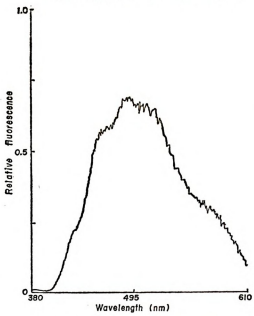


Fig. 1. Fluorescence emission spectrum of the filipin complex excited at 338 nm. The curve was obtained with 2 μg filipin complex per ml distilled water.

partial quantum efficiency (compare Fig. 2G to 2H). In contrast, the absorption spectrum, the corrected fluorescence, and the partial quantum efficiency of filipin plus cholesterol palmitate was very similar, if not identical, to that of filipin; compare Fig. 2A to 2C, 2D to 2F, and 2G to 2I. Thus, cholesterol, but not cholesterol palmitate, alters the absorption and fluorescence properties of the filipin complex in aqueous solution. None of the sterols tested fluoresced when excited at 338 nm.

Addition of methanol, final concentration 50 %, to the cuvette for the experiment of Fig. 2B, 2E, and 2H restored the absorbance, corrected fluorescence and partial quantum efficiency to that shown in Fig. 2A, 2D, and 2G. Thus, methanol prevents and reverses the interaction of filipin with cholesterol.

Other sterols, such as β-sitosterol, stigmasterol, and β-cholestanol, also quench the fluorescence of filipin and reduce the partial quantum efficiency. These sterols and cholesterol, but not cholesterol palmitate, prevent the antifungal effects of the filipin complex[9,13].

Discussion

The data show that cholsterol alters the fluorescence properies of the filipin complex and indicate that fluorescence could be a useful tool for probing the interactions of polyene antibiotics with sterols. The fluorescence properties of each polyene antibiotic may be different. This conclusion is supported by unpublished data which show that cholesterol, stigmasterol, sitosterol, and β-cholestanol all alter the fluorescence properties of pimaricin, but unlike the effect obtained with filipin, the partial quantum efficiency is greatly increased rather than decreased.

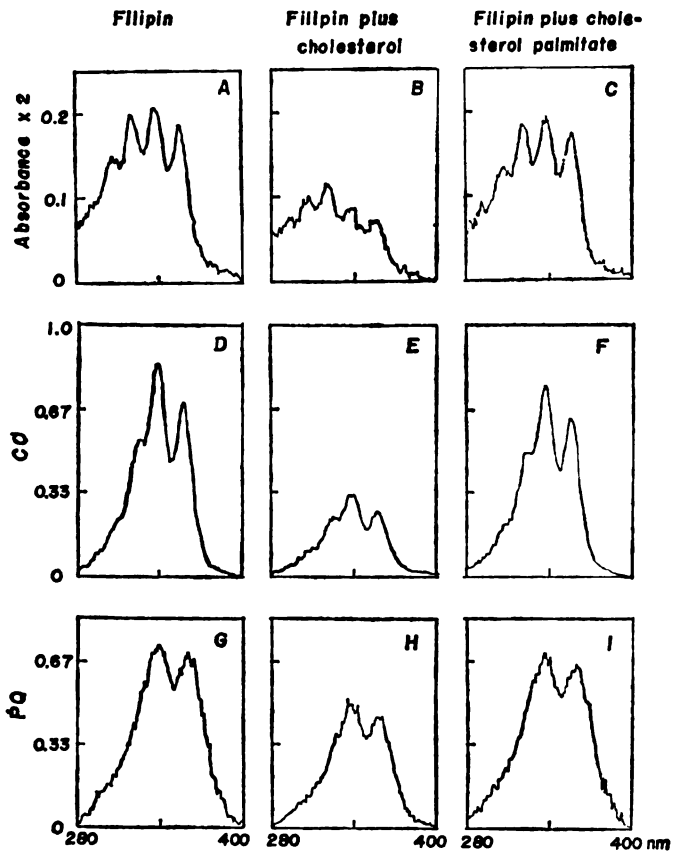
Our results correlate well with the results of Gottlieb and others[9,13] on the prevention of filipin toxicity to fungi by certain sterols. Sterols that prevent the fungicidal effects of filipin alter the fluorescence properties while compounds such as cholesterol palmitate, cortisone, and androstane-3β-ol-17-one which do not prevent the fungicidal effects of filipin also do not affect the corrected fluorescence or the partial quantum efficiency.

The reduction in the absorption intensity of filipin by addition of cholesterol agrees with the results of others[9,11]. A reduction of the absorption intensity of filipin by addition of sterols in aqueous solution might be due to a decrease of the concentration of the polyene in solution. Since partial quantum efficiency is independent of concentration, the change in partial quantum efficiency of the fluorescence of filipin caused by sterols is very strong evidence that filipins interact with certain sterols. The absorbance spectrum of filipin in the presence of cholesterol shows a large decrease in absorbance with no

detectabie change in the energy of the bands. This suggests a close approximation of the cholesterol molecule to filipin such that the charge distribution of the excited state orbitals of the filipin could be altered sufficiently to produce the observed loss in transition moment. It is of interest to note that the two bands, at 338 and 356 nm, are preferentially diminished when compared with the total absorbance spectrum. This could indicate close proximity of the cholesterol to the conjugated double bond system of the filipin. The observed decrease in the partial quantum efficiency strongly suggests pi*-pi interaction between the excited states of the fluorophore and the orbitals of the cholesterol. Failure to observe any shift in the energy of the excitation or emission spectra indicates that any strong interactions between the two molecules are not located in the vicinity of the chromophores or fluorophores.

Fig. 2. Effect of cholesterol and cholesterol palmitate on the corrected fluorescence, partial quantum efficiency, and absorption spectrum of the filipin complex in water.

The partial quantum efficiency, PQ, and the corrected fluorescence, CO, are identical to that defined by HOLLAND and TIMNICK[21]. Fluorescence, λ-emission, was monitored at 497 nm. The filipin complex, 2 μg/ml, in distilled water was incubated at 50°C for two hours: 1) without added sterol in A, D and G: 2) with 50 μg/ml chlolesterol in B, E, and H, and 3) with 50 μg/ml cholesterol palmitate in C, F, and I. Then absorbance, CO, and PQ were determined.



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Appendix II

Fluorometric Investigations of the Interaction of Polyene Antibiotics with Sterols

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Fluorometric Investigations of the Interaction of Polyene Antibiotics with Sterols†

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ABSTRACT: Changes in specific fluorescent properties, partial quantum efficiency (PQ), and corrected fluorescence (CO) can be used to monitor the interaction of various polyenes with sterols. The changes in PQ and CO, caused by addition of sterols, are different for each polyene antibiotic. Addition of cholesterol decreases the PQ and CO of filipin in aqueous solution 36 and 62%, respectively, but cholesterol increases the PQ and CO of pimaricin more than 80-fold. Addition of cholesterol to nystatin or amphotericin B has little effect on the fluorescence emission. The filipin complex as well as pimaricin strongly binds sterols that contain both a 3β-hydroxyl group and a long alkyl side chain that is attached to the D ring of sterols. The filipin complex and pimaricin interact weakly or not at all with cholesterol palmitate and 3-keto or 3α-hydroxy sterols. The methanol degradation product of filipin (a tetraene epoxide) does not interact with cholesterol. The stoichiometry of the filipin:cholesterol interaction is, within experimental error, 1:1. The interaction of filipin with cholesterol is independent of pH from pH 4.5 to 8, but is dependent on the method of isolation and preparation of the filipin complex. The filipin complex isolated from organic solvents does not interact with sterols—using fluorescence as a criteria of interaction—but it will interact with sterols after prolonged standing in aqueous solution or heating to 50° for 2 hr. These studies strongly indicate that filipins can exist in conformational states or bonded conditions such as dimers that do not bind sterols in a specific manner and that such forms undergo changes in aqueous solutions to a form or forms that can interact specifically with cholesterol and other 3β-hydroxy sterols.

Many polyene antibiotics can produce lethal permeability alterations in microorganisms containing sterols in their membranes (Whitfield *et al.*, 1955; Perritt *et al.*, 1960; Gottlieb *et al.*, 1960; Lampen and Arnow, 1961; Johnson *et al.*, 1962; Ghosh, 1963; Weber and Kinsky, 1965; Child *et al.*, 1969). These polyenes can also lyse erythrocytes (Kinsky *et al.*, 1962, 1967; Kinsky, 1963), and alter the structure and permeability of liposomes (Weissmann and Sessa, 1967; Sessa and Weissmann, 1968), and model membranes (Demel *et al.*, 1965; Van Zutphen *et al.*, 1966, 1971; Demel, 1968; Kinsky, 1970). Effects *in vitro* include stimulation of glucose utilization in mammalian fat cells (Kuo, 1968), decreased DNA synthesis and cellular respiration in Ehrlich ascites and Novikoff hepatoma tumor cells (Mondovi *et al.*, 1971), increased phospholipid turnover in beef thyroid slices (Larsen and Wolff, 1967), and rupture of beef spermatozoa (Morton and Lardy, 1967). Some polyenes, especially filipin, decrease serum cholesterol levels in dogs and chicks (Schaffner and Gordon, 1968; Fisher *et al.*, 1969), reduce canine prostatic hyperplasia (Gordon and Schaffner, 1968), mimic vitamin D mediated calcium transport in chick ileum (Adams *et al.*, 1970; Wong *et al.*, 1970), act as larvicides and chemosterilants in some insects (Sweeley *et al.*, 1970; Schroeder and Bieber, 1971), and are toxic to snails (Seneca and Bergendahl, 1955). The mechanism of polyene action that is suggested by these studies is the binding to sterols, free or membrane bound, by polyene macrolides. Spectrophotometric evidence for complex formation has been presented (Lampen *et al.*, 1960; Gottlieb *et al.*, 1961; Norman *et al.*, 1971, 1972) but interpretation of such data is difficult because reduction of the absorption peaks by sterols does not correlate with the ability of the sterol to alter the fungicidal activity of filipin (Gottlieb *et al.*, 1961). No spectral shifts were noted. Decreases in absorbance of filipin could indicate lowered solubility in aqueous solution (Kinsky, 1967), and, as shown herein, can be due to small differences in experi-

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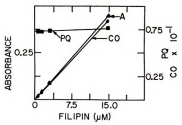

FIGURE 1: Affect of dilution on A, CO, and PQ of filipin. Filipin was prepared from a 14.8 μM stock solution in distilled water. Each filipin solution was excited at 338 nm and fluorescence emission was monitored at 496 nm. (Δ) A (absorbance), (○) CO (corrected fluorescence), and (□) PQ (partial quantum efficiency).

mental conditions. Fluorescence investigations, however, provide definitive evidence for the binding of cholesterol to the filipin complex (Schroeder *et al.*, 1971). Herein, the interaction of filipin and pimaricin with several sterols is demonstrated by simultaneous measurement of absorbance (A), corrected fluorescence (CO), and partial quantum efficiency (PQ) by means of a unique computer-centered fluorimeter.

