

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

## I. A FLUOROMETRIC AND SPECTROPHOTOMETRIC STUDY

ROBERTO STROM, CARLO CRIFÒ, *and* ARGANTE BOZZI

*From the Institutes of Biological Chemistry and of Applied Biochemistry, University of Rome, and Center for Molecular Biology, C.N.R., Rome, Italy*

**ABSTRACT** The increase of fluorescence emission of lucensomycin occurring upon interaction with cholesterol, either as colloidal suspension or included in phospholipid micelles or in erythrocyte membranes, was described. Colloidal cholesterol differed from that contained in membranes by the kinetic behavior of its interaction with lucensomycin, the reaction being very slow in the former case, very fast in the latter one. Variations of optical density also occurred, though neither the kinetics nor the titration curves were superimposable on those obtained fluorometrically. The stoichiometry was, however, the same.

### INTRODUCTION

In a previous communication (1) we had reported the enhancement of the fluorescence of the macrolide polyenic antibiotic lucensomycin (trade name: etruscomycin®) upon binding to erythrocyte membranes. The fluorescence quantum yield, which in aqueous solution was as low as  $0.3 \times 10^{-3}$ , increased, upon addition of membranes, to values around 0.25.

We report here experiments in model systems and in beef erythrocyte membranes indicating that cholesterol is the binding site, the combination of which with the polyene gives rise to the increase of fluorescence. Although the presence or absence of phospholipids around the cholesterol moieties influences largely the kinetics of fluorescence increase and, as shall be shown in the following paper, also the nature of the fluorescent complex, a preliminary evaluation of some binding parameters can be done, and the mobility of the lucensomycin-cholesterol complex evaluated.

### MATERIALS AND METHODS

Lucensomycin and *N*-acetyl-lucensomycin were kind gifts of Prof. F. Arcamone and Prof. M. Ghione of Farmitalia, Milano, Italy. Filipin III, 96% pure, was supplied by Dr. George B.

Whitfield of the Upjohn Co., Kalamazoo, Mich. The polyenes were kept as dry powders under nitrogen at 4°C until use, then dissolved at 5–15 mg/ml in dimethylsulfoxide, and this stock solution diluted in the appropriate solvent. Most experiments were performed in isotonic NaCl + isotonic phosphate buffer pH 6.8 (9:1, v/v); when different pHs were desired, isotonic buffers containing 0.03 M glycine,  $\text{KH}_2\text{PO}_4$ , and/or citric acid were used.

Proteins were determined according to Gornall et al. (2) and verified by use of a Coleman nitrogen analyzer (Coleman Instruments Div., Perkin Elmer Corp., Maywood, Ill.) and by their absorbance at 280 nm after addition of 3% (w/v, final concentration) sodium dodecylsulfate. Cholesterol was determined with the 15949 TCAA colorimetric test of Boehringer, Mannheim, Germany. Phospholipids were analyzed by thin-layer chromatography on silica gel, using chloroform-methanol-acetic acid-water (25:15:4:2, v/v) as the developing solvent (3), followed by a spray with potassium dichromate in sulfuric acid and charring in an oven at 100°C.

Crude egg lecithin, containing about 60% phosphatidylcholine and smaller amounts of phosphatidylethanolamine, phosphatidylserine, and lysophosphatidylcholine, was obtained from E. Merck, Darmstadt, Germany; purified phosphatidylcholine was obtained from Sigma Chemical Co. (St. Louis, Mo.). Lipid micelles were prepared by suspending the crude lecithin or the purified phosphorylcholine in the isotonic NaCl-phosphate solution; the suspension was then clarified by 15 min sonication with an MSE 100 W sonifier (Measuring and Scientific Equipment Ltd., London, England). If desired, cholesterol, dissolved in diethyl ether, was added during sonication; the sonication vessel was then brought to 37°C to allow evaporation of ether. A 10:1 proportion (w/w) between phospholipids and cholesterol was generally maintained.

Colloidal suspensions of cholesterol in twice-distilled water were prepared according to Stadtman (4), by slow addition on the boiling water of 0.12 vol of acetone in which 500 mg of cholesterol had been dissolved. After complete evaporation of acetone and subsequent cooling, the milky filtrate contained about 0.2 mg cholesterol/ml.

Erythrocyte ghosts were prepared according to Dodge et al. (5), with minor modifications, and resuspended by sonication in the isotonic NaCl-phosphate solution. Most experiments reported in the present paper were performed using the same membrane preparation, containing 2.64 mg protein/ml and 0.55 mg cholesterol/ml. Other preparations, however, behaved in an identical manner.

Mitochondria were prepared from rat liver, according to Schneider (6) and fragmented by repeated freezing and thawing in hypotonic (0.03 M) phosphate buffer pH 7.6, followed by 45 min centrifugation at 145,000 g.

The fluorescence spectra were recorded in an Aminco Bowman spectrophotofluorometer (American Instrument Co., Inc., Travenol Laboratories Inc., Silver Spring, Md.), using 1 cm cuvettes, excitation slit of 0.1 mm, and emission slit between 0.1 and 0.5 mm. The fluorescence unit was arbitrarily defined as corresponding to 0.4 mV at the recorder output. In some cases, quantum yield was determined from the area of the emission curve by comparison with that of a quinine disulfate standard (7).

