# THE INTERACTION OF THE POLYENE ANTIBIOTIC LUCENSOMYCIN WITH CHOLESTEROL IN ERYTHROCYTE MEMBRANES AND IN MODEL SYSTEMS

## III. Characterization of Spectral Parameters

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ABSTRACT The variations of optical density and fluorescence of lucensomycin are good indices of the binding of this polyenic antibiotic to membranes. The former parameter reflects more generally the binding to any site present in the membrane, while the latter is more specific for binding to cholesterol. The chromophore of the lucensomycin-cholesterol complex has a relatively long lifetime, is almost immobile in the membrane, and is not accessible to water-soluble fluorescence-quenching agents. The stoichiometry, evaluated fluorometrically, corresponds to about two cholesterol molecules per polyene. In colloidal cholesterol suspensions, the extent of binding as a function of free polyene concentration is described by rectangular hyperbolae, the dissociation constant being, however, dependent on the sterol concentration. In erythrocyte membranes, on the other hand, and even more markedly in model systems containing appropriate solvents, the combination between lucensomycin and the sterol sites is described by sigmoid titration curves, indicative of cooperative effects, and probably due to solvation of cholesterol.

### INTRODUCTION

The interaction of cyclic polyenic antibiotics with either biological or artificial membrane systems, leading to the formation of aqueous pores in the membrane and subsequent increase in permeability, is a well-known phenomenon (1–5). The sterol components of the membranes are the principal binding site for the polyenes (6–8), although in some cases an interaction with the phospholipid moieties has also been found (9).

Lucensomycin (trade name, Etruscomycin), a polyenic antibiotic (10) in which a mycosamine moiety is linked by a glycosidic bond to a $C_{25}$ lactone ring containing a tetraene sequence (see formula in Fig. 1), had been found in previous work (11, 12) to

become strongly fluorescent upon binding to erythrocyte membranes or to cholesterol-containing micelles. A similar phenomenon has also been observed (13) with pimaricin which is, like lucensomycin, an amphoteric tetraene. With other polyenes, instead, the variations of fluorescence are smaller, different authors reporting sometimes conflicting results (13–16). The degree of binding to natural or artificial membranes has therefore been estimated on the basis either of fluorescence polarization (15) or of modifications of the absorption spectrum (8, 17–19), or on the basis of the effects on permeability (20).

In the present work we report a study of the spectral characteristics of the fluorescent species which is formed when lucensomycin interacts with erythrocyte membranes, and we verify the validity of considering variations of optical density or of intensity of fluorescence as true binding parameters of lucensomycin to membrane components. We then consider the origin of the sigmoid curves which are sometimes obtained when fluorescence variations are expressed as a function of polyene concentration.

## MATERIALS AND METHODS

Lucensomycin and *N*-acetyl-lucensomycin were kind gifts of Prof. F. Arcamone and Prof. M. Ghione of Farmitalia, Milan, Italy. The methyl ester of *N*-acetyl-lucensomycin was prepared by reaction with diazomethane, which was bubbled for 30 min in a methanolic solution of the polyene at 0°C. The methyl ester was then purified by thick layer chromatography on silica gel, with ethyl acetate-methanol (7:3) as a solvent. With this method the original *N*-acetyl-lucensomycin remains at the origin, while the methyl ester runs almost with the front.

The various polyenes were kept as dry powders under nitrogen at 4°C until use, then dissolved without any further purification at 5–15 mg/ml in dimethylsulfoxide, and this stock solution was diluted in the appropriate solvent. Most experiments were performed in isotonic NaCl + isotonic phosphate buffer, pH 6.8 (9:1, vol/vol).

Colloidal suspensions of cholesterol in twice-distilled water were prepared according to Stadtman (21), by slow addition, on boiling water, of 0.12 vol acetone in which 50 mg/ml cholesterol had been dissolved. After complete evaporation of acetone and cooling of the mixture, the milky filtrate contained about 0.2 mg cholesterol per ml.

Crude egg lecithin, containing about 60% phosphatidylcholine and smaller amounts of phosphatidylethanolamine, phosphatidylserine, and lysophosphatidylcholine, was obtained from E. Merck, Darmstadt, Germany, and used after partial purification by acetone precipitation (22); purified egg phosphatidylcholine was prepared according to Pangborn (23). Lipid micelles were prepared by suspending the crude lecithin or the purified phosphatidylcholine that had been taken to dryness from a chloroform solution in isotonic NaCl-phosphate, and then clarifying the suspension by 120 s sonication with an MSE 100-W sonifier. Cholesterol, when desired, was either added to the chloroform solution, or added as a colloidal suspension in water to the final suspension of lecithin micelles.

Erythrocyte ghosts were prepared according to Dodge et al. (24), with minor modifications, and resuspended by sonication in the isotonic NaCl-phosphate solution. Cholesterol was usually determined with the 15949 TCAA colorimetric test of Boehringer (Mannheim, Germany). Phospholipids were analyzed by thin-layer chromatography on silica gel, with chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol) as the developing solvent, followed by a spray with potassium dichromate in sulfuric acid and charring in an oven at 100°C; quantitative determination of total phospholipids was effected according to Bartlett (25).

Absorption spectra were obtained on either a Beckman DK2 (Beckman Instruments, Inc.,

Fullerton, Calif.) or a Cary 14 (Cary Instruments, Monrovia, Calif.) spectrophotometer. Fluorescence was usually measured with an Aminco-Bowman spectrophotofluorimeter (American Instrument division of Travenol Laboratories Inc., Silver Spring, Md.), by using 1-cm cuvettes, excitation slits of 0.1–0.2 mm, and emission slits between 0.2 and 0.5 mm. In some cases, corrected excitation or emission spectra, as well as fluorescence polarization measurements, were taken with the luminance spectrograph as described by Blumberg et al. (26) for studies of distance determinations by intramolecular energy transfer (27). Fluorescence polarization is expressed, rather than as polarization

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}},$$

as the anisotropy coefficient

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{2p}{3 - p}$$

according to Jablonski (28).

All measurements were effected after attainment of equilibrium, i.e. 3–4 hr with model systems. In doubtful cases, the measurements were repeated until no further variation was found. The decay time of the fluorescent species was measured by a single photon pulse nanosecond technique, by using a low-pressure (0.5 atm) nitrogen lamp, triggered repetitively at 12 kHz, and storing the pulse information in a multichannel analyzer. The data, obtained in digital form, were then analyzed by a Fortran program from a time-shared graphic terminal connected to the multichannel scaler. The graphical best fit with one or two exponential decays was obtained by convoluting the independently measured decay curve of the lamp with these functions.