Experimental Section

Materials. Filipin, 86% pure, was donated by the Upjohn Co., Kalamazoo, Mich. The antibiotic was purified as described previously (Schroeder *et al.*, 1971) and dissolved in distilled water (2.95 μM). Pimaricin (1.37 μmoles/mg (91%)) was kindly provided by the American Cyanamid Co., Princeton, N. J. Nystatin (4300 μ/mg (78%)) and amphotericin B (1.06 μmoles/mg (97%)) were generous gifts of Squibb Laboratories, New Brunswick, N. J. Pimaricin and amphotericin B solutions were prepared by dissolving 1.37 and 1.06 μmoles, respectively, per 100 ml of distilled water. Nystatin was prepared by dissolving 0.839 μmole (1 mg) in 100 ml of pH 4 citrate–phosphate buffer (0.05 M) as described by Lampen *et al.* (1960). The methanol degradation product of filipin was prepared and purified as per Rickards *et al.* (1970). It was dissolved in distilled water (0.36 μmole/100 ml). Filipin isomers were separated by partition chromatography (Bergy and Eble, 1968), dissolved in distilled water, and treated identically with the filipin complex.

Steroids were purchased as follows: 5-cholesten-3β-ol (cholesterol), 5-cholesten-3β-ol 3-palmitate (cholesterol palmitate), ergosta-5,7,22-trien-3-ol (ergosterol), and 17-hydroxyl-11-dehydrocorticosterone (cortisone) from Sigma Chemical Co., St. Louis, Mo.; 5α-cholestan-3β-ol (cholestanol) from Chemed Inc., Odenton, Md.; androstan-3β-ol-17-one, 24-ethyl-5,22-cholestadien-β-ol (stigmasterol), and 24-ethyl-5-cholesten-3β-ol (β-sitosterol) from California Corp. for Biochemical Research, Los Angeles, Calif.; 5α-cholestan-3α-ol (epicholestanol) and 5-cholesten-3α-ol (epicholesterol) from Schwarz-Mann Co., Orangeburg, N. Y.

The following buffers were prepared using reagent grade chemicals 0.10 M: pH 2.6–7.0, citrate–phosphate; pH 8 and 11, phosphate; pH 9, Tris; and pH 10, glycine.

Methods. All polyene antibiotic solutions were stored in the dark at 4°. Nystatin, pimaricin, and amphotericin B solutions were used on the day of preparation. Unless otherwise stated, 10 ml of each polyene solution was incubated in the dark with 1 mg of steroid at 50° for 2 hr. Partial quantum efficiency (PQ), corrected fluorescence (CO), and absorption spectra were determined as described by Holland and Timnick (1972).

For the major part of this work, three output quantities from the computer were used. These quantities are defined as follows: (1) A = absorbance of the sample in solution and is equivalent to values obtained with conventional spectrophotometric instruments; (2) CO = corrected fluorescence. This quantity is a linear function of the number of quanta fluoresced per number of quanta in the excitation beam and is dependent upon the specific instrumental parameters used for the measurement; (3) PQ = partial quantum efficiency. This quantity is a linear function of the number of quanta fluoresced per number of quanta absorbed. PQ is obtained from excitation scans only. If the fraction of the total fluorescence detected for a specific fluorophore is known and if the instrumental parameters are accurately determined (Holland, 1971), this quantity can yield the total quantum efficiency. In practice, it has been more convenient to use PQ to determine relative quantum efficiency changes. Relative PQ measurements have been employed for this investigation.[1] Absorbance for filipin is expressed as A_{328}, an absorption maximum, or as a ratio of two absorbance maxima, A_{338}/A_{305} (Lampen *et al.*, 1960). Absorbance values for pimaricin are similarly expressed as A_{319}, or as a ratio of two absorbance maxima, A_{319}/A_{329}. All measurements were made at 23–24°.

Results

Effect of Dilution on Absorbance, Corrected Fluorescence, and Partial Quantum Efficiency of the Filipin Complex. Changes in A, CO, and PQ of the filipin complex in aqueous solution are monitored at 338 nm. This wavelength corresponds to a major absorbance maximum for filipin. Figure 1 shows that both A and CO decrease linearly with dilution through the range 14.8–0.74 μM; however, the PQ of filipin, as predicted by theory (Holland Timnick, 1972), is essentially independent of concentration. PQ decreased approximately 2%. Similar results are obtained when A, CO, and PQ are determined at 356 nm, the other major absorption maximum of filipin.

Effect of Cholesterol on Fluorescence Emission of Filipin, Nystatin, and Pimaricin. In the presence of cholesterol, the fluorescence emission spectrum of some polyenes is altered and these changes are different for each polyene. Cholesterol decreases the fluorescence emission of the filipin complex (Figure 2A), but apparently has little effect on its methanol degradation product which is a very weak fluorophore (Figure 2B). Cholesterol and cholesterol palmitate did not change the A, CO, and PQ of the methanolic degradation product. The fluorescence emission of nystatin decreases slightly in the presence of cholesterol (Figure 2C). The fluorescence emission of pimaricin, a polyene that fluoresces only weakly, is increased over 80-fold when cholesterol is added to the solution (see Figure 2D). The fluorescence emission of amphotericin (data not shown) is less than 1% that of the filipin complex, and fluorescence changes with cholesterol are small—decreases of approximately 10%.

Stoichiometry of the Filipin–Cholesterol Interaction. The purity of the filipin complex used was approximately 96% (Whitfield *et al.*, 1955; Bergy and Eble, 1968). The molecular

[1] Since the relationship between A and PQ is not linear and absorbance may occur by species other than the fluorophore, it should be noted that there exists no convenient mathematical correlation between the two quantities. In similar chemical systems, changes in CO represent the gross effects of changes in concentration, absorptivity, and quantum efficiency of the fluorophore, but changes in PQ represent changes in the ratio of absorbance between the fluorophore and any chromophore that may be present.

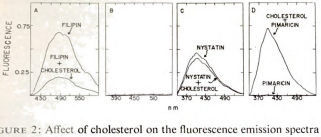

FIGURE 2: Affect of cholesterol on the fluorescence emission spectra of filipin, the methanol degradation product of filipin, nystatin, and pimaricin. Ten-milliliter aliquots of the following polyenes were incubated for 2 hr at 50°, with 2.56 μmoles of cholesterol as described in the Experimental Section: (A) filipin (2.95 μM in distilled water), (B) methanol degradation product of filipin (3.6 μM in distilled water) with cholesterol (lower curve) and without cholesterol (upper curve), (C) nystatin (8.39 μM in distilled water), and (D) pimaricin (13.7 μM in distilled water). Filipin and the methanol degradation product of filipin were excited at 338 nm, nystatin at 323 nm, and pimaricin at 292 nm.

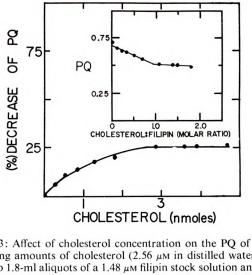

FIGURE 3: Affect of cholesterol concentration on the PQ of filipin. Increasing amounts of cholesterol (2.56 μM in distilled water) were added to 1.8-ml aliquots of a 1.48 μM filipin stock solution and incubated at 37° for 2 hr in the dark. Decreases in PQ and CO were determined at 338 nm. CO was corrected for dilution. Fluorescence emission was monitored at 496 nm. The insert shows the effect of increasing molar ratios of cholesterol:filipin on PQ. A molecular weight of 650.14 for the filipin complex (Schroeder and Bieber, 1971) was used to calculate the molar cholesterol:filipin ratio.

weight of the filipin complex used for these calculations in these experiments was an average of the molecular weights of the isomers based on their per cent composition in the complex; see Pandey and Rinehart (1970) for the per cent composition and Schroeder and Bieber (1971) for the calculation of average molecular weight. A known amount of filipin complex in water was titrated with cholesterol, and the per cent change in PQ and CO at 338 nm was determined. As shown in Figure 3, PQ decreased until 0.95 nmole of cholesterol was added per nmole of filipin. Similar results were obtained when the per cent change in CO *vs.* cholesterol concentration was determined. The stoichiometry, nmole of cholesterol to nmole of filipin, as shown in the insert of Figure 3, was 0.95. In three experiments, using 1.5 μM filipin, the stoichiometry varied between 0.8 and 0.95. When 3.0 and 6.0 μM filipin were used, the stoichiometry was 1.0 and 0.8, respectively.

Interaction of the Individual Filipin Components with Cholesterol. Filipin is a mixture of four components, two of which are conformational isomers (isomers III and IV), and two of which differ by one and two hydroxyl groups, respectively (isomers II and I) (see Pandey and Rinehart, 1970). The four major filipin components separated by the column chromatography system of Bergy and Eble (1968) interact with cholesterol, but components II, III, and IV, which comprise 96% of the filipin complex, decrease CO 53, 43, and 65%, respectively, and also decrease PQ 35, 48, and 49%, respectively, when incubated with cholesterol (see Table I). Component I apparently interacts slightly with cholesterol as shown by decreases in CO and PQ of 3 and 11%, respectively. An average of the PQ values of the four filipin components (normalized for per cent composition of each component in the filipin

TABLE I: Interaction of Filipin Components with Cholesterol.[a]

Expt	% Decrease in CO (at 338 nm)	% Decrease in PQ (at 338 nm)
Filipin component I + cholesterol	3	11
Filipin component II + cholesterol	53	35
Filipin component III + cholesterol	43	48
Filipin component IV + cholesterol	65	49

[a] Filipin components were separated by partition chromatography, as described in the Experimental Section. Components I, II, III, and IV were dissolved in distilled water 0.5, 1.4, 1.4, and 0.85 μM, respectively. Aliquots (10 ml) of each solution were incubated with and without 2.59 μmoles of cholesterol at 50° for 2 hr. Changes in CO and PQ were measured at 338 nm. Fluorescence emission was monitored at 496 nm.