The degree of polarization  $p$  was evaluated according to the well-known formula

$$p = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp}),$$

correcting for measurement artifacts by repeated measurements and according to Burns (8). Absorption spectra were obtained on a Beckman DK2 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), and circular dichroism (CD) spectra on a Cary 60 spectropolarimeter (Cary Instruments, Monrovia, Calif.) equipped with a model 6002 CD attachment. In all cases the background was subtracted.

All measurements were effected after attainment of equilibrium, as checked by repeated measurements or by previous investigation of the kinetics of the reaction; generally, a delay of 3–4 h or even more between preparation and reading of the samples was observed.

## RESULTS

### *Fluorescence Increase upon Binding to Cholesterol Molecules*

As previously reported (1), in the absence of membranes the fluorescence of lucensomycin in aqueous solution at neutral pH was extremely low, and increased very sensibly upon addition of membranes. Excitation spectra showed, like absorption ones, maxima at 291, 305, and 320 nm. The emission spectrum had a peak centered at 410 nm, which, under the experimental conditions used, was structureless. Typical titration curves can be obtained.

Preliminary kinetics experiments indicated that, in the observable range (lucensomycin  $\geq 0.3 \mu\text{M}$ , membrane cholesterol  $\geq 1 \mu\text{M}$ ), the fluorescence increase occurred at a rate too high to be followed with conventional mixing techniques, being practically complete within 6–10 s. Fluorescence of lucensomycin-membrane complex showed (Fig. 1), upon variation of pH, a wide maximum in the neutral range, decreasing at pH  $< 3$  ( $pK \simeq 2$ ) and at pH  $> 8$  ( $pK \simeq 9.3$ ). These results are consistent with the amphoteric nature of lucensomycin, which has (9) a car-

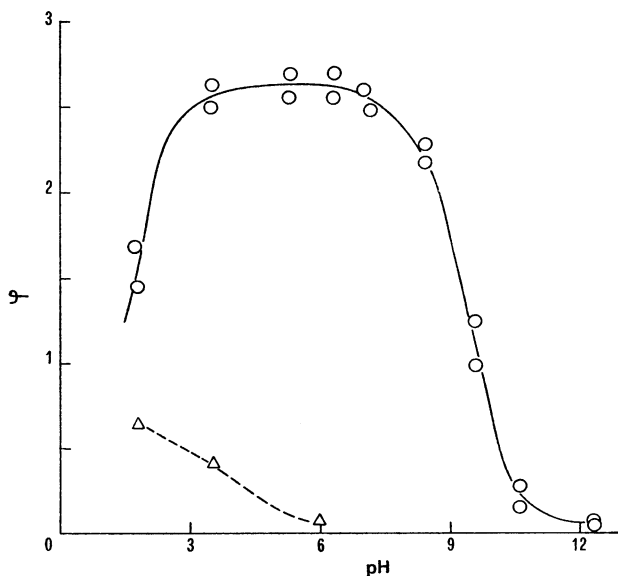


FIGURE 1 pH dependence of the fluorescence intensity of membrane-lucensomycin complex. Measurements were performed with a  $1.6 \mu\text{M}$  aqueous solution of lucensomycin ( $\circ$ — $\circ$ ) or *N*-acetyl-lucensomycin ( $\Delta$ — $\Delta$ ) in the presence of an amount of erythrocyte membranes corresponding to 32 nmol cholesterol/ml.

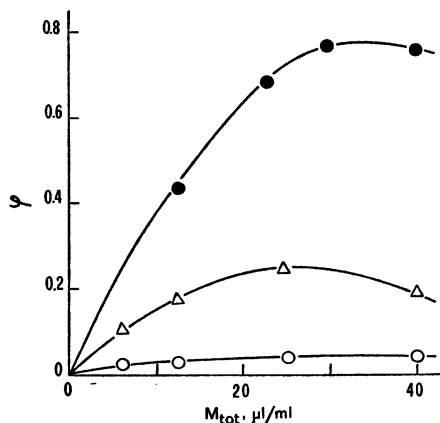


FIGURE 2 Fluorometric titration of 4  $\mu\text{M}$  lucensomycin with a 1 mg/ml suspension of egg lecithin, without (O) or with 25  $\mu\text{g/ml}$  ( $\Delta$ ) or 100  $\mu\text{g/ml}$  ( $\bullet$ ) cholesterol.