## RESULTS

### *Modification of the Spectral Characteristics of Lucensomycin upon Binding to Cholesterol-Containing Membranes*

As shown in Fig. 1, the absorption spectrum of lucensomycin in aqueous solution presents three characteristic maxima at 320 nm, 305 nm, and 293 nm (±1 nm), with extinction coefficients of 60.5, 70.0, and 49.0 $cm^2 \cdot \mu mol^{-1}$, respectively, and shoulders at 282 nm and 270 nm. Upon addition of colloidal cholesterol or of cholesterol-containing liposomes, there was a 5–6 nm red shift and a decrease of intensity over this portion of the spectrum, the long absorption maximum at the longest wavelength being the one most affected. With beef erythrocyte ghosts a similar pattern was obtained, except that the red shift was somewhat less marked; the extinction coefficients at saturating membrane concentrations became 14.9 $cm^2 \cdot \mu mol^{-1}$ at 320 nm and 29.7 $cm^2 \cdot \mu mol^{-1}$ at 305 nm.

Another phenomenon occurring upon combination of lucensomycin with cholesterol-containing liposomes or with erythrocyte ghosts was the appearance of an intense fluorescence, with a broad emission at 410 nm (11, 12). As shown in Fig. 2, the excitation spectrum closely resembled the absorption spectrum of the bound polyene, but the emission spectrum did not exhibit, even when narrow bandwidths were used, any fine structure.

An accurate direct measurement of the anisotropy coefficient $r$, obtained as de-

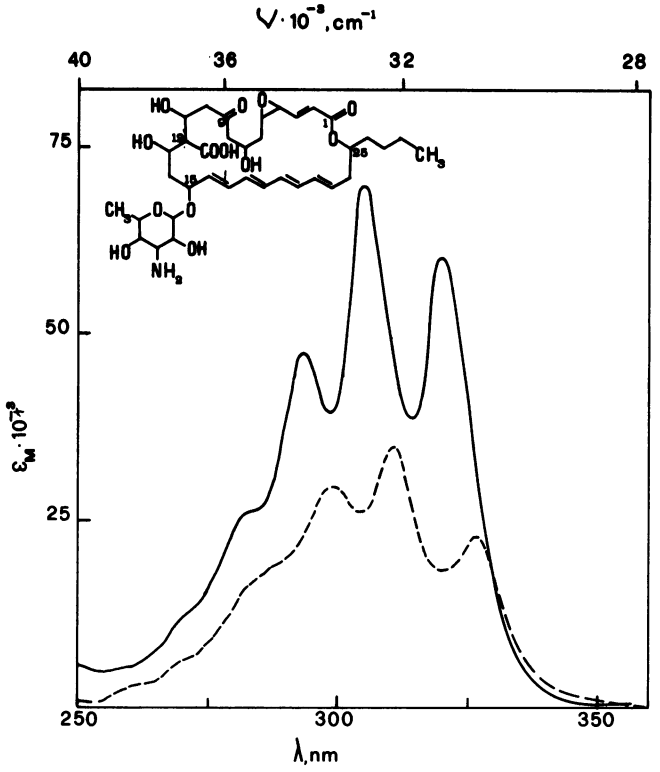


FIGURE 1 Chemical formula of lucensomycin and absorption spectra of the polyene in $H_2O$ in the absence (——) or presence (- - - -) of colloidal cholesterol in threefold excess.

scribed by Blumberg et al. (26), yielded a value between 0.34 and 0.35—corresponding to a degree of polarization $p$ around 0.44. This value was practically constant throughout the excitation region from 290 nm to 328 nm (Fig. 2). At longer wavelengths a slight decrease of $r$ occurred, possibly because of the presence in the sample of other more mobile fluorophores. Such a high value of $r$ is similar to that obtained from previous, less accurate, measurements (11).

Experimental measurements of the actual lifetime of fluorescence $\tau$ indicated the presence of at least two fluorescent species; the fluorescence decay curves could be satisfactorily fitted (Fig. 3) by assuming the presence of a major component (88–97%) with a long lifetime (28–35 ns), and of a minor one (3–12%) with a short lifetime (4–8 ns). In the absence of membranes, i.e. when the quantum yield of lucensomycin is very low (11), the component with longer lifetime vanishes and a component with very short lifetime ($\cong$0.5 ns) appears.

It seems, therefore, that the minor component with the 4–8 ns lifetime is to be ascribed to the presence of some impurity, the fluorescent membrane-bound polyene having a lifetime around 30 ns. This value remained practically constant when excitation was varied from 290 to 330 nm.

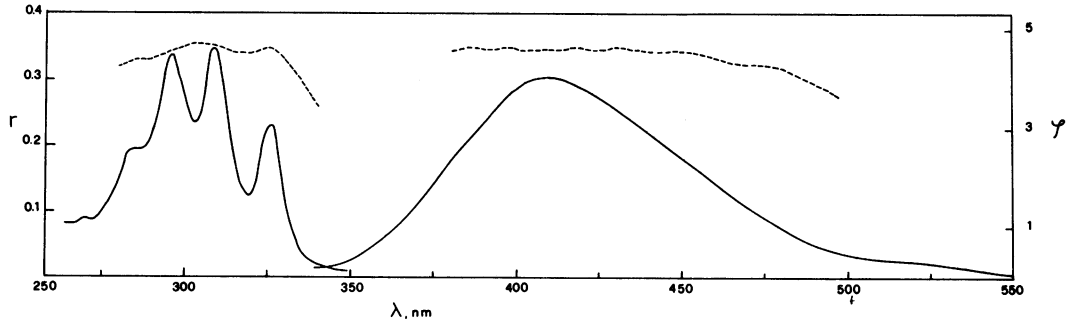


FIGURE 2 Excitation, emission, and fluorescence anisotropy spectra of lucensomycin bound to erythrocyte ghosts. Fluorescence intensity $\phi$, expressed in arbitrary units, is corrected for the photomultiplier response. The excitation spectrum was recorded by setting the emission at 410 nm, the emission spectrum setting the excitation at 307 nm. Excitation and emission bandwidths were 3.3 and 3.2 nm, respectively. Fluorescence anisotropy $r$ (dotted line) was recorded directly.

