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

II. Cooperative Effects in Erythrocyte Membranes

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ABSTRACT Fluorometric titration curves of erythrocyte membranes with increasing lucensomycin have a sigmoid shape. This behavior, which was not present when colloidal cholesterol suspensions were used, is, however, not peculiar to the membrane structure, being also present in cholesterol-containing phospholipid micelles. Addition of acetic acid induced or increased sigmoidicity. This behavior can either be due to a true cooperativity in binding or to different fluorescence yields of the various lucensomycin-membrane complexes. The latter hypothesis appears to be slightly favored.

INTRODUCTION

In the accompanying paper (1) we have described the variations of fluorescence emission and of absorbancy of lucensomycin upon interaction with cholesterol molecules. With colloidal cholesterol suspensions, although cholesterol determinations indicated the formation of LC_2 complexes (L being the polyene and C the binding site), both fluorescence and spectrophotometric titrations gave curves compatible with a simple $L + C \leftrightharpoons LC$ mechanism or with the equivalence of the two binding sites. We report here evidence indicating that, in cholesterol-containing lipid micelles or in erythrocyte membranes, the reaction is more complex, either because of cooperativity, in the micelle or in the membrane, between the binding sites for the polyene or because of a cooperative effect restricted to the ability to fluoresce.

MATERIALS AND METHODS

As previously described (1).

RESULTS

Fluorometric Measurements of the Binding to Erythrocyte Membranes

Fig. 1 illustrates how fluorescence intensity depended, at various lucensomycin concentrations, upon the amount of membrane added. Using polyene concentrations > 0.6 μM, the fluorescence reached a maximal value corresponding to a quantum yield of about 0.25. At lower lucensomycin concentrations, however, the maximal fluorescence was significantly less, as if not all polyene molecules had become fluorescent. Fig. 2 indicates how the maximal fluorescence reached depended upon lucensomycin concentration. In some cases (i.e., at the lowest concentrations) it would even be shown that fluorescence, after having reached a maximum, decreased upon further addition of membranes (see insert of Fig. 1); this decrease could not be attributed solely to an enhanced light-scattering effect of membranes.

Titration curves of known amounts of membranes with varying lucensomycin

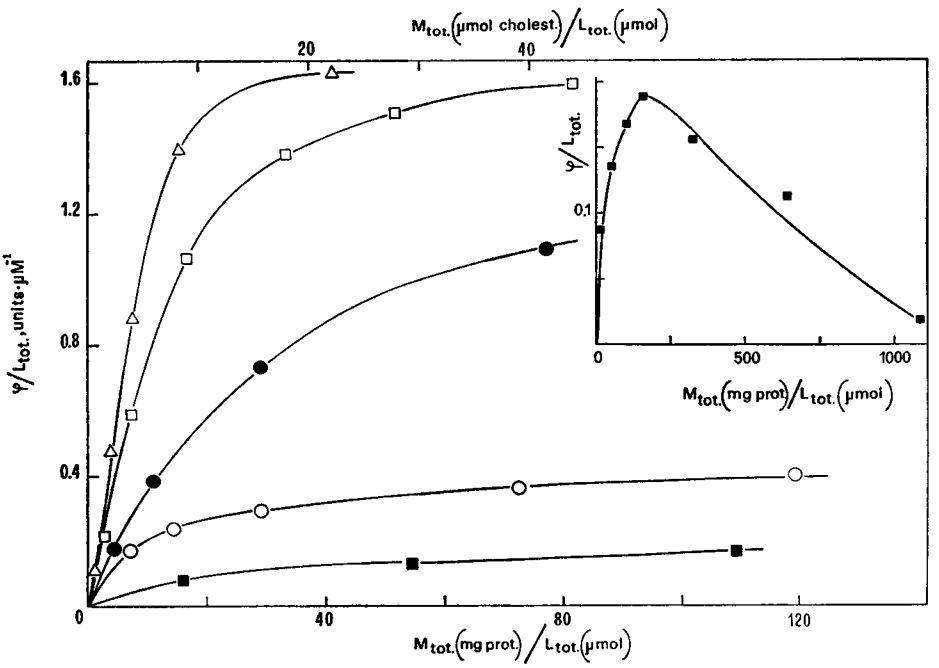

FIGURE 1 Fluorometric titration of lucensomycin with erythrocyte membranes. Five different concentrations of the polyene were used: 0.24 μM (■); 0.36 μM (○); 0.5 μM (●); 0.8 μM (□); 2.3 μM (△). Fluorescence (φ) is expressed as arbitrary units of emission at 410 nm upon excitation at 308 nm (see Methods). All values are normalized for lucensomycin concentration. Membranes are expressed either as milligrams of protein (lower scale) or micromoles of cholesterol (upper scale). In the insert, the full titration curve, extending to high M_{tot}/L_{tot} ratios, of 0.24 μM lucensomycin (■) is reported.

