THE USE OF A FLUORESCENT STEROL TO INVESTIGATE THE MODE OF ACTION OF AMPHOTERICIN METHYL ESTER, A POLYENE ANTIBIOTIC

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Summary: A fluorescent sterol, dehydroergosterol, was synthesized and shown to support the growth of Mycoplasma mycoides subsp. capri. Dehydroergosterol was incorporated into lecithin vesicles and its interaction with amphotericin methyl ester investigated by measuring the fluorescence emission of the sterol in the presence of the antibiotic. The reaction between dehydroergosterol and amphotericin methyl ester was shown to be of the type $A + B \rightleftharpoons AB$ with a dissociation constant of 1.05 ± 0.05 μM.

Introduction

Estimates of a 1:1 (molar) stoichiometric association of cholesterol with filipin, a polyene antibiotic, have been made from measurements of the changes in the fluorescence emission of filipin upon binding to free cholesterol (1) and sarcoplasmic reticulum vesicles (2). Values for the stoichiometry of the association between cholesterol and amphotericin B vary between 0.7 and 3.9 according to the technique used, although a value of unity seems likely (3). The interaction of membrane sterol with a polyene antibiotic has not previously been determined directly, although polyenes have been shown to remove cholesterol from its interaction with other membrane lipids (4,5). A highly fluorescent sterol, dehydroergosterol, was chosen to investigate its interaction in lecithin vesicles with amphotericin methyl ester (AME).

Methods

Materials: AME hydrochloride was a gift from Dr. C.P. Schaffner. The preparation and purification of dehydroergosterol (Fig. 1) (6) and egg lecithin (7) have been described previously. Dehydroergosterol was characterized by U.V. and I.R. spectroscopy, melting point determination and molecular weight determination by mass spectrometry.

Growth test: Mycoplasma mycoides subsp. capri was grown in a modified Edward medium (8) containing dehydroergosterol as the sole sterol source. After growth in this medium, lipid extracts were prepared from the mycoplasma and assayed for dehydroergosterol. The growth conditions, lipid extraction procedures and chromatographic procedures have been described previously (9). Dehydroergosterol was assayed quantitatively by measuring its absorption at 327 nm in solution in chloroform.

Preparation of Sonicated Vesicles: Egg lecithin and dehydroergosterol (2:1, molar ratio) were mixed in chloroform and the solvent removed by rotary evaporation. 0.05 M Tris/HCl (pH 8.0) containing 0.125 M sucrose was then added to give a final lipid concentration of 1%. The lipid was resuspended by vortexing with a few glass beads. This macrovesicular suspension was then placed in a sonic bath (Dawe Sonicleaner Type 6441A; Dawe Instruments Ltd., London) operating at 50 kHz and 80W for 1 h under N_2. A uniform preparation of vesicles (diameter < 0.05 μm) resulted, with a composition identical to the initial lipid composition.

Fluorescence Studies: Fluorescence emission was estimated over the range 350-600 nm on an Aminco-Bowman Spectrophotofluorometer (American Instrument Co. Inc., U.S.A.) connected to an autoplotter. The optimum excitation wavelength of 329 nm (on the scale) was the same for both dehydroergosterol and AME although their maximum emissions occurred at 385 nm and 450 nm respectively. The final molar concentration of dehydroergosterol was obtained by diluting the vesicle preparation with 0.05 M Tris/HCl (pH 8.0) containing 0.125 M sucrose. AME was added to the suspension of vesicles from a stock solution in distilled water.

Results and Discussion

Dehydroergosterol supported the growth of Mycoplasma mycoides subsp. capri, an organism which will not grow in the absence of sterol, and was recovered unmodified from the plasma membrane of the organism. This confirms that dehydroergosterol is incorporated into a natural membrane and can serve as a useful tool in the study of the role of membrane sterol. Dehydroergosterol-containing vesicles were used in preference to dehydroergosterol-containing mycoplasmas in this study because with whole cells it is more difficult to show a specific interaction between two molecules.

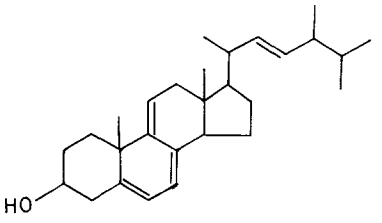

Fig. 1 The proposed structure of dehydroergosterol

The absorption and fluorescence spectra of dehydroergosterol are illustrated in Fig. 2. The fluorescence emission of AME did not overlap that of dehydroergosterol although the fluorescence emission of filipin completely obscured the 385 nm emission of dehydroergosterol. The absorption of AME at 385 nm was very small in comparison with the fluorescence emission of dehydroergosterol. The high fluorescence of dehydroergosterol in vesicles was due not only to the hydrophobic environment, as the emission was much greater than when in solution in chloroform, but also to an ordered arrangement of the sterol in the bilayer. Addition of AME to the vesicles reduced the emission of dehydroergosterol, probably due to the formation of a complex with a reduced quantum yield. The reduction of fluorescence of vesicular dehydroergosterol in the presence of AME for four dilutions of the vesicle preparation is shown in Fig. 3. When plotted as a percentage reduction in emission the values conform to a simple binding curve (Fig. 4) indicating a reversible polyene/sterol interaction. This is the case when the reagents are present in concentrations above the value for the dissociation constant.