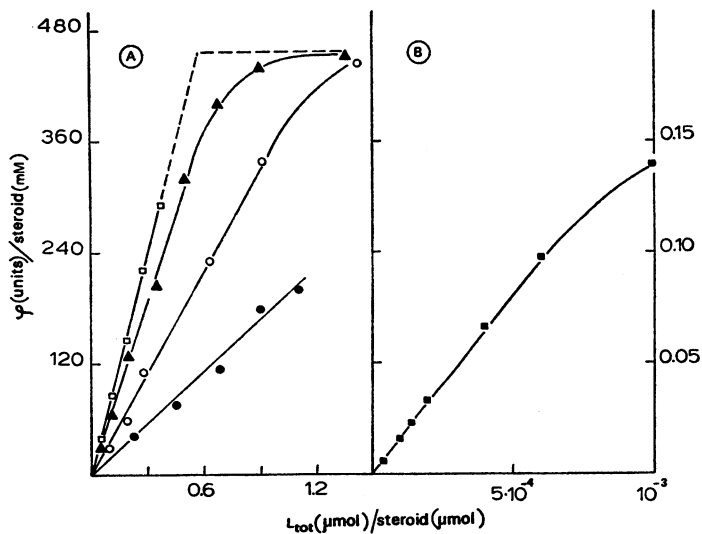


FIGURE 3 Fluorometric titration of cholesterol (A) or deoxycholate (B) with increasing amounts of lucensomycin. Cholesterol concentrations: 0.52  $\mu\text{M}$  ( $\bullet$ — $\bullet$ ); 1.3  $\mu\text{M}$  ( $\circ$ — $\circ$ ); 5.2  $\mu\text{M}$  ( $\Delta$ — $\Delta$ ); 13  $\mu\text{M}$  ( $\square$ — $\square$ ). Na deoxycholate ( $\blacksquare$ — $\blacksquare$ ): 6 mM. All data are normalized to the same steroid concentration. The 13  $\mu\text{M}$  cholesterol curve is extrapolated (----) to the expected asymptote.

boxyl group linked to the macrolide lactone ring and an amino group in the glycosidic moiety. The *N*-acetylated derivative became, instead, fluorescent, in the presence of membranes, only at acidic pH, confirming the requirement of the absence of a net charge for formation of a fluorescent complex.

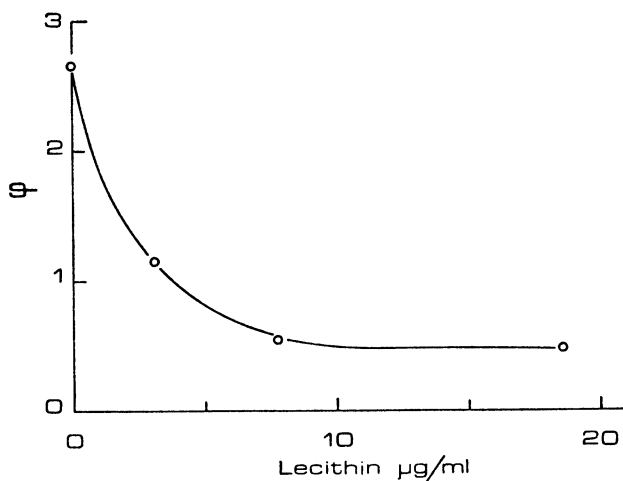


FIGURE 4 Decrease of fluorescence upon addition of lecithin to a lucensomycin-cholesterol aqueous suspension. Cholesterol was  $20 \mu\text{M}$ , added as a colloidal aqueous suspension. Lucensomycin was  $1.5 \mu\text{M}$ . Fluorescence is expressed as conventional units. The experimental points correspond to lecithin:cholesterol ratios, on a weight basis, of 0, 0.4, 1, and 2.4.

#### *Lucensomycin Fluorescence in Model Systems*

Addition of micelles prepared with the total lipid fraction from beef erythrocytes to lucensomycin in aqueous solution at neutral pH gave a fluorescence increase, with titration curves identical, on a cholesterol basis, with those obtained with whole erythrocyte ghosts. If, to the polyene in aqueous solution, rat liver mitochondrial fragments or phospholipid (egg lecithin) micelles were added, a moderate increase of fluorescence occurred. As previously reported (1), lucensomycin also exhibited some fluorescence when dissolved in a number of organic solvents. No direct relation between the intensity of emission and the polarity of the solvent, expressed either as dielectric constant or as  $Z$  value (10), could be evidenced, the fluorescence being stronger in dimethylsulfoxide or even in formamide than in *n*-hexane. The quantum yield reached, at best (i.e., in dioxane), a value of 0.026, which is only  $\frac{1}{10}$  of that obtained with membranes in water (1). With dioxane or chloroform as solvents, addition of membranes or of cholesterol did not cause any further increase of fluorescence; when, instead, cholesterol was included in phospholipid micelles suspended in aqueous medium, fluorescence reached values comparable with those obtained with erythrocyte membranes, the maximal fluorescence reached apparently depending on the cholesterol:phospholipid ratio (Fig. 2).