TABLE II: Effect of the Length of Storage in Aqueous Solution and Temperature on PQ Changes of Filipin.[c]

Filipin Age	Absorbance Ratio of Filipin	% Decrease in PQ with Cholesterol[a]	% Decrease in PQ with Cholesterol[b]
2 hr	1.96	4	24
12 hr	1.82	2	35
2 days	1.55	40	38
7 days	1.40	38	39
4 months	0.73	8	9

[a] 10-ml aliquots of each solution were treated with 2.59 μmoles of cholesterol for 2 hr in the dark at 24°. [b] 10-ml aliquots of each solution were treated with 2.59 μmoles of cholesterol for 2 hr in the dark at 50°. The per cent decrease in PQ of filipin treated with cholesterol was determined at 338 nm. Fluorescence emission was monitored at 496 nm. [c] Filipin solutions (2.95 μM in distilled water) were kept in the dark at 4° for the times indicated. The absorbance ratio (A at 338 nm/A at 305 nm) of filipin was measured as described in the Experimental Section.

TABLE III: Effect of Temperature on A, CO, and PQ of Filipin and Filipin Plus Cholesterol in Aqueous Solutions.[a]

Expt	Expt Conditions	Temperature of the Principal Incubation: 4°, 72 hr A	CO	PQ	24°, 72 hr A	CO	PQ	4°, 64 hr; Then 37°, 8 hr A	CO	PQ	4°, 70 hr; Then 50°, 2 hr A	CO	PQ
1	Filipin	0.138	1.07	0.78	0.144	1.26	0.79	0.117	1.03	0.76	0.102	0.79	0.72
2	Filipin: 2 hr at 50° (dark)	0.103	0.84	0.72	0.098	0.85	0.72	0.103	0.87	0.72	0.102	0.85	0.72
3	Filipin + cholesterol: 2 hr at 50° (dark)	0.055	0.46	0.38	0.059	0.46	0.38	0.054	0.46	0.37	0.059	0.46	0.38

[a] In expt 1 for the principal incubation, each of four 10-ml aliquots of filipin (2.95 μM in distilled water) was incubated in the dark at one of the following conditions: 4°, 72 hr; 24°, 72 hr; 4°, 64 hr, followed by 37°, 8 hr; 4°, 70 hr, followed by 50°, 2 hr. A, CO, and PQ were determined at 338 nm. Fluorescence emission was monitored at 496 nm. For expt 2, the four solutions treated from expt 1 were incubated an additional 2 hr in the dark at 50°. A, CO, and PQ were determined as above. In expt 3, the four solutions treated from expt 1 were incubated with 2.59 μmoles of cholesterol at 50°, 2 hr in the dark. A, CO, and PQ were determined as above.

complex) differs from the measured PQ of the total mixture by only 5%, indicating that there is little, if any, interactions between the individual filipin components in solution which affect fluorescence efficiency.

Relation of the Absorbance Ratio at 338–305 nm to the Cholesterol Binding Capacity of Filipin. The capacity of an aqueous solution of filipin to elicit changes in PQ due to a specific interaction of filipin with cholesterol can be monitored by measuring the absorbance ratio. Table II shows that the absorbance ratio (ratio of absorbance at 338 nm to that at 305 nm) of filipin in aqueous solutions decreases from 1.96 for newly prepared aqueous solutions to 0.73 after storage for four months at 4°. Cholesterol binding ability of filipin—determined by changes in PQ—increases to a maximum in 2 days, remains constant for several weeks, and then decreases slowly, as shown in Table II. Thus, the potential binding ability of filipin can be estimated from the absorbance ratio. Incubation of the apparent noncholesterol binding form of filipin at 50° for 2 hr converts filipin to a form that readily binds cholesterol (see Table II, last column). It is important to note that the filipin used in these experiments was a standard preparation that had been isolated and purified using organic solvents.

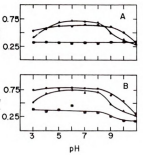

FIGURE 4: Effect of pH on the cholesterol-induced PQ changes of filipin. Filipin (2.95 μM) at pH 3–11 was prepared by diluting filipin (14.8 μM in distilled water) with buffer to a final buffer concentration of 0.05 M. Buffers were prepared as described in the Experimental Section. The solutions were then incubated for 2 hr at 50° with and without cholesterol as described in the Experimental Section A, CO, and PQ were determined at 338 nm. Fluorescence emission was monitored at 496 nm. (○) Unincubated filipin, (△) incubated filipin, and (□) filipin incubated with cholesterol.

Effect of Temperature on A, CO, and PQ of Filipin and Mixtures of Filipin Plus Cholesterol in Aqueous Solutions. The absorbance and corrected fluorescence values of aqueous filipin solutions vary as much as 30 and 26%, respectively. The variations are dependent on temperature and other experimental conditions. For example, filipin incubated in water for the time intervals given in Table III, expt 1 at 4, 24, 37, and 50° has CO values of 1.07, 1.26, 1.03, and 0.79, respectively. Similar responses to temperature in PQ were obtained (see Table III, expt 1). Incubation at 50° for 2 hr of filipin solutions used for expt 1 gives a form of filipin with almost identical A, CO, and PQ values (see Table III, expt 2). Thus, aqueous filipin solutions that have different spectral properties—hence, different forms—can be converted to a form that has reproducible spectral properties. This form of filipin is the form that interacts with cholesterol as shown by equal decreases in A, CO, and PQ (Table III, expt 3).

Effect of pH on Filipin–Cholesterol Interaction. Corrected fluorescence and partial quantum efficiency changes of filipin interacting with cholesterol are independent of pH over the range pH 4.5–8.0, as shown in Figure 4. At high pH, the CO of the filipin complex is decreased until no interaction with cholesterol can be detected by CO changes (Figure 4A); however, PQ shows that even at pH 11, cholesterol interacts with filipin (Figure 4B). At pH's lower than 4.5, CO and PQ values of filipin, but not of cholesterol-bound filipin, decrease.

Factors Influencing the Filipin–Cholesterol Complex. Other experiments have shown that organic solvents and extraction of filipin from the filipin–cholesterol complex affect the absorbance ratio, CO, and PQ of filipin. Methanol (50% final concentration) disrupts the filipin–cholesterol complex and CO increases to 80% of the value for unbound filipin, and PQ is the same as that of unbound filipin. The PQ of filipin, without cholesterol, in 50% methanol increases only 2%. This indicates that methanol disrupts the complex and some of the filipin was lost from solution without binding to cholesterol. Extraction of the filipin–cholesterol complex with 1-butanol, followed by methanol extraction of the residue after removal of 12-butanol gives a product that has, in distilled water, the same absorbance ratio, CO, and PQ as unbound filipin. Thus,

TABLE IV: Interaction of Filipin with Sterols.[a]

Steroid Added	% Decrease: Absorbance Ratio	In A	In CO	In PQ
None	1.38			
Cholesterol (3β-OH)	0.92	55	62	32
Epicholesterol (3α-OH)	1.32	24	36	13
Cholestanol (3β-OH)	0.96	33	57	36
Epicholestanol (3α-OH)	1.40	31	27	2
β-Sitosterol (3β-OH)	0.58	41	85	52
Stigmasterol (3β-OH)	0.79	39	71	62
Ergosterol (3β-OH)	1.02	35	28	5
Cholesterol palmitate	1.50	9	5	4
Cortisone	1.70	6	2	3
Androstan-3β-ol-17-one	1.34	3	14	4

[a] Ten-milliliter aliquots of filipin (2.95 μM in distilled water) were incubated with and without 1 mg of sterol at 50° for 2 hr. Absorbance ratio, A, CO, and PQ were determined at 338 nm. Fluorescence emission was monitored at 496 nm.

TABLE V: Interaction of Pimaricin with Sterols.[a]

Steroid Added	Absorbance Ratio	% of Pimaricin + Cholesterol CO	% of Pimaricin + Cholesterol PQ
None (unincubated)	2.85	1	1
None	3.02	1	1
Cholesterol (3β-OH)	1.39	100	100
Stigmasterol (3β-OH)	1.34	81	80
β-Sitosterol (3β-OH)	1.14	69	76
Cholesterol palmitate	2.76	2	1
Cortisone	2.76	2	1
Epicholesterol (3α-OH)	1.55	10	12
Epicholestanol (3α-OH)	1.53	11	7

[a] Ten-milliliter aliquots of pimaricin (13.7 μM in distilled water) were incubated with and without 1 mg of sterol at 50° for 2 hr. Pimaricin interacting with cholesterol fluoresces strongly and is arbitrarily assigned CO and PQ values of 100%. Absorbance ratio, CO, and PQ were determined at 319 nm as described in the Experimental Section. Fluorescence emission was monitored at 402 nm.

the filipin–cholesterol complex can be disrupted and the reisolated filipin exhibits properties of the original filipin. If filipin is extracted from aqueous solutions, reduced to dryness *in vacuo*, and dissolved in *tert*-butyl alcohol, lyophilized, and then dissolved in distilled water, the absorbance ratio, CO, PQ are the same as unbound, unreactive filipin; but the absorbance ratio differs by 28% from that of the extracted filipin. Thus, the absorbance ratio of filipin is affected by organic solvents and by the treatment after extraction from the filipin–cholesterol complex, but PQ is affected only by whether filipin is in the bound or unbound form.

Interaction of Filipin with Sterols. Filipin interacts strongly with 3β-hydroxy sterols as shown in Table IV. Cholesterol, cholestanol, β-sitosterol, and stigmasterol decrease the absorbance ratio, A, CO, and PQ of filipin by more than 30%. β-Sitosterol and stigmasterol, which contain one more double bond than cholesterol, interact more strongly with filipin (52 and 62% decreases in PQ, respectively) than any of the 3β-hydroxy sterols tested. Both sterols also contain C-24 ethyl groups. Ergosterol interacts only weakly as shown by the 5% decrease in PQ. This may be due to cleavage of the ring structure of ergosterol by ultraviolet irradiation, which could prevent interaction with filipin. Epicholesterol (3α-hydroxy) has much less of an effect on PQ. Cholesterol palmitate (the 3β-ester of cholesterol and palmitic acid), cortisone (3-keto), and androstane (3β-ol-17-one) do not, or only weakly, interact with filipin as shown by the small decreases in A, CO, and PQ. Sterols such as α-cholestanol do not decrease PQ, but reduce A and CO 31 and 27%, respectively.