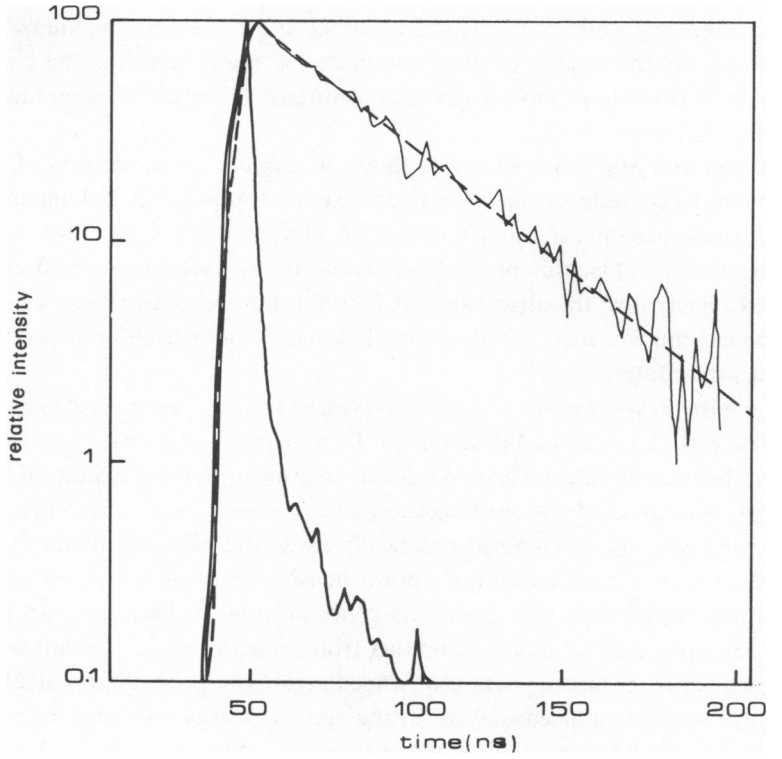


FIGURE 3 Fluorescence decay of lucensomycin bound to erythrocyte ghosts. Membrane concentration: 7 $\mu$M cholesterol; lucensomycin concentration: 5 $\mu$M. Excitation at 312 nm, emission at 410 nm (thin line, solid curve). Thick line, solid curve: decay curve of the lamp (excitation and emission both at 410 nm). Dashed line: calculated best fit curve, assuming two fluorescent species, 95% with $\tau$ = 35 ns, 5% with $\tau$ = 5 ns.

TABLE I

SPECTROPHOTOMETRIC AND FLUOROMETRIC TITRATION, WITH INCREASING LUCENSOMYCIN, OF COLLOIDAL CHOLESTEROL AND OF EGG LECITHIN

| | +cholesterol 18.52 $\mu$M | | +crude egg lecithin, 20 $\mu$g/ml | | +purified egg lecithin, 50 $\mu$g/ml | |
|---|---|---|---|---|---|---|
| $L_{tot}$, $\mu M$ | $-\Delta OD_{320} \times 10^3$ | $\Delta\varphi$ | $-\Delta OD_{320} \times 10^3$ | $\Delta\varphi$ | $-\Delta OD_{320} \times 10^3$ | $\Delta\varphi$ |
| 0.364 | 8.03 | 194 | 9.21 | 6.7 | 0.04 | 2.3 |
| 0.724 | 17.24 | 320 | 14.09 | 8.2 | 1.02 | 2.8 |
| 1.265 | 24.33 | 549 | 20.79 | 19.0 | 1.58 | 3.5 |
| 1.810 | 44.11 | 678 | 43.32 | 24.8 | 3.17 | 5.2 |
| 2.350 | 49.68 | 866 | 51.98 | 27.8 | 4.12 | 6.7 |

$L_{tot}$ indicates the total lucensomycin concentration (final concentration) in the sample, $\Delta$OD and $\Delta\varphi$ represent the differences in absorbance and in fluorescence intensity, respectively, between the samples containing colloidal cholesterol (or egg lecithin) and those not containing it.

*Validity of the Spectral Changes as True Binding Parameters*

As mentioned in previous papers (12, 29), a certain degree of discrepancy was found when the changes in absorbance and in fluorescence intensity were compared. In order to verify separately the validity of these parameters as really reflecting the binding of lucensomycin to cholesterol moieties in the membrane, three sets of experiments were performed.

*Assays with Model Systems.* As shown in Table I, upon addition of lucensomycin to colloidal cholesterol there was a decrease of absorption at 320 nm and an increase of fluorescence intensity at 410 nm (when excited at 306 nm). With liposomes prepared from different lecithin preparations without any cholesterol, the fluorescence remained extremely low; the absorbance at 320 nm, however, decreased significantly when crude lecithin was used, although no cholesterol could have been present after the acetone precipitation.

*Lucensomicin Titration of Formalin-Treated Ghosts.* Beef erythrocyte ghosts were treated with 3% vol/vol Formalin for 18 h at room temperature according to Butler (30), then extensively washed. After this treatment, the sedimentation behavior of the ghosts was changed. A well-packed pellet was more easily obtained, but the light-scattering properties remained practically invariant, allowing reliable measurements of fluorescence intensity and of optical density. Sets of assays were prepared with or without membranes and increasing concentrations of lucensomycin in water. After 2 h their optical densities at 320 nm (net from background at 360 nm) were measured; subsequently, each assay was centrifuged at 200,000 *g* for 30 min at 21°C, and the concentration of free lucensomycin in the supernate was estimated from the absorbance at 305 nm. The extent of binding was evaluated as (*a*) difference between the concentrations of free lucensomycin in centrifugation supernates from assays with or without membranes; (*b*) difference of absorbance at 320 nm of the assays with or without membranes before centrifugation); (*c*) fluorescence of the assays with membranes.