Indeed, addition of a colloidal suspension of cholesterol in water was also able to cause the increase of lucensomycin fluorescence in phospholipid-free aqueous

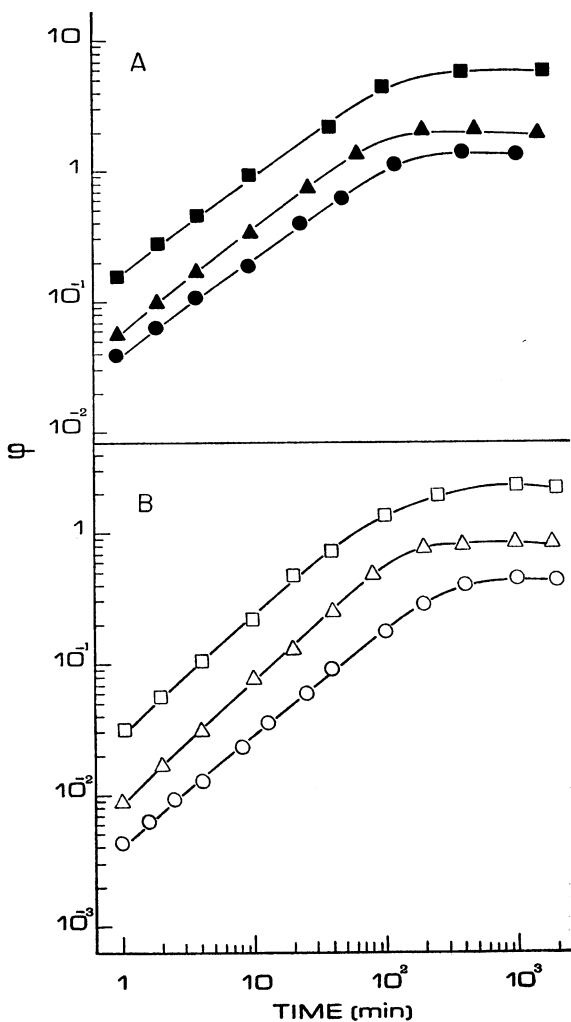


FIGURE 5 Kinetics of the fluorescence increase upon addition, to lucensomycin in aqueous medium, of cholesterol in colloidal suspension. Both scales are logarithmic. In A, lucensomycin was  $8.5 \mu\text{M}$ , and cholesterol  $2 \mu\text{M}$  (●—●),  $5 \mu\text{M}$  (▲—▲) or  $20 \mu\text{M}$  (■—■). In B, lucensomycin was  $2 \mu\text{M}$ , and cholesterol  $1 \mu\text{M}$  (○—○),  $2.5 \mu\text{M}$  (△—△) or  $10 \mu\text{M}$  (□—□). Fluorescence is expressed in arbitrary units.

media. Typical titration curves can thus be obtained (Fig. 3 A). As a matter of fact, addition of phospholipids decreased, sometimes to a considerable extent, the fluorescence of lucensomycin-cholesterol complexes (Fig. 4). The reason for this behavior shall be discussed in the second paper of this series (11).

A considerable difference could be evidenced when the kinetics of fluorescence increase due to the presence of phospholipid-free colloidal cholesterol or to erythrocyte membrane were compared. In the former case, a half-time between 40 min and

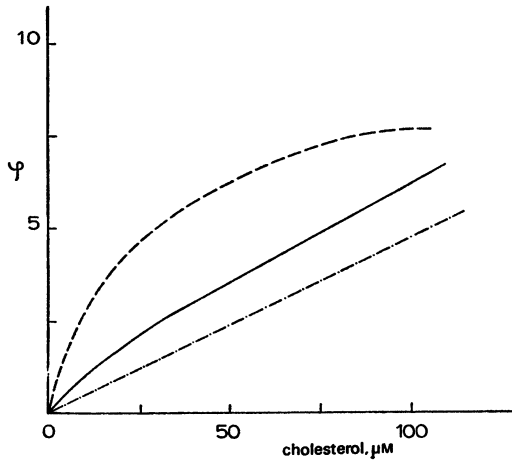


FIGURE 6 Time-dependent variation of fluorometric titration curves, with  $5.4 \mu\text{M}$  lucensomycin and increasing cholesterol. The various solutions were mixed at time 0, and the fluorescence intensity measured, on duplicate samples, after 25–30 min (— · — · —), 120–125 min (—), and 960–965 min (---). (No further variations of shape of the titration curves occurred with time up to 3 days.) Experimental points are omitted for sake of clarity.