Binding of Sterols by Pimaricin. Addition of 3β-hydroxy sterols such as cholesterol, stigmasterol, sitosterol, and cholestanol to pimaricin enhances its fluorescence 80- to 100-fold, as shown in Table V. β-Cholestanol increases CO and PQ more than 10 times as much as cholesterol (data not shown). Epicholesterol (3α-hydroxy), epicholestanol (3α-hydroxy), cholesterol palmitate, and cortisone interact only weakly (12, 7, 1, and 1% increases in PQ, respectively). It should be noted that sterols which interact with pimaricin also interact with filipin.

Interaction of Other Polyenes with Cholesterol. Table VI indicates that nystatin and amphotericin B also interact with cholesterol. PQ decreases 33 and 22%, respectively. Cholesterol palmitate decreases the PQ of amphotericin B by 5% while the PQ of nystatin is lowered 1%.

Discussion

Since much of this research embodies measurements of absorbance, corrected fluorescence, and a unique quantity, partial quantum efficiency, it would be appropriate to discuss the nature of these measurements and their significance. These parameters have been obtained from a single instru-

TABLE VI: Interaction of Nystatin and Amphotericin B with Cholesterol.[a]

Polyene	Sterol Added	% Decrease in PQ: At 323 nm	At 355 nm
Nystatin	Cholesterol	33	
Nystatin	Cholesterol palmitate	1	
Amphotericin B	Cholesterol		22
Amphotericin B	Cholesterol palmitate		5

[a] Ten-milliliter aliquots of nystatin (8.39 μM in pH 4 citrate–phosphate buffer, 0.05 M) were incubated with and without sterol for 2 hr at 30°. Each solution contained 0.3% dimethyl sulfoxide. PQ was determined at 323 nm. Fluorescence emission was monitored at 402 nm. Ten-milliliter aliquots of amphotericin B (10.6 μM in distilled water) were incubated with and without 1 mg of sterol for 2 hr at 50°. PQ was determined at 355 nm. Fluorescence emission was monitored at 475 nm.

ment system. This system is a computer-centered combination spectrophoto-spectrofluorimeter that is capable of simultaneous absorption and fluorescence measurements (see Holland (1971) and Holland and Timnick (1972)). PQ, when monitored as a function of excitation wavelength, produces a value that is linearly related to the total quantum efficiency. For a single fluorophore, this value will be constant across an excitation band and independent of concentration within the measuring capability of the instrument system. This concentration independence presents a powerful new approach to detection of conformational, bonding, or solvation changes.

For fluorophores in the presence of nonfluorescing chromophores, the PQ will deviate from the theoretical linear shape and tend to reveal the absorption bands of the fluorophore (Holland, 1971). This is precisely the case encountered in the filipin complex investigation (Schroeder *et al.*, 1971).

In mixtures of fluorophores that absorb and fluoresce within the same wavelength windows, the total PQ of the system will be the sum of the concentration normalized components of the mixture provided no interaction occurs between the various components, as illustrated by the components of the filipin complex (Table I).

The data show that changes in PQ, the partial quantum efficiency, of filipins and pimaricin on addition of sterols can be used to study an interaction of these polyene antibiotics with sterols. *Interaction in this discussion refers to the specific interaction between polyenes and sterols that alters fluorescence.* Since PQ is independent of concentration, this eliminates many of the problems inherent in quantitating changes in fluorescence or absorption of compounds that are sparingly soluble in water.

The specific interaction of filipin with cholesterol, that is detected fluorometrically, depends on how filipin was treated prior to exposure to sterols. Freshly prepared aqueous solutions of the filipin complex that were isolated using organic solvents or dissolved in solvents such as methanol do not show changes in PQ immediately. Thus, these solutions of filipin apparently do not interact with sterols. If such solutions are allowed to stand for long periods of time or are heated for 2 hr at 50°, the ratio of the absorption maxima at 338–305 nm changes from 1.96 to 1.55. Such solutions readily interact with sterols as shown by changes in PQ (see Table II). Thus, the absorbance ratio is a sensitive measure of the capability of filipin to bind sterols in a specific manner that affects fluorescence properties. Interactions between filipin and sterols, specific or nonspecific, that do not alter fluorescence properties of the system would not be detected.

A likely explanation of these results is that filipin, isolated using organic solvents, dissolved in alcohols or lyophilized from *tert*-butyl alcohol, acquires a conformation or bonding that is unfavorable for complexing with sterols in a specific manner.

The above-mentioned results appear to be entirely consistent with the results of Amman *et al.* (1955) and Lampen *et al.* (1960) and provide a possible explanation for their findings. These investigators showed greater than an 18-hr lag in the fungicidal activity of filipin toward *Candida albicans* and *Saccharomyces cerevisiae*. Weber and Kinsky, (1965) also observed a 36-hr lag in the inhibition of growth of *Mycoplasma laidlawii;* however, no growth inhibition of *Mycoplasma* was observed in previous studies (Kinsky (1963, 1964)). Our results indicate that such a lag could be partly, if not entirely, caused by starting with a form of filipin that does not bind sterols. In the aqueous media, this could be slowly converted to a sterol-binding form.

It must be emphasized that each family of polyene antibiotics apparently responds differently to sterols. As shown in Figure 2, cholesterol causes a decrease in PQ of filipin, but pimaricin, another polyene, fluoresces very little in aqueous solution. Addition of cholesterol to pimaricin in water causes at least an 80-fold increase in PQ as well as a marked increase in fluorescence. In contrast, changes in PQ and fluorescence of nystatin and amphotericin B were small when sterols were added.

We have concluded that the higher the affinity of the sterol for filipin, the greater will be the decrease in PQ. This conclusion appears to be supported by the biological studies of Gottlieb and coworkers (1960). They found that certain sterols were very effective in overcoming or preventing the fungicidal effects of the filipin on *S. cerevisiae*, but other sterols were less effective and some were ineffective. Our results, as well as the recent results of Norman *et al.* (1972), correlate well with their studies. Sterols such as cholesterol, cholestanol, stigmasterol, and β-sitosterol, which decrease the PQ of filipin greatly were effective in preventing the fungicidal effects of filipin. Sterols such as cortisone and androstan-3β-ol-17-one, did not cause much change in PQ. Gottlieb and coworkers (1960) found that their preparation of androstan-3β-ol-17-one prevented fungicidal effects of filipin. They noted that this finding was not consistent with the remainder of their biological data.

The one exception is ergosterol. It can prevent fungicidal effects of filipin but does not alter PQ. The failure to alter PQ is most likely caused by the well-known rapid photolytic cleavage of the β ring of ergosterol by ultraviolet light (Fieser and Fieser, 1959). Ergosterol should be destroyed in the cuvette during our assay.

Esterification of the β-hydroxyl group of cholesterol with palmitate prevented interaction with filipin. The results strongly indicate that the 3β-hydroxyl group of sterols is of prime importance for interacting with filipin. This agrees with conclusions of others (see Gottlieb *et al.*, 1960; Norman *et al.*, 1972). If the 3-hydroxyl group is α, smaller changes in PQ occur. For example, cholestanol, a 3β-hydroxy sterol, reduced PQ 36% but the α isomer, α-cholestanol, reduced PQ 2%. Similarly, cholesterol reduced PQ 32% and epicholesterol, the α isomer, reduced PQ 13% (see Table IV). The 3β-hydroxyl group also appears to be required for the interaction of pimaricin with sterols. The two α isomers investigated affected PQ only 10% that of the β isomers; see Table V.

The alkyl side chain of sterols appears to be essential for interaction of sterols with filipins. Compounds such as cortisone and androstan-3β-ol-17-one which do not have the long alkyl hydrophobic side chain do not alter PQ significantly. The conjugated pentaene double bonds appear to be essential for binding sterols because the methanol degradation product—a tetraene containing epoxide (see Rickards *et al.*, 1970)—does not show changes in PQ on addition of sterols nor does it have fungicidal activity (see Whitfield *et al.*, 1955). The lactone of filipin is also required for interaction with sterols. Cleavage of the lactone destroys the fungicidal effects of filipin (Kinsky *et al.*, 1967). Filipin loses 74% of its antifungal activity at pH 2.5 and 81% at pH 10.3 after storage for 18 hours (see Amman *et al.*, 1955). Conditions which should cleave the lactone, high and low pH's prevented PQ and fluorescence changes caused by cholesterol (see Figure 4).

The above-mentioned interactions, the reversal of the cholesterol–filipin interaction by methanol, indicate that specific hydrogen and hydrophobic bonds are major forces involved in the interaction of filipin with cholesterol. The fact

that the absorption maxima of filipin are decreased but *not* shifted on interaction with cholesterol indicates that no bond changes are occurring in the vicinity of the fluorophore; rather, the concentration of the fluorophore is reduced or the absorptivity of the molecule has been diminished. The change in the ratio of the absorption peaks indicates that a simple reduction in concentration is not an adequate explanation; however, analysis of these changes does not clearly delineate any interactions that may be involved. Since PQ is independent of concentration, it does present a measure of any interactions between the molecules of filipin and the various sterols occurring in the solution itself. For example, an analysis of the measured PQ for increasing amounts of cholesterol when added to an aqueous solution of filipin reveals a continuing change until a molar ratio of approximately 1:1 is attained.

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Appendix III

Reversible Interconversions of Sterol-Binding and Sterol-Nonbinding Forms of Filipin as Determined by Fluorimetric and Light Scattering Properties

by

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REVERSIBLE INTERCONVERSIONS OF STEROL-BINDING AND STEROL NONBINDING FORMS OF FILIPIN AS DETERMINED BY FLUORIMETRIC AND LIGHT SCATTERING PROPERTIES[†]

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RUNNING TITLE: Active and Inactive Forms of Filipin

FOOTNOTES

[1]This method has been detailed by Schroeder et al. (1971, 1972) and was initially presented at the American Society of Biological Chemists Meeting, June, 1971. Copies of the presentation are available from L. L. Bieber or F. Schroeder. Use of this method to confirm the presence of predominantly active or inactive filipin in aqueous systems has been successful in every application that we have tried. Recently, this method apparently has been adopted by others (Bittman and Fischkoff, 1972).