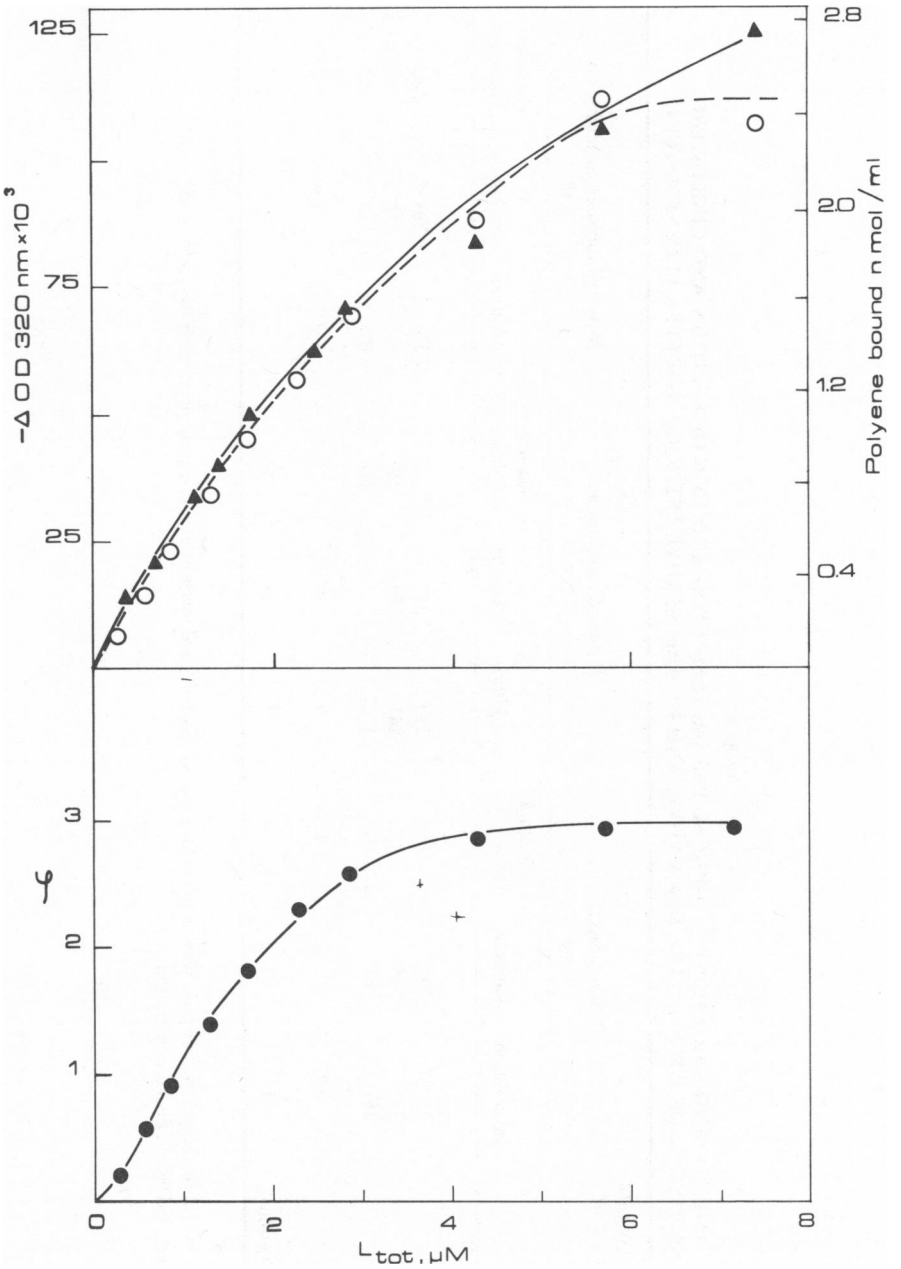


FIGURE 4 Evaluation of the binding of lucensomycin to Formalin-treated erythrocyte ghosts. The curves in the upper graph were obtained: (*a*) by differential centrifugation, followed by measurement of the residual polyene in the supernate by its absorption at 305 nm (right-hand scale: ▲—▲—; (*b*) by measurements of the absorption at 320 nm of the whole mixture (left-hand scale: ○—○—). The lower curve (●—●—) results from fluorometric measurements, in arbitrary units, with excitation at 308 nm and emission at 410 nm. Membrane concentration: 2.9 $\mu$M cholesterol.

TABLE II

HEMOLYTIC POTENCY AND VARIATIONS OF OPTICAL PARAMETERS, UPON ADDITION OF LECITHIN AND CHOLESTEROL, OF LUCENSOMYCIN, OF ITS *N*-ACETYL DERIVATIVE, AND OF THE METHYL ESTER OF *N*-ACETYL LUCENSOMYCIN

| Concentration of polyene ($\mu$M) causing 50% lysis of $7.3 \times 10^6$ erythrocytes/ml . . . | Lucensomycin 1.286 | | | *N*-Acetyllucensomycin 21.4 | | | *N*-Acetyllucensomycin methyl ester 8.58 | | |
|---|---|---|---|---|---|---|---|---|---|
| | no addition | +lecithin | +lecithin +cholesterol | no addition | +lecithin | +lecithin +cholesterol | no addition | +lecithin | +lecithin +cholesterol |
| Optical parameters | | | | | | | | | |
| $OD_{320} \times 10^3$ | 212 | 209 | 64 | 210 | 217 | 123 | 215 | 220 | 140 |
| $OD_{305} \times 10^3$ | 245 | 245 | 92 | 241 | 250 | 163 | 247 | 251 | 189 |
| $OD_{293} \times 10^3$ | 167 | 161 | 80 | 176 | 152 | 118 | 185 | 175 | 152 |
| $R \frac{293}{320}$ | 0.79 | 0.77 | 1.25 | 0.84 | 0.70 | 1.04 | 0.86 | 0.80 | 1.09 |
| Fluorescence (arbitrary units) | 2 | 15 | 5,313 | 7 | 10 | 70 | 8 | 28 | 2,263 |

All polyenes were 3.5 $\mu$M.

The lecithin preparation was chromatographically pure phosphatidylcholine from egg yolk, and was used at a final concentration of 100 $\mu$g/ml (= 135 $\mu$M).

Cholesterol, when present, was 90 $\mu$M final concentration.

It was verified that after centrifugation less than 5% fluorescence remained in the supernate, indicating that all the fluorescent species sedimented with membranes. The results are shown in Fig. 4. It can be seen that the estimates of binding by the centrifugation method and by the variations of absorbance at 320 nm could be superimposed on each other, assuming the difference of extinction coefficients at 320 nm between free and bound lucensomycin to be 45.6 $cm^2 \cdot \mu mol^{-1}$ (which corresponds exactly to the difference of the two extinction coefficients given above for the free and bound polyene). The titration curve obtained by fluorescence measurements had, however, a quite different shape with a small but definite sigmoid inflection.