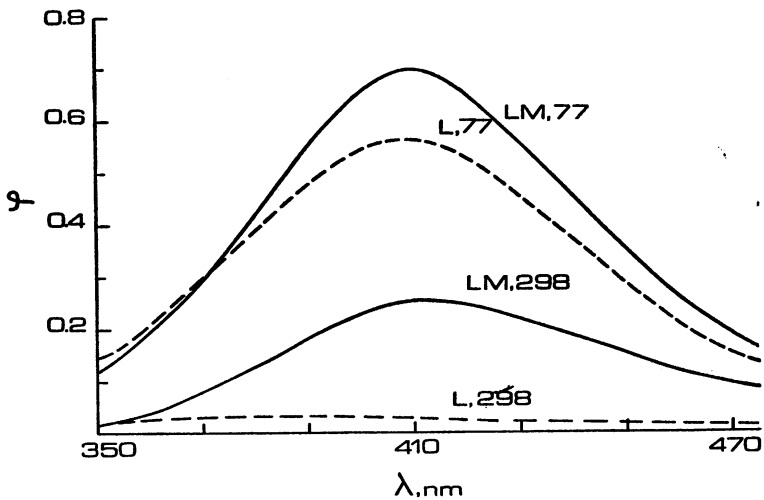


FIGURE 7 Fluorescence spectra of lucensomycin at liquid nitrogen ( $77^\circ\text{K}$ ) or at room temperature ( $298^\circ\text{K}$ ), in the absence (---: L) or in the presence (—: LM) of erythrocyte membranes. The spectra were recorded on the Aminco-Bowman spectrophotofluorometer equipped for phosphorimetry, but without inserting the chopper. The sample, containing  $3 \mu\text{M}$  lucensomycin and, if the case,  $28 \mu\text{M}$  membrane cholesterol in ethylene glycol-water 1:1 (v/v), was put in a quartz tube (2 mm, internal diameter) surrounded by an optically transparent Dewar with ( $77^\circ\text{K}$ ) or without ( $298^\circ\text{K}$ ) liquid nitrogen.

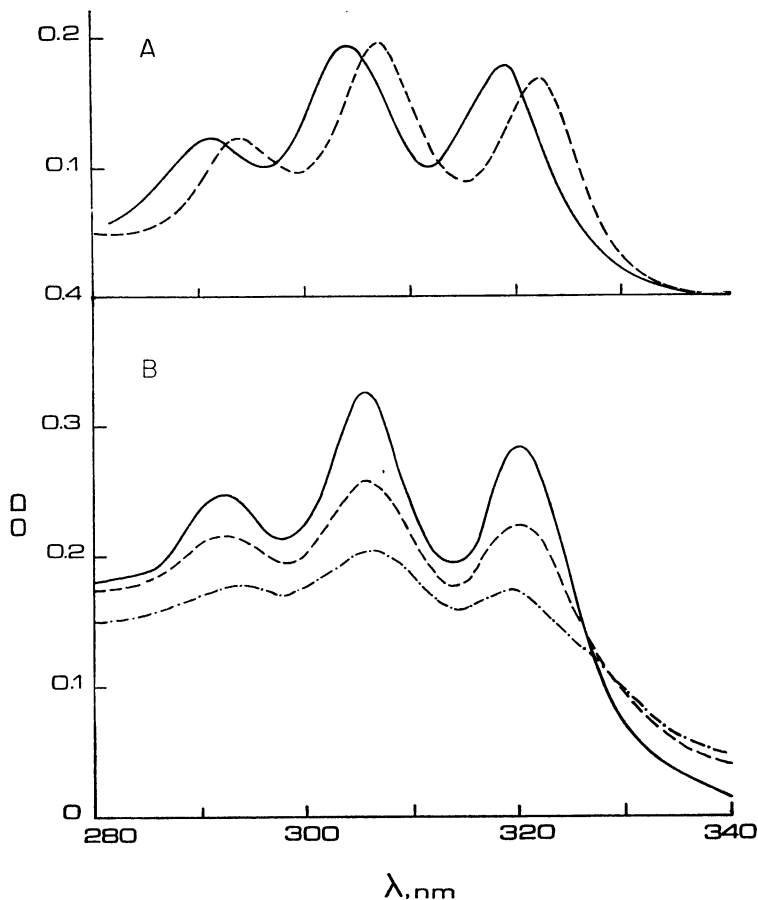


FIGURE 8 Variations in absorption spectra of lucensomycin in aqueous medium upon addition of colloidal cholesterol (B) or of sodium deoxycholate (A). Above (A): lucensomycin 2  $\mu\text{M}$  in the absence (—) or presence (---) of 12 mM sodium deoxycholate. Below (B): lucensomycin 5.4  $\mu\text{M}$  in the absence or presence of 2.0  $\mu\text{M}$  (-----) or 5.5  $\mu\text{M}$  (-·-·-·-) colloidal cholesterol. In both cases, spectra were recorded at equilibrium.

130 min was found in the range of our experimental conditions: in the latter one (Fig. 5), as already mentioned, the reaction was complete within the first minute. Such a behavior could lead to miscalculations of titration curves (Fig. 6). Lucensomycin fluorescence in aqueous solution could also be enhanced by addition of steroids such as cholate, deoxycholate, or digitonin. Ouabain was, instead, ineffective. Fig. 3 B illustrates the titration curve of 6 mM deoxycholate with increasing amounts of lucensomycin: the maximal fluorescence is sensibly lower, on a steroid basis, than that obtained with cholesterol or with erythrocyte ghosts.

The shapes of the excitation and emission spectra of lucensomycin-cholesterol



and lucensomycin-membrane complexes are identical and do not depend on the lucensomycin:steroid ratio. Except for a small difference in the relative height of the 305 and 320 nm peaks, they are also close, apart from the intensity, to those obtained with lucensomycin alone in dioxane or chloroform (see also reference 1).