ABSTRACT: Combined fluorimetric and Tyndall light scattering measurements have confirmed the existence of different forms of filipin; filipin herein refers to the filipin complex. In these studies, the ratio of two of the absorbance peaks of the filipin molecule A_{338}/A_{305} is a consistent indicator of the presence of active and inactive forms. The partial quantum efficiency (PQ) was used to measure the interaction between filipin and sterols. Light scattering (R_{90}) was used to monitor changes in the aggregate size of the polyene. These analyses have shown that filipin can occur in "active forms" -- those that interact with cholesterol -- and "inactive forms" -- those that do not interact with cholesterol -- in aqueous solutions [Schroeder, F., Holland, J. F., and Bieber, L. L. (1972) Biochemistry 11, 3105]. Inactive filipin is converted to the active form by incubation at 50°C for two hours. The conversion of the nonbinding to the binding form in aqueous systems follows a first-order rate law and appears to involve conformational changes that are time- and temperature-dependent. The conversion of active to inactive forms of filipin is reversible and independent of the presence or absence of large micelles of filipin. Methods using nonaqueous solvents to isolate and purify filipin consistently produce this nonbinding form. Short-chain alcohols disrupt aqueous filipin micelles in the order of effectiveness: Propanol > ethanol > methanol. Light scattering of filipin systems is decreased approximately by an order of magnitude with addition of excess cholesterol. Many of the apparent discrepancies in the literature concerning the interaction of filipin with sterols and sterol-containing membranes may result from the use of the sterol-nonbinding form of filipin and the use of concentrations so high that the observations are independent of sterol-binding properties.

INTRODUCTION

Several groups of investigators using filipin at a concentration of 10^{-5} to 10^{-6} M concluded that the antibiotic had no significant effect on subcellular organelles such as mitochondria isolated from fungal (Gottlieb et al., 1961; Kinsky et al., 1965) or mammalian sources (Lardy et al., 1958; Morton and Lardy, 1967). At 10^{-2} to 10^{-4} M, filipin bound extensively to mitochondria and inhibited mitochondrial respiration, ruptured lysosomes, and also inhibited cancer cell growth (Balcavage et al., 1968; Haksar and Peron, 1972; Mondovi et al., 1971). Experiments on phospholipid spherules (liposomes) indicated that, at concentrations of 10^{-3} to 10^{-5} M, the filipin complex interacted equally well with liposomes whether or not cholesterol was present (Weissmann and Sessa, 1967; Sessa and Weissman, 1968). In contrast, other investigators who used filipin at 10^{-5} to 10^{-6} M (Kinsky et al., 1968; Kleinschmidt et al., 1972; Norman et al., 1972a) found a requirement for the presence of cholesterol. At 10^{-8} to 10^{-5} M concentrations, filipin interacts preferentially with monolayers and bilayers containing cholesterol (Demel et al., 1965; Van Zutphen et al., 1971), but at 10^{-2} to 10^{-4} M filipin concentrations, the antibiotic is able to interact with monolayers of pure lipid in the absence of cholesterol (Demel et al., 1968; Kinsky et al., 1968; Van Zutphen et al., 1971).

It has been suggested that filipin and other polyenes may exist as micelles or aggregates in aqueous solution (Lampen et al., 1959 Kinsky, 1967; Norman et al., 1972b; Schroeder et al., 1972). When the concentration of a micelle-forming molecule is much greater than the critical micelle concentration (CMC), the molecule could act as a detergent (Fendler and Fendler, 1970). The ability of filipin, a neutral amphipathic compound, to act as a detergent at concentrations much higher than the CMC could

explain some of the paradoxical results obtained by previous workers who have used extreme differences in concentrations.

Fluorescence changes have been used to demonstrate the occurrence of sterol-binding (herein referred to as the active form) and sterol-nonbinding (inactive form) forms of filipin (Schroeder et al., 1971, 1972) and to monitor the interaction of filipin with sterols (Schroeder et al., 1971, 1972). This technique has recently been used to study the interaction of filipin with membranes (Drabikowski et al., 1973) and to monitor the interaction of other polyenes with sterols (Schroeder et al., 1972; Bittman and Fischkoff, 1972; Strom et al., 1972).

It is apparent that the capacity of filipin to interact with sterols is dependent in a complex manner on temperature, age of solution, and other variables (Sessa and Weissman, 1968; Norman et al., 1972; Schroeder, et al., 1972). Herein, we show that the ability of the filipin complex to interact with cholesterol is independent of the presence of large filipin micelles, and that the conversion of the sterol-nonbinding form to the sterol-binding form is a first order process which may be indicative of a conformational change.

Materials and Methods

The filipin complex was a gift of the Upjohn Company, Kalamazoo, Michigan. This material was 86% pure; therefore, the antibiotic was further purified as previously described (Whitfield et al., 1953; Schroeder et al., 1971, 1972). The filipin used herein is a complex of several components (Bergy and Eble, 1968). The three principal components (96% of the filipin) all interact with cholesterol (Schroeder et al., 1972).

Cholesterol was purchased from Sigma Chemical Co., St. Louis, Missouri. Purity of the sterol was checked by thin-layer chromatography and gas

chromatography.

Filipin solutions were stored at 4°C in the dark for the times indicated in the figure legends. The cholesterol-binding ability of the antibiotic was measured as described earlier (Schroeder et al., 1971, 1972). Unless otherwise stated, absorbance, corrected fluorescence (CO), partial quantum efficiency (PQ), and Tyndall light scattering (R_{90}) were determined at 50°C on 3 ml of the filipin solution (2.95 μM) before and after mixing with 20-fold molar excess cholesterol as previously described (Schroeder et al., 1972). After 2 hours at 24°C in the dark (Jones and Gordon, 1972), the absorbance, CO, PQ, and R_{90} were determined at 24°C on all filipin solutions containing methanol, ethanol, propanol, p-dioxane, or DMF. These solutions were also used for determination of CMC. Light scattering due to solvent was corrected (Krescheck et al., 1966). The dimensionless light scattering factor (R_{90}), absorbance ratios, CO, and PQ were measured on the computer-centered spectrofluorimeter of Holland et al. (1972). The absorbances were measured at 305 and 338 nm, the light scattering at 338 nm, and the CO and PQ were measured at an emission wavelength of 496 nm with the excitation wavelength at 338 nm. This instrument treats scattered radiation in an analogous manner to fluorescence. The routine used for obtaining R_{90} values was the corrected fluorescence routine, F_{co}; the only difference being that the two monochromators were scanning in wavelength synchrony. Hence, R_{90} was determined and is equivalent to R_0, where 0 = 90° (Tinker and Saunders, 1968). R_{90} is corrected for the inner filter effect, and all intensities are measured in terms of quanta. The instrument was calibrated against water and benzene.

Results

Light Scattering and PQ Measurements of Sterol-binding and Sterol-Nonbinding Forms of Filipin

A freshly prepared aqueous solution of filipin, as shown in Figure 1A, has an absorbance ratio (A_{338}/A_{305}) of 1.96 and light scattering at 338 nm is 41 x 10^{-6}. The interaction of this freshly prepared filipin -- isolated using organic solvents (Whitfield et al., 1965) -- with cholesterol in aqueous solution is delayed until the filipin is converted into an active form, as shown by the PQ curve of Figure 1A. PQ, which is altered by interaction of filipin with cholesterol, does not decrease until the antibiotic has been incubated for 35 minutes at 50°C. Maximal interaction, as measured by per cent decrease in PQ, requires 2 hours. Longer incubation of filipin with cholesterol does not further decrease PQ. In Figure 1A, filipin was incubated at 50°C for the times indicated by each point. Cholesterol was then added and values were determined after two minutes' delay. A similar PQ curve is obtained when cholesterol is added to filipin prior to the preincubation, as shown in Figure 1B. The conversion of inactive to active filipin follows first-order kinetics, as indicated in Figure 2. The data in Figure 2 were obtained form the PQ data of Figures 1A and 1B. The conversion of the inactive to the active form is temperature-dependent. As shown in Table I, at 24°, only 7% of the filipin is converted to the active form in two hours, while at 50°C, the conversion is completed during this interval.

The initial value of the light scattering in Figure 1A indicates that the filipin exists in an aggregated form. Incubation at 50°C for 35 minutes decreases light scattering by 77%, showing partial disruption of the large aggregates. This incubation also reduces the absorbance ratio from 1.96 to 1.7; however, PQ does not decrease upon the addition of cholesterol; hence the antibiotic is not binding cholesterol at this point. After 1.5 to 2.0 hours, light scattering of filipin is decreased by more than 90%,

the absorbance ratio is decreased to 1.5, indicating presence of the active form and the interaction with cholesterol is maximized as demonstrated by a 34% decrease in PQ.

As shown in Figure 1C, filipin stored for 60 days at 4°C in the dark immediately interacts with cholesterol, indicated by the 32% decrease in PQ. The low value of the absorbance ratio signifies that the active form is present and the high initial value of light scattering indicates that active filipin as well as inactive filipin can exist in micellar or aggregated form.

Effect of Temperature on the Activity and Light Scattering of Freshly Prepared Filipin Solutions. Incubation at 24°C instead of 50°C increases the time necessary for formation of the active form of filipin. This decrease in incubation temperature of inactive filipin results in a 12-fold increase in time required to produce the active form (see Table I).

Effect of Dimethylformamide on Activity and Light Scattering of Filipin. Dimethylformamide (DMF) is frequently used as a solvent for filipin, but as shown in Table II, filipin dossolved in DMF is initially inactive as is indicated by the absorbance ratio of 2.3. However, since the light scattering at 338 nm is only 1×10^{-6}, the filipin freshly prepared in DMF is not in the micellar form. If the DMF stock solution is stored for 2.5 days at 4° in the dark, the absorbance ratio indicates that the antibiotic is now active, as confirmed by PQ. The increase in the light scattering indicates that the filipin has aggregated.

Effect of Alcohols on the Activity and Light Scattering of Filipin. Methanol solubilizes filipin and also inactivates the antibiotic (Gottlieb et al., 1961; Norman et al., 1971). As the methanol concentration is increased, the absorbance ratio increases from 1.4 to 2.1, indicating that

filipin is being transformed from an active to an inactive form. Light scattering at 338 nm decreases from 50 x 10^{-6} to almost zero (see Figure 3A). As shown in Figure 3B, CO (corrected fluorescence) and PQ also increase as the methanol concentration is changed from 0 to 25%.