*Spectral Changes in Lucensomycin Derivatives.* Table II shows the results of parallel experiments performed with lucensomycin, *N*-acetyl-lucensomycin, and the methyl ester of *N*-acetyl-lucensomycin. It can be seen that there is a direct relationship between increase in fluorescence and effectiveness in causing hemolysis, while the decrease in absorbance at 320 nm and even the ratio between the absorbances at 320 nm and 290 nm are much less reliable indexes.

*Shape of Titration Curves and Evaluation of the Equilibrium Constant*

Knowing the values of the extinction coefficients of free and bound polyene, it was possible to plot the binding of lucensomycin to cholesterol-containing membranes vs. the concentration of the free polyene. With colloidal cholesterol, as shown in Fig. 5, rectangular hyperbolae were obtained by both spectrophotometric and fluorometric techniques. The dissociation constant was, however, found to depend on the concentration of cholesterol, being around 0.16 $\mu$M when the sterol was 3.6 $\mu$M and over 1 $\mu$M (possibly as high as 8 $\mu$M) when the sterol was 32.2 $\mu$M.

With beef erythrocyte ghosts (7.5 nmol cholesterol/ml), on the other hand, the fluorometric titration curves had, as shown in Fig. 4 and Fig. 6, a sigmoid shape, which was not apparent in spectrophotometric titrations. The concentration of free lucensomycin giving 50% of binding was 0.52 $\mu$M for the spectrophotometric titration and 0.3 $\mu$M for the fluorometric one. These values remained practically constant when the membrane concentration was varied by a factor of four. In Formalin-treated ghosts (Fig. 4) the values were both increased by a factor of two.

The stoichiometry of the reaction between cholesterol and lucensomycin can easily be derived from the spectrophotometric titration curves by comparing the maximal amount of polyene bound with the concentration of sterol. With colloidal cholesterol (Fig. 5) the lucensomycin:cholesterol ratio reached a maximum value of 0.6. With erythrocyte ghosts (Figs. 4 and 6) the evaluation was complicated by the discrepancy between the spectrophotometric and fluorometric titrations; the former gave a maximum ratio of 1–1.3, the latter of 0.5–0.6 (assuming, in this case, a superposition of the ascending portions of the two ascending curves).

*On the Nature of the Sigmoid Shape of Fluorometric Titration Curves*

As described in previous work (29) and as can also be seen in Fig. 4 and in Fig. 6, the fluorometric titration curves of erythrocyte ghosts with increasing lucensomycin have a sigmoid shape. This behavior was not found upon titration of colloidal

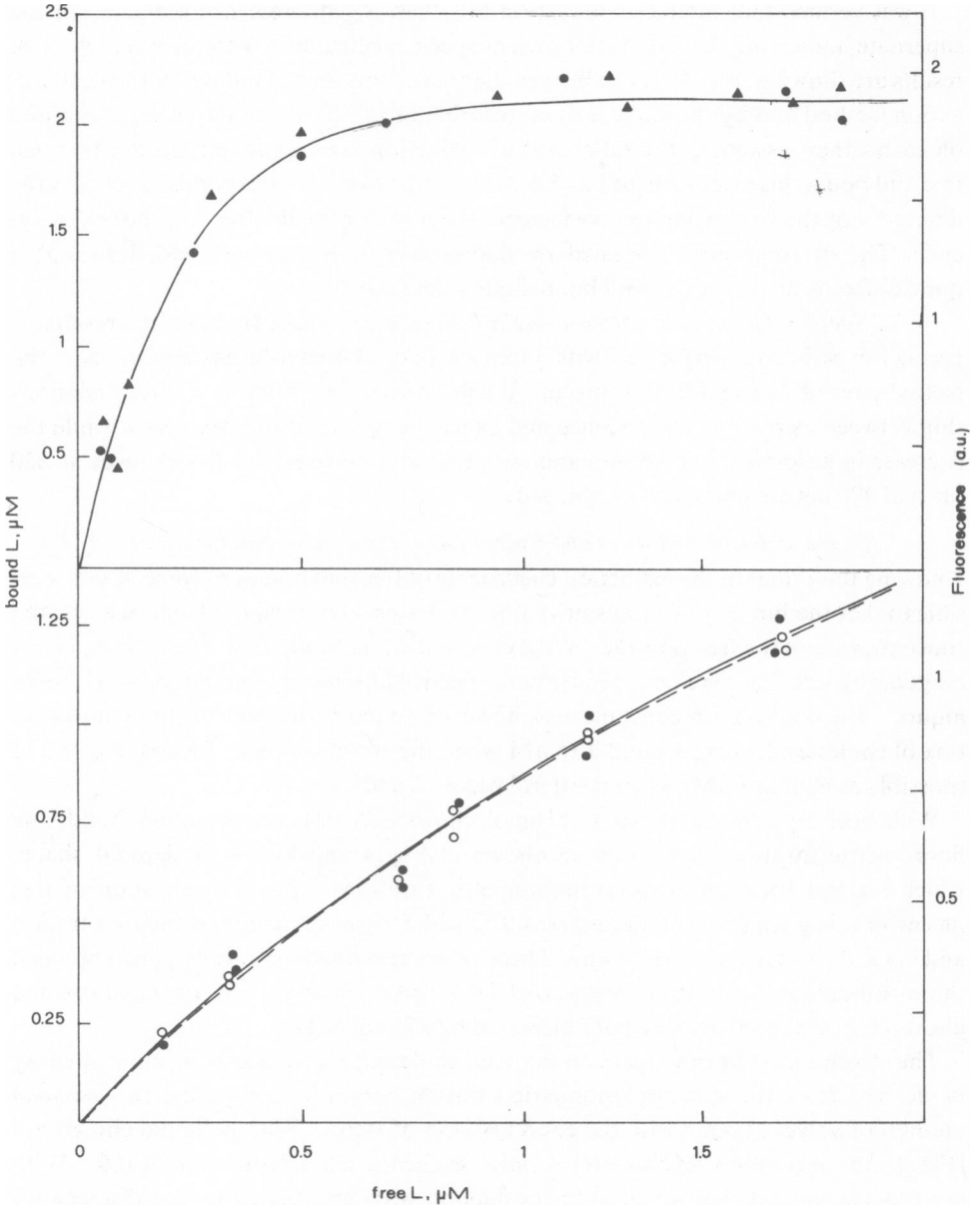


FIGURE 5 Spectrophotometric (left scale: ●—●—) and fluorometric (right scale: ○—○— and ▲—▲—) titrations of colloidal cholesterol suspension with lucensomycin. Cholesterol concentrations: upper curve, 3.6 μM; lower curve, 32.2 μM.

cholesterol suspensions or with cholesterol-containing liposomes formed with pure phosphatidylcholine (either natural or from egg, or synthetic dipalmitoyllecithin), but was present if the cholesterol-containing liposomes were formed with "crude" lecithin preparations. The phospholipid component responsible for it was not identified.