At liquid nitrogen temperature (77°K), in an ethylene glycol-water (1:1, v/v) glass, lucensomycin alone showed a well-measurable fluorescence, centered at  $405 \pm 5$  nm (Fig. 7). Addition of erythrocyte membranes did not lead to a significant increase of fluorescence intensity. At room temperature, under otherwise

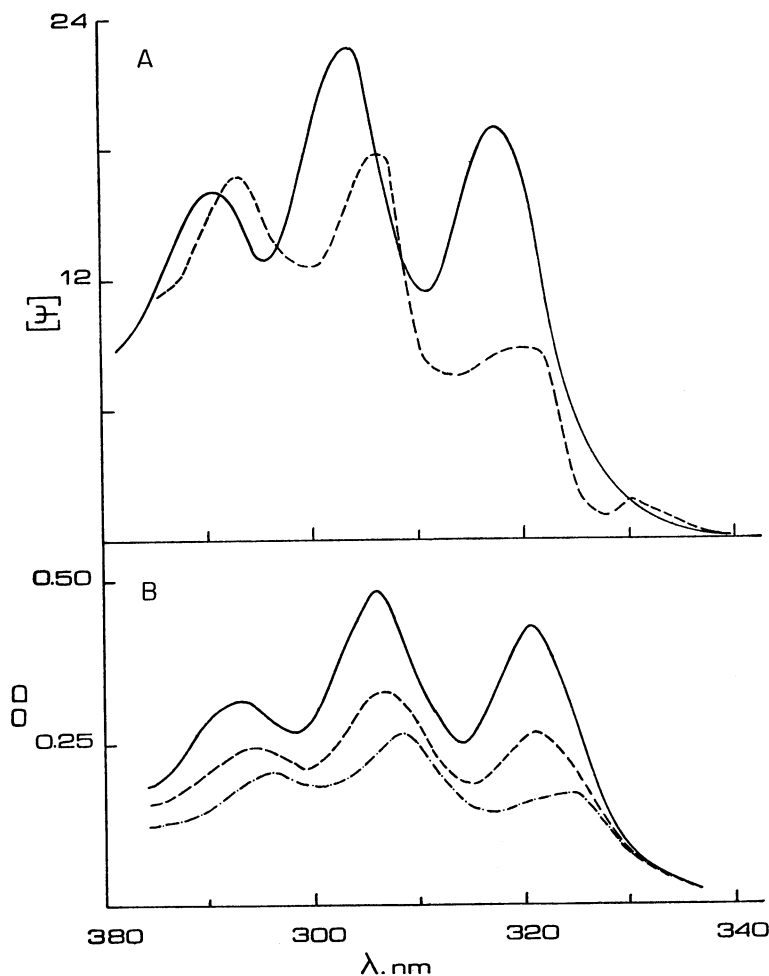


FIGURE 9 Above (A): CD spectra of 30  $\mu$ M lucensomycin in the absence (—) or presence (---) of 35.6  $\mu$ M erythrocyte membrane cholesterol.  $[\psi]$  is expressed as millidegrees. Below (B): absorption spectra of 7  $\mu$ M lucensomycin in the absence (—) or the presence of 3.1  $\mu$ M (---) or 12.4  $\mu$ M (-·-·-) erythrocyte membrane cholesterol. In the reference cuvettes, blanks without the polyene.

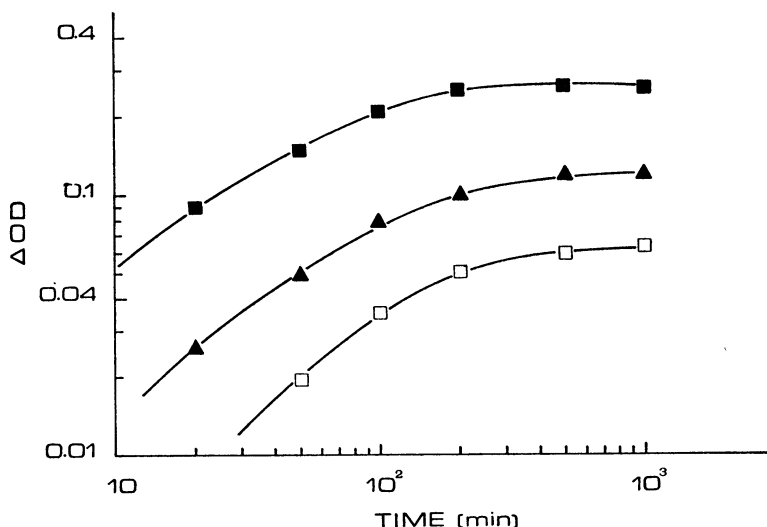


FIGURE 10 Kinetics of the variations of the optical density at 320 nm upon addition of cholesterol in colloidal suspensions to lucensomycin in aqueous medium. Both scales are logarithmic. Symbols are the same as in Fig. 5:  $\square$ — $\square$  lucensomycin 2  $\mu\text{M}$  + cholesterol 10  $\mu\text{M}$ ;  $\blacktriangle$ — $\blacktriangle$  lucensomycin 8.5  $\mu\text{M}$  + cholesterol 5  $\mu\text{M}$ ;  $\blacksquare$ — $\blacksquare$  lucensomycin 8.5  $\mu\text{M}$  + cholesterol 20  $\mu\text{M}$ . Fluorescence as arbitrary units.

identical conditions, the intensity of lucensomycin alone was too low to be measured and increased sensibly, though not as much as in the absence of ethylene glycol, upon addition of membranes. Phosphorescence at 77°K was very weak, detectable only if fluorescence had been suppressed by use of rotating windows, and it did not vary upon addition of membranes.