CMC values for filipin in 25% methanol were calculated from the data given in Figure 3 as well as by a determination of R_{90} versus increasing concentration of filipin in 25% methanol. The values obtained are 3.1 and 3.0 μM, respectively. The CMC of filipin in aqueous solution is 2.7 μM as shown in Figure 4.

Other experiments (data not shown) show that the minimum per cent alcohol required for disruption of both the active and inactive filipin micelle is linearly related to the number of methylene groups: propanol < ethanol < methanol. Increasing hydrophobicity of the alcohol increases its ability to solubilize filipin micelles. These results agree with those predicted by others (Emerson and Holtzer, 1967) that the micelle is held together by hydrophobic forces and its disruption is dependent on the solvent's power to solvate the hydrophobic monomeric species. The dielectric constant of the solvent appears to have little effect on the ability of the solvent to break up filipin micelles since the minimum per cent solvent required to break up the micelles is 29% w/w for p-dioxane (dielectric constant ε = 2.2) while that of methanol is 25% w/w (ε = 32.6).

Discussion

The results are consistent with our previous observations that filipin can occur in forms that bind and others that do not bind 3-β-hydroxy sterols and that these forms are interconvertible depending on the experimental conditions (Schroeder et al., 1971 & 1972).

The sterol-binding activity of filipin, as measured by its capacity

to immediately alter PQ when exposed to cholesterol, appears to be independent of the presence or absence of micelles. Nonmicellar, active filipin -- either monomers or small aggregates -- interacts with cholesterol; however, since micelles are not static, but exist in equilibrium with monomers (Krescheck et al., 1966; Jaycock and Ottewill, 1967; Bennion et al., 1969), with rate constants yielding half lives in the millisecond range (Bennion et al., 1969), any possible interaction of micelles of active filipin with sterols would not be detected with the present methodology.

The results of our investigations can be summarized as indicated in Scheme 1. It is logical to assume that inactive monomers or small molecular aggregates that are not detectable by light scattering are in equilibrium with micelles of inactive filipin. The decrease in absorbance ratio during incubation or long periods of standing suggests that filipin undergoes a time- and temperature-dependent change, most likely conformational, to active filipin form(s) which can also form micelles. The finding that the conversion of the sterol-nonbinding to the sterol-binding form follows first-order kinetics is consistent with a conformational change. Conformational changes of antibiotics dissolved in organic or aqueous solvents have been previously demonstrated with the cyclic peptides valinomycin and polymyxin B (Patel, 1973; Patel and Tonelli, 1973; Chapman and Golden, 1972). As is indicated by the dashed arrows in Scheme 1, alcohols disrupt the filipin:cholesterol complex and break up both types of micelles, always resulting in sterol-nonbinding forms of filipin, possibly monomers. Conversion to the inactive form is shown by the increase in absorbance ratio from less than 1.5 to about 2.1.

Although the data presented herein and previously by Schroeder et al. (1971, 1972) and by others (Bittman and Fischkoff, 1972; Norman et al., 1972; Drabikowski et al., 1973) are generally consistent with the model shown

in Scheme 1, some contradictions do exist and, thus, it seems appropriate to mention some potential problems that may occur with the sensitive fluorescence techniques.

1. Changes in Concentration of the Polyene. Any process that changes the concentration of the polyene in solution, such as precipitation or absorption to a solid surface or to a particle, will alter the intensity of fluorescence and absorption, thereby producing apparent, but not necessarily real, changes in these parameters, making these parameters of questionable value for studying molecular interactions unless the exact nature of the concentration changes are known. It is of importance to note that the parameter, PQ, is dependent upon the quantum efficiency of the fluorophore and independent of its concentration. In a study of this type where micelle formation, precipitation, and denaturation processes are prevalent, such a parameter has unique capability in the detection of binding or conformational changes within the vicinity of the fluorophore.

2. Use of High Concentrations of the Polyene. (a) Detergent Effects. Amphipathic compounds have a low CMC, which is 2.7 μM for filipin. At concentrations two to three orders of magnitude above the CMC, 10^{-4} M and greater, these compounds can be detergents. Detergent effects would make the interaction of the polyene non-specific rather than specific for sterols.

(b) Absorption Artifacts. At high concentrations of the polyene, the absorption of the primary excitation beam is so great that the observed fluorescence would actually increase with dilution, a classical example of the inner filter effect (Holland et al., 1973). With such conditions, it is easy to obtain apparent, but artifactual, increases in fluorescence by simple dilution or any other process which results in a lower total absorption in the sample cell. The instrument used for our studies corrects

for this artifact (Holland et al., 1972 & 1973), always presenting a more accurate measure of the actual fluorescence emission.

3. Use of the Inactive Form of Filipin. We have shown that, depending on the experimental conditions, filipin can exist in either a sterol-binding or a non-binding form. Use of the inactive form of filipin has produced anomalous results in the literature. For example, it has recently been reported that filipin interacts about equally well with cholesterol as epicholesterol, but a 50-fold molar excess of cholesterol and epicholesterol was needed to obtain maximum effects (Bittman and Fischkoff, 1972). This result is puzzling and appears to be contrary to previous results which have demonstrated a 1:1 stoichiometry between filipin and cholesterol interaction (Schroeder et al., 1972) and also appears to be inconsistent with results which show that a slight molar excess of cholesterol in the diet can prevent the larvicidal effects of filipin towards Musca domestica (Schroeder and Bieber, 1971). We have found that excess cholesterol produces little enhancement of fluorescence when compared to the situation where cholesterol equals the filipin concentration. Much of this discrepancy could also again be due to use of an inactive form of filipin. Although their samples were incubated for 2.5 hours, the temperature apparently was 15°. Our results indicate that incubating the inactive form of filipin in water for 2.5 hours at 50° is adequate to convert it to the sterol-binding form, but 2.5 hours at 24° is not adequate. At 15°, the rate of conversion to the active form would be much slower. The effect of sterol concentration on potentiating the conversion of inactive filipin to the sterol-binding form is not known, but the sterol appears to assist the conversion when present in large molar excess.

To preclude use of the inactive form, the absorbance ratio A_{338}/A_{308} can

be used to confirm the presence of predominantly active filipin. In aqueous solution, the ratio should not be greater than 1.6.[1]

4. Effects by Non-sterol Molecules on Filipin-produced Fluorescence. It seems likely that in complex systems such as membrane preparations, many factors would be present that could alter the fluorescence properties of filipin. For example, it has been reported that lecithin, in the absence of cholesterol, enhances the fluorescence intensity of filipin (Bittman and Reddy, 1971). This effect of lecithin would be in direct competition with the effect of cholesterol and could lead to paradoxical results if both species were to act on filipin simultaneously.

In another instance, it has been reported that the fluorescence of the tetraene polyent antibiotic, lucensomycin, is greatly altered by various organic solvents in the absence of sterols (Crifo et al., 1971). These and other interactive effects demand great care in the selection of experimental parameters and in the interpretation of the data obtained.

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TABLE I: Effect of Temperature on the Conversion of Filipin[a] from Inactive to Active Forms.

Incubation		R_{90} at 338 nm x 10^6	A_{338} nm/A_{305} nm	% Decrease in PQ_{338} nm with Cholesterol
Temperature (°C)	Time (hrs.)			
24	0.33	41	1.96	0
24	2.0	23	1.78	2
24	24.0	2	1.56	33
50	2.0	2	1.50	36

[a]Filipin (2.95 μM) was freshly prepared and incubated at 24°C or 50°C, as described in Methods. R_{90}, A_{338}/A_{305}, and PQ were determined as stated in Methods.

TABLE II: Effect of Dimethylformamide on R_{90} and Activity of Filipin[a]

Time at 4°C (days)	R_{90} at 338 nm x 10^6	A_{338} nm/A_{305} nm	% Decrease in PQ_{338} nm with Cholesterol
0	1	2.3	3
2.5	40	1.0	25

[a]Filipin (2.9 μM) solutions were prepared from fresh and 2.5-day old stock solutions of filipin in DMF (dimethylformamide), as described in Methods. R_{90}, A_{338}/A_{305}, and PQ were determined at 24°C as stated in Methods.

Fig. 1 - Time-Course for Conversion of Inactive Filipin to Active Filipin.

In A, filipin (2.95 μM) was freshly prepared. Three-ml aliquots containing 9 nmoles filipin were incubated at 50°C in a heated cuvette. At the times indicated by each point, the solution was mixed with 180 nmoles cholesterol and 2 min and values were determined. A_{338}/A_{305}, R_{90}, and PQ were determined at 50°C before and after addition of cholesterol, as described in Methods. In B, filipin (2.95 μM) was prepared as in A and incubated continuously at 50°C with cholesterol. PQ was determined two minutes after the times indicated by each point. In C, filipin (2.95 μM) was prepared from a 60-day old aqueous filipin solution stored at 40°C in the dark. A_{338}/A_{305}, R_{90}, and PQ were determined before and after addition of cholesterol as in A. ●——● = R_{90} at 338 nm; ▲——▲ = A_{338} nm/A_{305} nm; ■——■ = PQ_{338} nm.