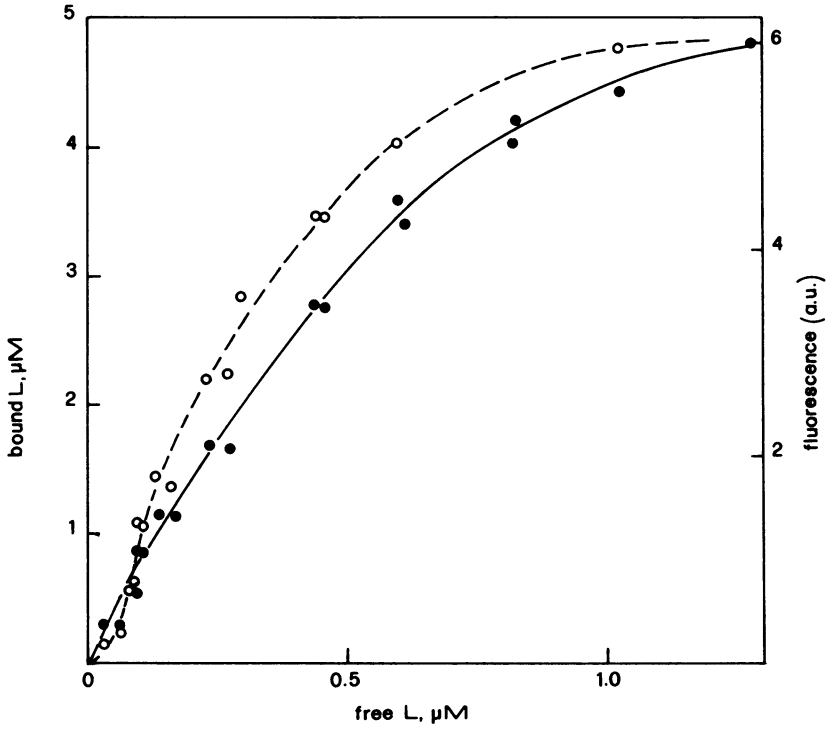


FIGURE 6 Spectrophotometric (left scale: ●—●—) and fluorometric (right scale: ○—○—) titrations of erythrocyte ghosts. Membrane concentration: 7.5 μM cholesterol.

The same phenomenon had also been found to occur, even more markedly, upon addition of a relatively small amount of acetic acid (29). This effect was not due to changes in pH, since addition of equivalent amounts of HCl was ineffective. Moreover, acetic acid could be substituted by methanol or by tetrahydrofuran, but by neither dimethylsulfoxide nor dioxane.

In previous work (29), the nature of this phenomenon had been discussed in terms of two alternative hypotheses: a real cooperativity, the binding of a lucensomycin molecule to a membrane site being favored if other neighboring sites are also occupied; or an effect limited to the appearance of fluorescence emission, a certain number of lucensomycin molecules bound to one site being required to form a fluorescent species. In the latter case several cholesterol-lucensomycin complexes would be formed in the membrane, but fluorescence would not be proportional to the number of lucensomycin molecules in the complex. An increasing heterogeneity of lifetimes would then be expected upon addition of increasing amounts of a sigmoidicity-inducing agent. As shown in Fig. 7, no such phenomenon could be observed: addition of acetic acid in assays containing fixed amounts of lucensomycin and erythrocyte ghosts decreased the total fluorescence by decreasing the amount of fluorescent species with a 30-ns lifetime; the value of this lifetime was not appreciably modified, nor did any new

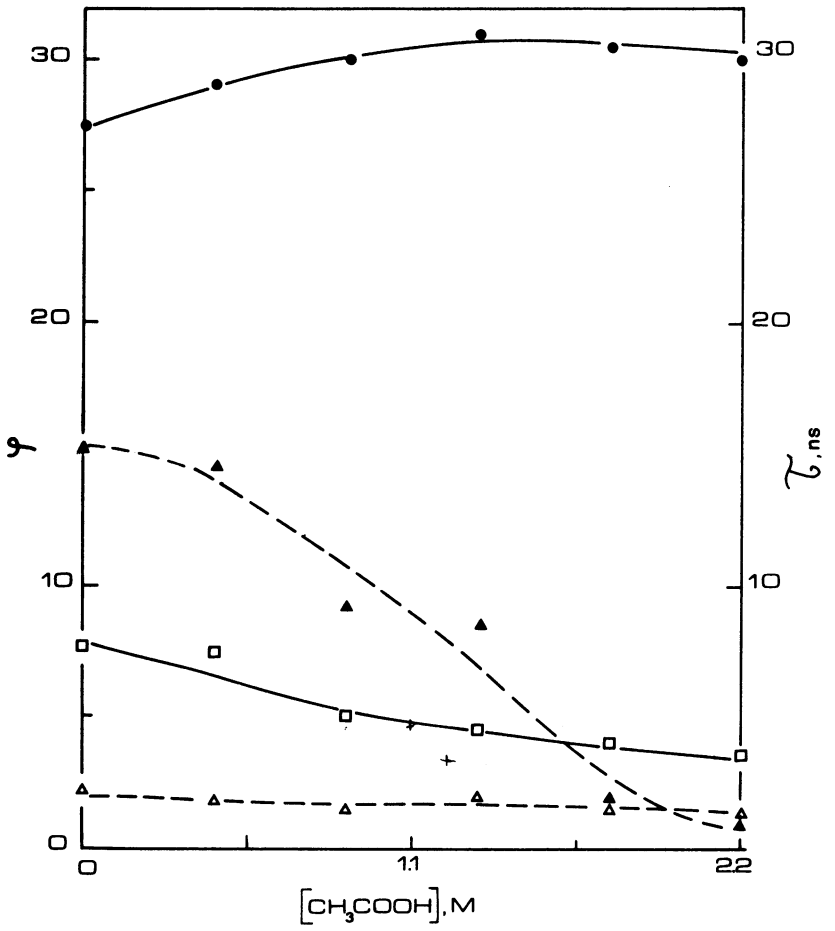


FIGURE 7 Effect of acetic acid in the intensities of emission ($\phi$, left-hand scale) and on the decay times ($\tau$, right-hand scale) of the fluorescent species present in a mixture of 5 $\mu$M lucensomycin of erythrocyte ghosts (7 $\mu$M cholesterol). Full lines: variations of the two decay times $\tau$(●,□). Broken lines: emission intensities $\phi$ of the species with $\tau \simeq 30$ ns (▲) and $\tau \simeq 5$ ns (△). Other conditions as in Fig. 3.

fluorescent species appear. It may therefore be concluded that fluorescence intensity depends upon the presence of a single fluorescent species, and that the sigmoid shape of the titration curve is a direct measure of the formation of this species, no complex with other measurable fluorescence being present.