The mobility of the fluorescent species at room temperature was investigated by use of fluorescence polarization measurements. In 90–100% dioxane, lucensomycin alone had  $p = 0.21$  and did not vary upon addition of membranes. In aqueous medium and in the presence of colloidal cholesterol we found  $p = 0.31$ – $0.32$ , without any variation due to increased cholesterol:lucensomycin ratio or to the progress of the reaction. In erythrocyte membranes, either before or after sonication, we found  $p = 0.40$ – $0.42$ .

The binding of lucensomycin to free steroids or to erythrocyte membranes was accompanied by changes in the absorption spectrum of the polyene. In the presence of colloidal cholesterol, at equilibrium, there was a decrease of the whole absorption spectrum; in the presence of cholate or deoxycholate there was, instead, mainly a red shift, with little or no variation of extinction coefficient (Fig. 8). Upon addition of erythrocyte membranes, a small red shift was associated with a reduced intensity of the absorption bands of the polyene, especially of that at 320 nm. This variation was also detectable, with even greater ease due to noninterference, in this region of the relatively small light scattering of the membranes, in CD spectra (Fig. 9).

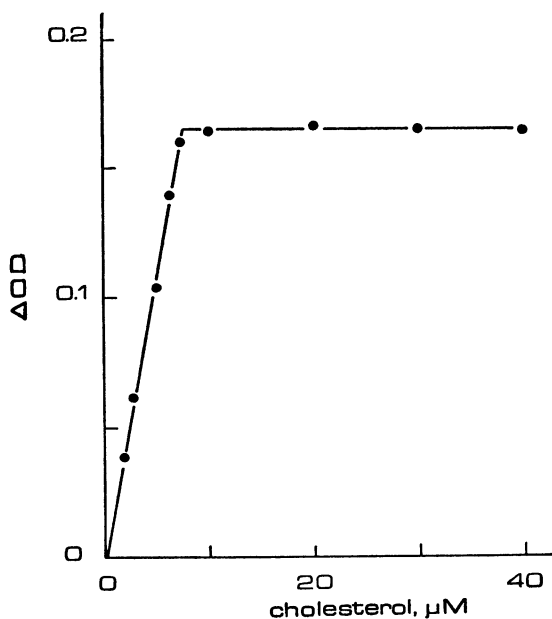


FIGURE 11 Spectrophotometric titration of  $5.4 \mu\text{M}$  lucensomycin with increasing amounts of cholesterol. The absorption at  $320 \text{ nm}$  of samples to which various amounts of colloidal cholesterol had been added was measured  $960 \text{ min}$  after mixing. It should be noted that this curve, belonging to the same experiment, corresponds to the final curve of the fluorometric titration illustrated in Fig. 6.

Kinetically, the variations of lucensomycin absorption spectra upon addition of membranes occurred within the first minute after mixing, while upon addition of colloidal cholesterol the reaction required several minutes (Fig. 10). Some differences between the fluorescence and the absorption variations were, however, present and are apparent from a careful comparison of Figs. 5 and 10: the curves at  $8.5 \mu\text{M}$  lucensomycin and  $5 \mu\text{M}$  cholesterol ( $\blacktriangle$ — $\blacktriangle$ ) and at  $2 \mu\text{M}$  lucensomycin and  $10 \mu\text{M}$  cholesterol ( $\square$ — $\square$ ) are almost superimposable in Fig. 5, and differ to a large extent in Fig. 10. Waiting for equilibrium to be reached, a titration of a known amount of lucensomycin with varying cholesterol concentration can be performed, as shown in Fig. 11.

#### DISCUSSION

The results presented in this paper confirm previous reports (12–14) indicating that the main binding site of polyenic antibiotics to membranes is the cholesterol moiety. When this work had almost been completed, two reports appeared: one (15) reporting modifications of the absorption spectrum of the related macrolide polyene filipin upon addition of cholesterol or of membranes, the other (16) reporting similar

modifications, as well as fluorescence increase, upon binding to cholesterol of a third polyene, pimaricin.

The reason for the increased fluorescence of lucensomycin upon addition of cholesterol or of erythrocyte membranes does not appear to be due to formation of an exciplex, i.e., to arise from the excitation of the lucensomycin-cholesterol complex. In fact, in such a case the emission spectrum of the cholesterol-bound polyene should have been shifted to longer wavelengths as compared with that of the free polyene in organic solvents, and this is not the case. It appears more likely that the fluorescence of free lucensomycin is quenched, at room temperature, by the aqueous solvent, through internal or collisional energy loss. Either when it is at liquid nitrogen temperature or when it is bound to cholesterol, this quenching effect decreases, and fluorescence appears. At 77°K addition of membranes does not bring about any further increase of fluorescence; no thorough investigation of the temperature dependence of the binding of lucensomycin to membranes was, however, performed. The absence of a net charge on the polyene molecule appears to be a prerequisite for fluorescence.