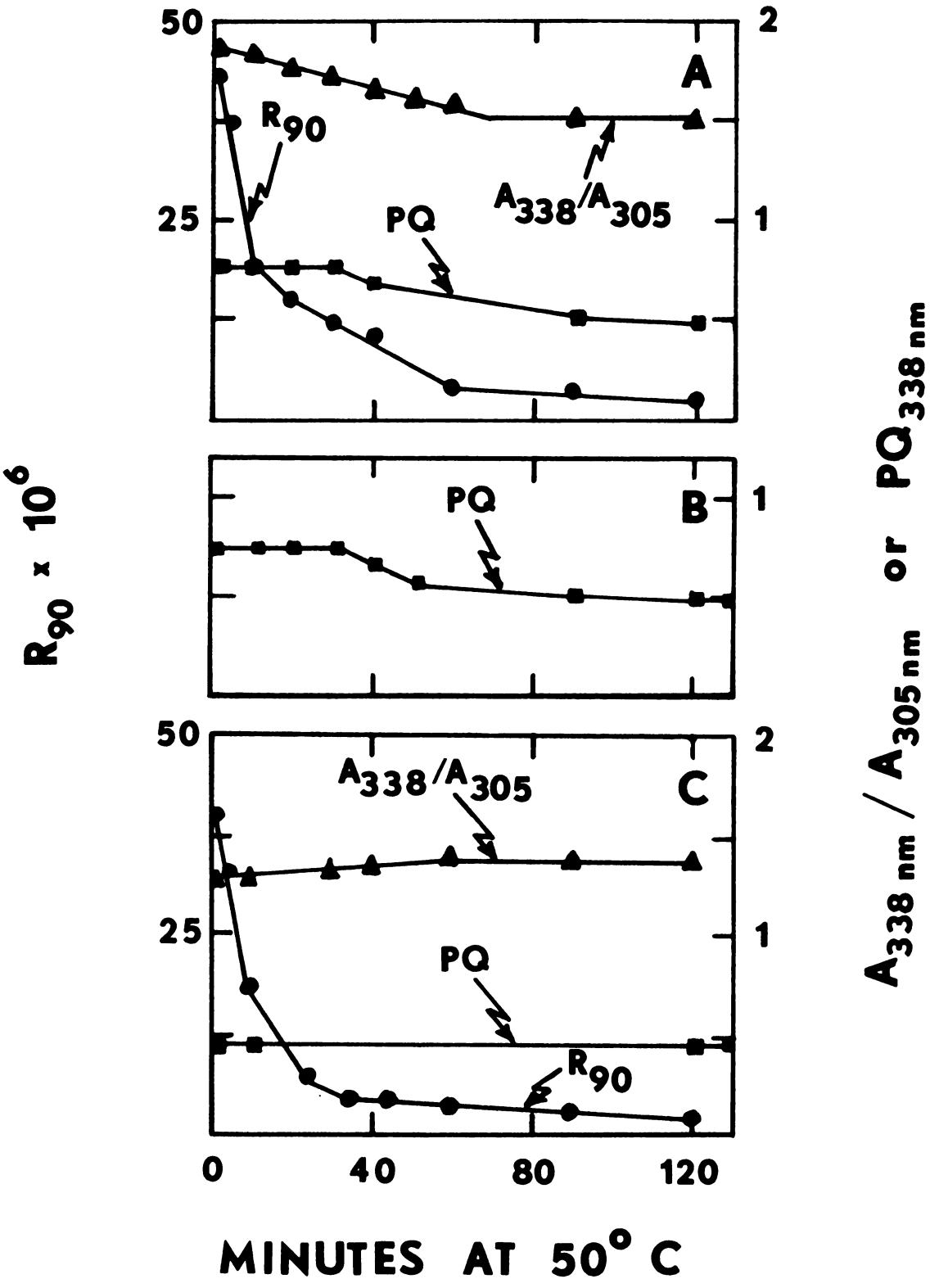

50
25
2
1
A
R_{90}
A_{338}/A_{305}
PQ
B
PQ
1
C
A_{338}/A_{305}
PQ
R_{90}
0
40
80
120
MINUTES AT 50° C
$R_{90} \times 10^6$
$A_{338\,nm} / A_{305\,nm}$ or $PQ_{338\,nm}$

Fig. 2 - First-order Rate of Conversion of Inactive to Active Filipin.

The % inactive filipin remaining at the indicated times was calculated from the PQ data of Figure 1A and 1B, as follows: % inactive filipin remaining = 100X [(maximal decrease in PQ of filipin interacting with cholesterol) - (decrease in PQ of filipin interacting with cholesterol at indicated time)] ÷ [maximal decrease in PQ of filipin interacting with cholesterol].

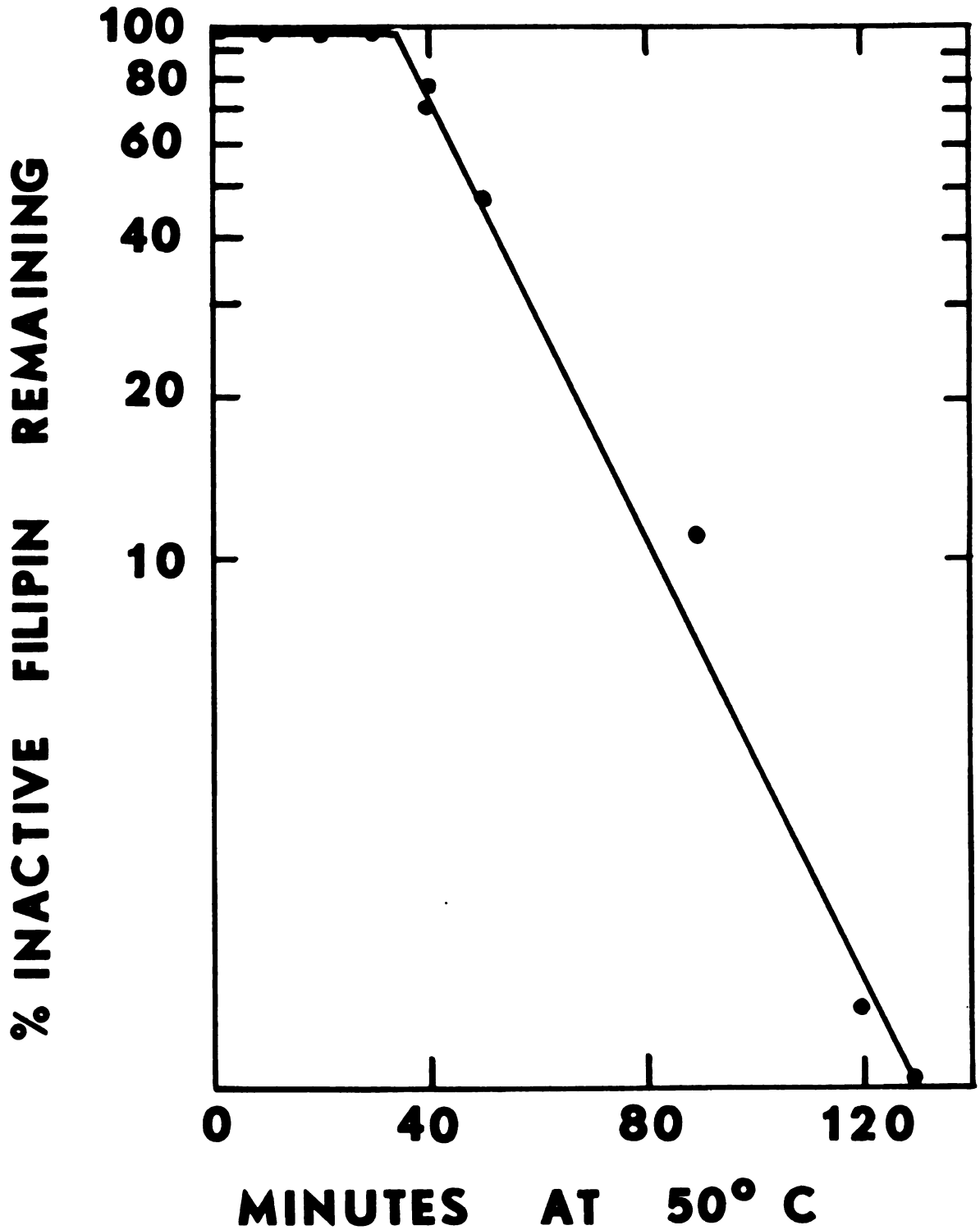

100
80
60
40
20
10
% INACTIVE FILIPIN REMAINING
0
40
80
120
MINUTES AT 50° C

Fig. 3 - Effect of Methanol on R_{90}, A_{338}/A_{305}, CO, and PQ of Filipin.

A_{338}/A_{305}, R_{90}, CO, and PQ were determined at 24°C. Filipin (2.95 μM) was prepared from a 2-day old stock solution, as described in Methods. ●——● = R_{90} at 338 nm; ▲——▲ = A_{338} nm/A_{305} nm; O——O = CO_{338} nm; ■——■ = PQ_{338} nm.