Fig. 8 shows the results of a titration in the presence of acetic acid, where fluorescence and absorbance at 320 nm were measured on the same set of assays. Within experimental limits the two curves had a very similar shape, indicating that the presence of acetic acid really interferes with the binding of lucensomycin to the membrane cholesterol.

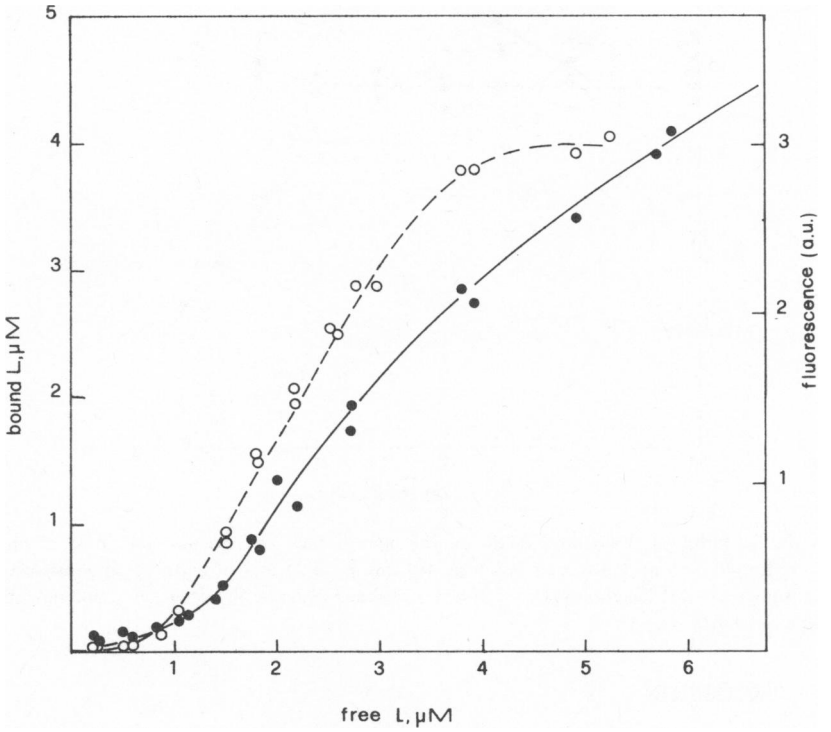


FIGURE 8 Spectrophotometric (left scale: ●—●—) and fluorometric (right scale: ○—○—) titrations of erythrocyte ghosts in the presence of 1.65 M acetic acid. Membrane concentration: 7.5 μM cholesterol.

*Location in the Membrane of the Polyene*

Lucensomycin appears to be firmly bound to the cholesterol-containing membranes: it can be sedimented together with the membranes (Fig. 4), even upon centrifugation through a 5–40% sucrose gradient (29). Moreover, the high polarization of fluorescence, together with the relatively high value of the lifetime, indicates a considerable restriction of the mobility of the fluorophore.

Further information could be obtained by studying the effects on fluorescence of a water-soluble quenching agent. Iodide, known to be a drastic quencher of fluorophores exposed to aqueous solvents (31, 32), probably by enhancing the intersystem crossing rate (33), failed to cause any effect when added to membrane-bound lucensomycin in concentrations up to 1 M (Fig. 9). This result indicates that, although lucensomycin causes in the membrane the formation of aqueous pores, the fluorophore itself is shielded from the aqueous medium. This conclusion would be strengthened if it could be shown that iodide exerted a quenching effect on the free polyene. Since free lucensomycin is not fluorescent in water this could be verified, as shown in Fig. 2, only with the related polyenic antibiotic filipin.

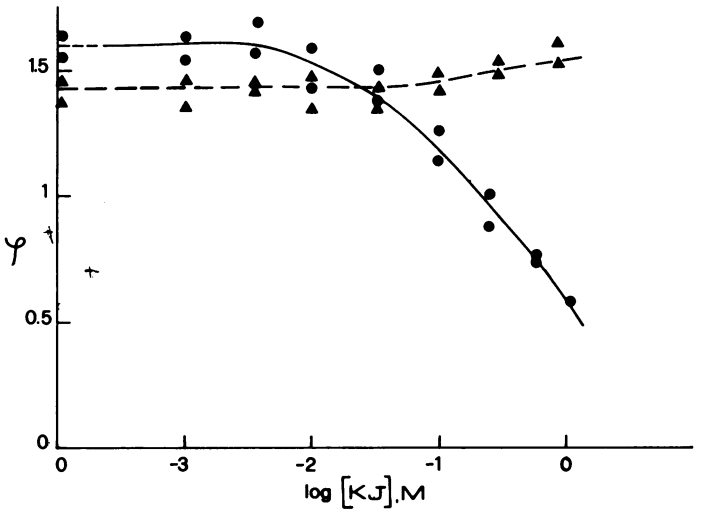


FIGURE 9 Effect of potassium iodide on the fluorescence of aqueous solutions of filipin (2.5 $\mu$M; excitation at 355 nm, emission at 480 nm: ●—●—) and of erythrocyte ghosts-bound lucensomycin (2 $\mu$M lucensomycin + 200 $\mu$M membrane cholesterol; excitation at 307 nm, emission at 410 nm: ▲—▲—).