These curves are characteristic of a reversible, bimolecular reaction of the type $A + B \rightleftharpoons AB$, but analysis using the Michaelis relation is not possible due to the relatively high concentrations of both reagents. It is possible that one AME molecule associates

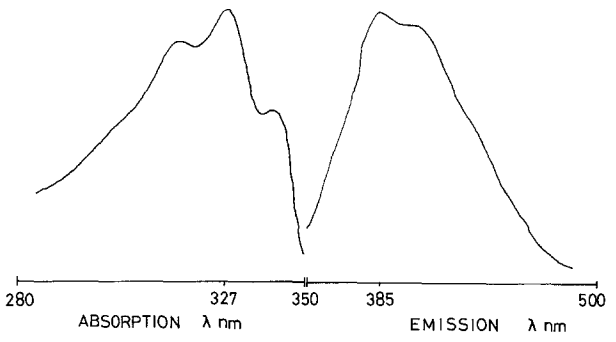

Fig. 2 The absorption and fluorescence emission spectra of dehydroergosterol. Fluorescence emission measurements in arbitrary units were made on the emission maximum of 385 nm.

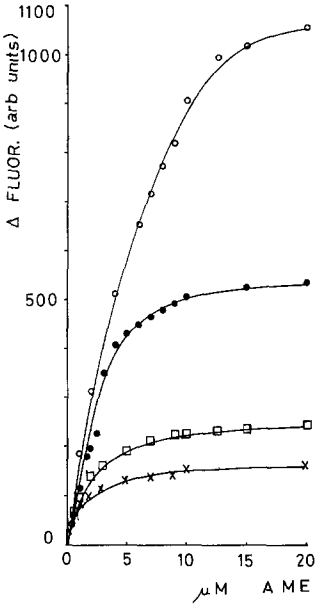

Fig. 3 The change in fluorescence emission (in arbitrary units) of vesicular dehydroergosterol (lecithin: dehydroergosterol, 2:1 molar ratio) caused by different concentrations of amphotericin methyl ester (AME). The fluorescence emission was determined at 385 nm and subtracted from the emission in the absence of AME. o—o 10 μM dehydroergosterol, ●—● 5 μM dehydroergosterol, □—□ 2 μM dehydroergosterol, X—X 1 μM dehydroergosterol.

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with more than one dehydroergosterol provided that the dissociation constants for each addition are identical, but the nature of the

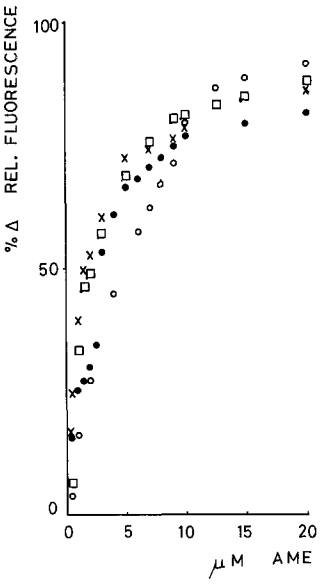

Fig. 4 The change in fluorescence emission (in arbitrary units) of vesicular dehydroergosterol (lecithin: dehydroergosterol, 2:1 molar ratio) caused by different concentrations of amphotericin methyl ester (AME) expressed as a percentage of the fluorescence emission of dehydroergosterol in the absence of AME. O—O 10 μM dehydroergosterol, ●—● 5 μM dehydroergosterol, □—□ 2 μM dehydroergosterol, X—X 1 μM dehydroergosterol.

association makes this unlikely. A graphical determination of the equilibrium constant for this association is available (10). Calculation of the dissociation constant for the binding of vesicular dehydroergosterol to AME from each of the four curves in Fig. 3 gives a value of 1.05 ± 0.05 μM.

The experimental determination of a dissociation constant for a polyene antibiotic/membrane sterol association provides a satisfactory basis for the reversibility of polyene action observed with thin lipid membranes (11), red cells (12) and HeLa cells (13) although the presence of a cell wall in polyene-sensitive fungal cells may account for the failure to detect reversibility in these organisms (14). AME action on sterol-containing membranes involves incorpora-

tion of AME into the membrane, association with sterol and removal of the sterol from its association with other membrane lipids, followed by pore formation. The higher affinity of AME for ergosterol than for cholesterol, both in solution and in natural membranes (15), coupled to the lower affinity of the lecithin/ergosterol interaction in comparison with the lecithin/cholesterol interaction (16) may account for the high sensitivity to AME of fungal cells compared to animal cells (17). The dynamic nature of polyene/sterol pores (18) may also be sterol-dependent. Cells containing membrane sterols with a high affinity for AME may result in pores with a relatively long life which would also increase sensitivity to AME. Determination of the dissociation constants for the reaction of AME with different sterols may thus provide the basis for obtaining a better understanding of the mode of action of polyene antibiotics. It must be added that this study does not preclude mechanisms proposed to account for the polyene-sensitivity of sterol-free liposomes (19).

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