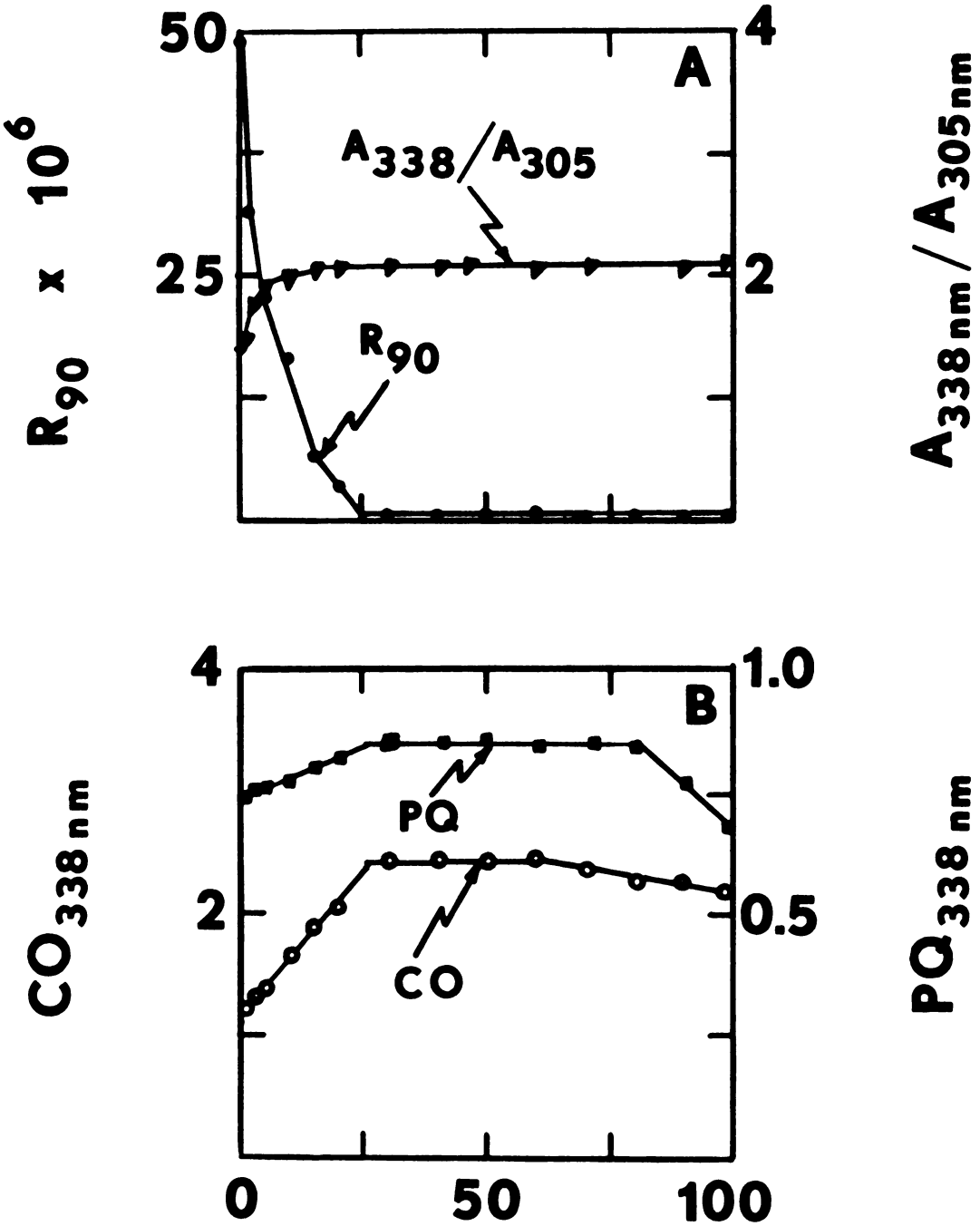
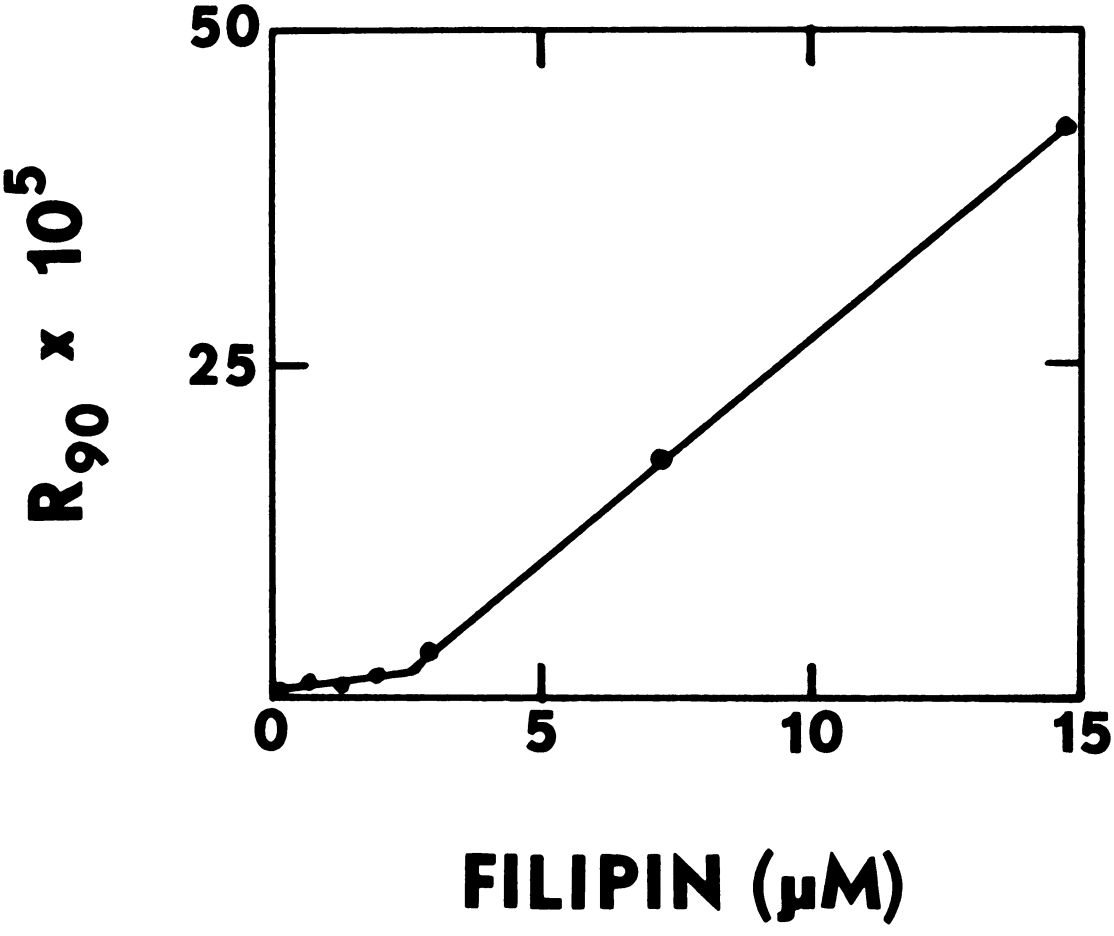

50
25
4
2
A
A_{338}/A_{305}
R_{90}
R_{90} x 10^6
A_{338nm}/A_{305nm}
4
2
1.0
0.5
B
PQ
CO
CO_{338nm}
PQ_{338nm}
0
50
100
% METHANOL

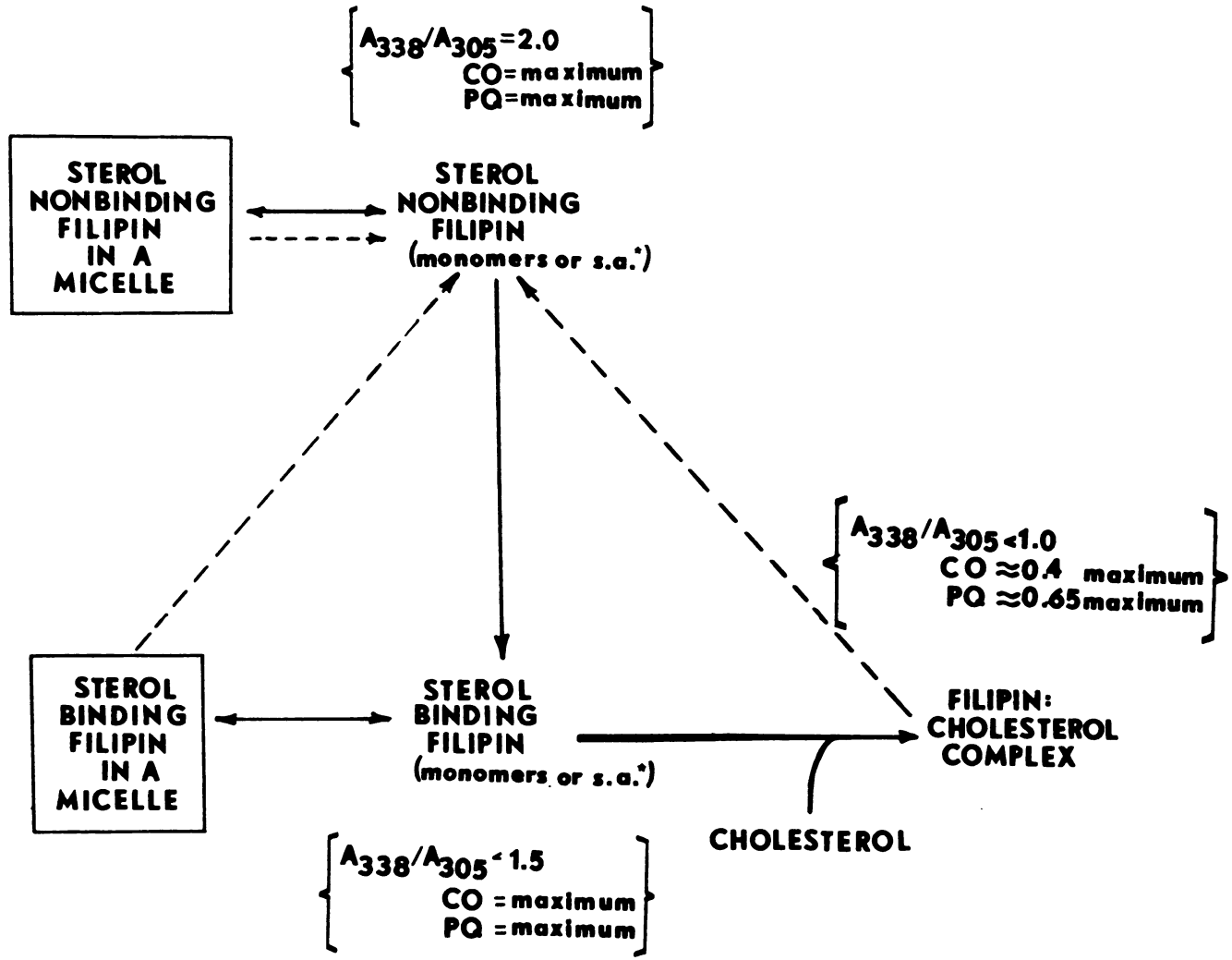
Fig. 4 - Determination of Critical Micelle Concentration of Filipin in Water.

Two-day old filipin (14.7 μM) was diluted with distilled water to the concentrations indicated. After mixing and incubation at 24°C for 2 hrs, R_{90} was determined as described in Methods.


50
25
0
5
10
15
$R_{90} \times 10^5$
FILIPIN (μM)

Scheme 1. Characteristics and Proposed Model for Some States of the Sterol-binding and the Inactive Forms of Filipin.

Solid arrows (——→) refer to processes occurring in aqueous solutions. Broken arrows (- - →) refer to effects of organic solvents such as methanol, ethanol, and propanol added to aqueous solutions. A_{ratio} = absorbance at 338 nm/absorbance at 305 nm; CO = corrected fluorescence; PQ = partial quantum efficiency. S.A. = small aggregates.


A_{338}/A_{305}=2.0
CO=maximum
PQ=maximum
STEROL NONBINDING FILIPIN IN A MICELLE
STEROL NONBINDING FILIPIN (monomers or s.a.*)
A_{338}/A_{305}<1.0
CO ≈0.4 maximum
PQ ≈0.65maximum
STEROL BINDING FILIPIN IN A MICELLE
STEROL BINDING FILIPIN (monomers or s.a.*)
FILIPIN: CHOLESTEROL COMPLEX
CHOLESTEROL
A_{338}/A_{305}< 1.5
CO =maximum
PQ =maximum