## DISCUSSION

The data reported in the present work on the spectral properties of lucensomycin indicate that the absorption spectrum in the 260–340-nm region is presumably due to a single transition of the tetraene chromophore, with vibrational fine structure. In fact the peak at longest wavelength is located at 320 nm (31,250 $cm^{-1}$), the other following at higher energies being separated from it by a progression of about 1,500 $cm^{-1}$, as has been described for polyenes in general (34, 35). On the other hand, the uniqueness of the transition is confirmed by the following observations: (*a*) the CD spectrum has the same pattern and sign as the absorption spectrum (12); (*b*) when lucensomycin is bound to membranes all the absorption peaks induce the appearance of fluorescence with similar quantum yields. This is confirmed by the constancy of the lifetime of the fluorescent species over all the excitation peaks; (*c*) the polarization of fluorescence is constant over all the excitation peaks. This result is at variance with that obtained by Bittman and Fischkoff (15) with filipin, where there was interference by the different fluorescence intensity of the free polyene. In our case this interference is absent because the free polyene is practically nonfluorescent, and therefore the data concern only the bound polyene.

Upon binding to cholesterol or to cholesterol-containing liposomes or membranes, there is an increase of fluorescence intensity and a decrease of absorbance. The latter phenomenon is more marked at the lowest frequency; the hypothesis that it is due to a difference of the distribution of intensity among vibrational bands, as required by the Franck-Condon principle, is, however, to be discarded, since the relative intensity of the shoulders at 282 nm and 270 nm does not increase accordingly. There is, in fact,

a true decrease of the oscillator strength of the transition. As a first hypothesis, the interaction with cholesterol can be visualized as distorting the all-*trans* fundamental configuration of the tetraene.

A fairly large change in the chromophore configuration is likely to occur in the excited state; this conclusion is supported by the lack of mirror image symmetry between excitation and emission spectra, by the large Stokes shift (i.e. the wide distance between excitation and emission maxima), and by the fact that the experimental value of the actual lifetime $\tau$ is much larger than the value which can be derived for the natural lifetime $\tau_0$ by application to the absorption spectrum of the Strickler-Berg equation (36, 37). Interaction with cholesterol appears to protect the excited state against radiationless conversion to the fundamental state: as shown in previous work (12), at liquid nitrogen temperature fluorescence emission can be observed, with identical excitation and emission wavelengths, even in the absence of cholesterol.

As for the validity of the variations in absorbance and in fluorescence to evaluate the binding between lucensomycin and cholesterol, it should be remembered that Gottlieb et al. (7) had already seen a bad correlation between reduction of absorption peaks and fungicidal activity of filipin. In our hands, the decrease in absorbance of lucensomycin occurred also with cholesterol-free crude egg lecithin, while fluorescence was strictly dependent upon the presence of cholesterol. In Formalin-treated erythrocyte ghosts the binding, estimated by differential centrifugation, corresponded to that which could be evaluated through the decrease of absorbance; it is therefore plausible that fluorescence reflects only the binding to cholesterol, while the variations of optical density indicate the binding of the polyene both to cholesterol and to other membrane receptors.

From a technical point of view, precise spectrophotometric measurements are often difficult to perform in turbid samples, the whole variation being within a four-fold range. Variations of fluorescence intensity cover instead an approximately 1,000-fold range and are, moreover, specific for the interaction with the sterol. Since they are expressed in arbitrary units, they indicate only the fraction of total sterol sites to which the polyene is bound. Other optical parameters, such as the ratio between absorption peaks or polarization of fluorescence, are of much more doubtful validity. The former, used by a number of authors (8, 17, 18), is difficult to determine with good reliability in turbid samples; moreover, in the case of lucensomycin it suffers sometimes from the superposition, in the 280–290 nm region, of membrane protein chromophores. Finally, such a parameter could have some validity only if the intensity of the peak at the lower wavelength remained constant, since otherwise the additivity of this parameter would be lost; this is not the case with lucensomycin.

In the case of lucensomycin, the polarization of fluorescence, used by Bittman and Fischkoff (15), reflects only the cholesterol-bound polyene and therefore does not vary in the course of the titration. More generally, however, this parameter would maintain the property of additivity only if expressed as anisotropy coefficient $r$ (28, 36), and even in that case only if the intensity of fluorescence did not vary.

The possibility of measuring spectrophotometrically the concentrations of free and

bound lucensomycin allowed the evaluation of the dissociation constant and of the stoichiometry of the complex. The former appeared to be in the micromolar range as described for filipin (19); with colloidal cholesterol, however, the value obtained was found to be dependent on the sterol concentration; this can be due to the aggregation of the sterol in large micelles so that a disaggregation process is needed to allow binding of the polyene. This interpretation is supported by the extremely slow rate at which the binding occurs under these conditions (12).

As for the stoichiometry, previous work (12, 29) had given results in favor of two cholesterol molecules per molecule of polyene. Similar results had also been reported by Norman et al. (8, 17) for various polyene antibiotics, while other authors (16, 20) have taken as most probable a one-to-one ratio. From the data reported in the present work, the cholesterol:lucensomycin ratio was, for colloidal cholesterol and for the cholesterol sites in the erythrocyte ghosts (as measured by fluorimetric titrations), well over 1, though somewhat lower than 2. The ratio appeared to decrease to values around 1 in membranes if binding was estimated spectrophotometrically. Under these conditions, however, it appears that in the phospholipid component of the membrane—as well as in crude lecithin—a number of sites are revealed to which the polyene binds with changes in absorbance but with no increase in fluorescence intensity. The fluorometric titration curves reflect, therefore, only the binding of the polyene to the sterol component of the membrane, while the spectrophotometric curves indicate a more general binding to any site in the membrane. The sigmoid shape would be due to the necessity for two cholesterol molecules to be freed from their interaction with phospholipids before they form a polyene-binding site able to give the fluorescent complex. This interpretation is supported by the observation that this sigmoid shape is induced (in colloidal cholesterol) or increased (in erythrocyte ghosts) by addition of substances which are good solvents for the sterol. On the other hand, comparative evaluation of the ability of added cholesterol to inhibit lucensomycin- or digitonin-induced hemolysis indicates that half as much cholesterol is needed to displace digitonin than to displace lucensomycin (Strom et al., unpublished results). Since digitonin is known to form one-to-one complexes with cholesterol (38), this result supports the view of a 1:2 lucensomycin-cholesterol stoichiometry.

As for the position of the lucensomycin-cholesterol complex in the membrane, the high value of the fluorescence anisotropy, together with the long lifetime of the excited state, indicates that the complex is practically immobile within the membrane. The ineffectiveness of iodide ions as quenching agents indicates that the fluorophore is buried deep in the membrane, and casts some doubt on the possibility of visualizing the aqueous pores, induced by polyenes in natural membranes, as formed by the polyene-cholesterol complexes themselves (20).

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