It may be of interest that increase of fluorescence upon binding to cholesterol has also been found by Schroeder et al. (16) with pimaricin, which is, like lucensomycin, an amphoteric tetraene. Filipin, which has no ionizable groups, is fluorescent per se; upon binding to cholesterol containing membranes its fluorescence did not, in our hands, vary, although Schroeder et al. (16) have reported a decrease and Lagwiaska et al. (18) an increase of fluorescence. The size of macrolide ring can also be of importance: preliminary experiments with nystatin and amphotericin B, amphoteric polyenes with very large rings, indicated that only a minor increase of fluorescence intensity occurred.

Binding of the polyene to cholesterol or to membranes causes a conspicuous immobilization of the molecule, the polarization factor increasing from 0.21 (in dioxane) to 0.31 and to 0.41, respectively. This shows that the macrolide ring, which is always relatively rigid, becomes almost fixed when included in a membrane structure, it may be recalled that the maximum value for  $p$  is 0.50.

In the accompanying paper (11), it shall be shown that at high cholesterol, or membrane, concentration, the titration curve with increasing amounts of lucensomycin is a straight line starting from the origin and reaching the horizontal asymptote. The abscissa of the intersection with the asymptote gives the number of polyene molecules which combine, under optimal conditions, to each sterol molecule. From the data of Fig. 3 the fluorescent complex appears to be of the type  $LC_2$ . A similar ratio is also obtained from the spectrophotometric titration of Fig. 11. This result is at variance with those obtained with other polyenes by Norman et al. (15) and by Schroeder et al. (16); reasons for this discrepancy are not known.

As for the equilibrium constant of the reaction, the discrepancies between fluorometric (Fig. 6) and spectrophotometric (Fig. 11) titrations do not afford to evaluate

it. In contrast to what shall be shown with erythrocyte membranes in the accompanying paper (11), with colloidal cholesterol both spectrophotometric and fluorometric titration curves, though different, would allow us to assume that the two binding sites are equivalent, both as an intrinsic equilibrium constant and as a fluorescence quantum yield. From the fluorometric curves, the equilibrium constant would be of the order of 0.1–0.3  $\mu\text{M}$ ; from the spectrophotometric ones, it would be at least 10 times smaller.

*Received for publication 5 June 1972 and in revised form 24 January 1973.*

## REFERENCES

1. CRIFÒ, C., R. STROM, A. SCIOSCIA SANTORO, and B. MONDOVI. 1971. *FEBS (Fed. Eur.) Lett.* **17**:121.
2. GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID. 1949. *J. Biol. Chem.* **177**:751.
3. SKIPSKI, V. P., and M. BARCLAY. 1969. *Methods Enzymol.* **14**:530.
4. STADTMAN, T. C. 1957. *Methods Enzymol.* **3**:392.
5. DODGE, J., C. MITCHELL, and D. T. HANAHAN. 1963. *Arch. Biochem. Biophys.* **100**:119.
6. SCHNEIDER, W. C. 1948. *J. Biol. Chem.* **176**:259.
7. UDENFRIEND, S. 1969. *Fluorescence Assay in Biology and Medicine*. Academic Press, Inc., New York.
8. BURNS, V. W. 1971. *Arch. Biochem. Biophys.* **145**:248.
9. GAUDIANO, G., P. BRAVO, A. QUILICO, B. T. GOLDING, and R. W. RICHARDS. 1966. *Gazz. Chim. Ital.* **96**:1470.
10. KOSOWER, E. M. 1958. *J. Am. Chem. Soc.* **80**:253.
11. STROM, R., C. CRIFÒ, and A. SCIOSCIA SANTORO. 1973. *Biophys. J.* **13**:581.
12. DEMEL, R. A., L. L. M. VAN DEENEN, and S. C. KINSKY. 1965. *J. Biol. Chem.* **240**:2749.
13. KINSKY, S. C., S. A. LUCE, D. ZOFF, L. L. M. VAN DEENEN, and J. HAXBY. 1967. *Biochim. Biophys. Acta.* **135**:844.
14. ZUTPHEN, M. VAN, L. L. M. VAN DEENEN, and S. C. KINSKY. 1966. *Biochim. Biophys. Res. Commun.* **22**:393.
15. NORMAN, A. W., R. A. DEMEL, B. DE KRUYFF, and L. L. M. VAN DEENEN. 1972. *J. Biol. Chem.* **247**:1918.
16. SCHROEDER, F., J. F. HOLLAND, and L. L. BIEBER. 1972. *Biochemistry.* **11**:3105.
17. MATAGA, N., and T. KUBOTA. 1970. *Molecular Interactions and Electronic Spectra*. Marcel Dekker, Inc., New York. Chap. 9.
18. LAGWIASKA, E., M. G. SARZALA, and W. DRABIKOWSKI. 1972. Abstracts of the 4th International Biophysical Congress, E IX b I/5, Moscow. **3**:54. (Abstr.)