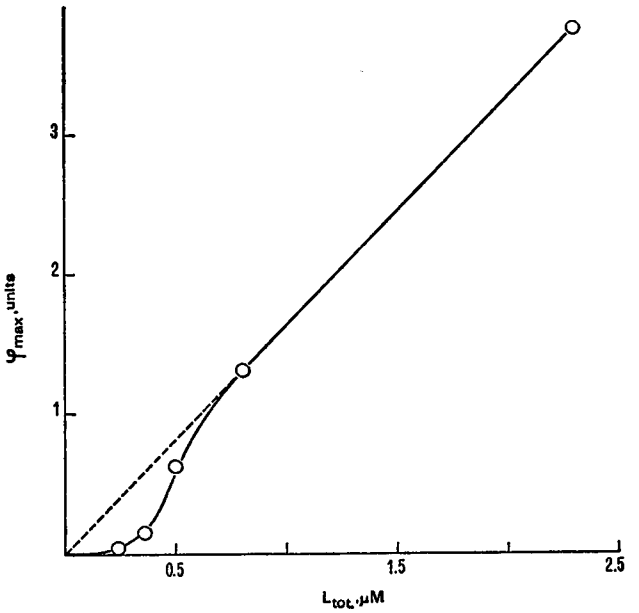

FIGURE 2 Relation between lucensomycin concentration and maximal fluorescence which may be attained upon addition of erythrocyte membranes.

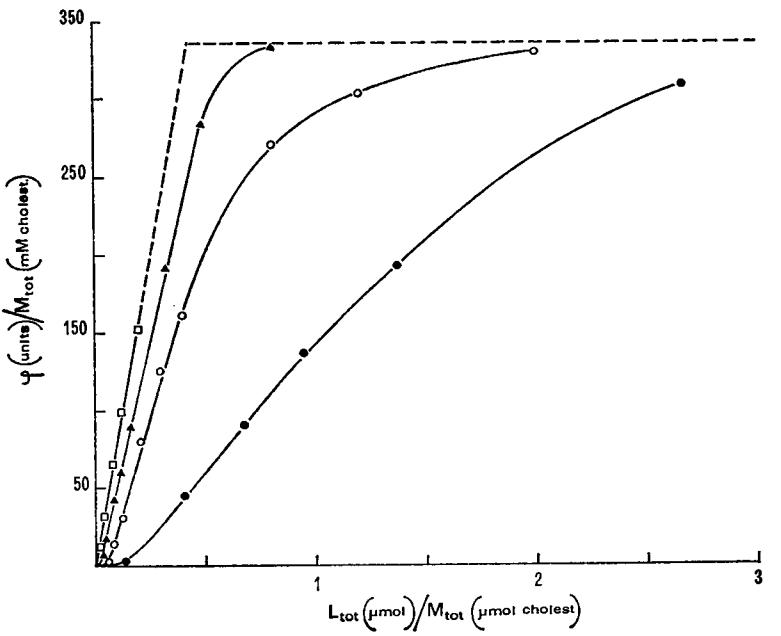

FIGURE 3 Fluorometric titration of erythrocyte membranes with lucensomycin. Five different concentrations of membranes were used, which, expressed as cholesterol, were: 0.95 μM (●); 3.15 μM (○); 8 μM (▲); 31.5 μM (□). The dotted line indicates the ideal behavior of the titration curve at very high membrane concentrations. All values are normalized for membrane concentration.

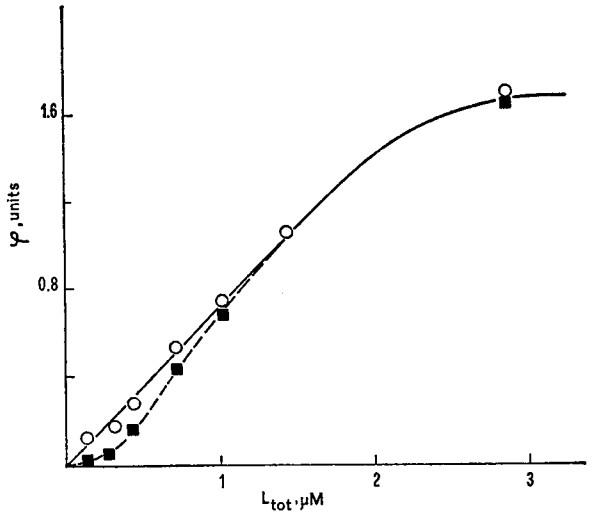

FIGURE 4 Fluorometric titration of erythrocyte ghosts with lucensomycin in the absence (■—■) or presence (○——○) of 3.7 μM *N*-acetyl-lucensomycin. The membrane concentration, expressed as cholesterol, was 3.6 μM.

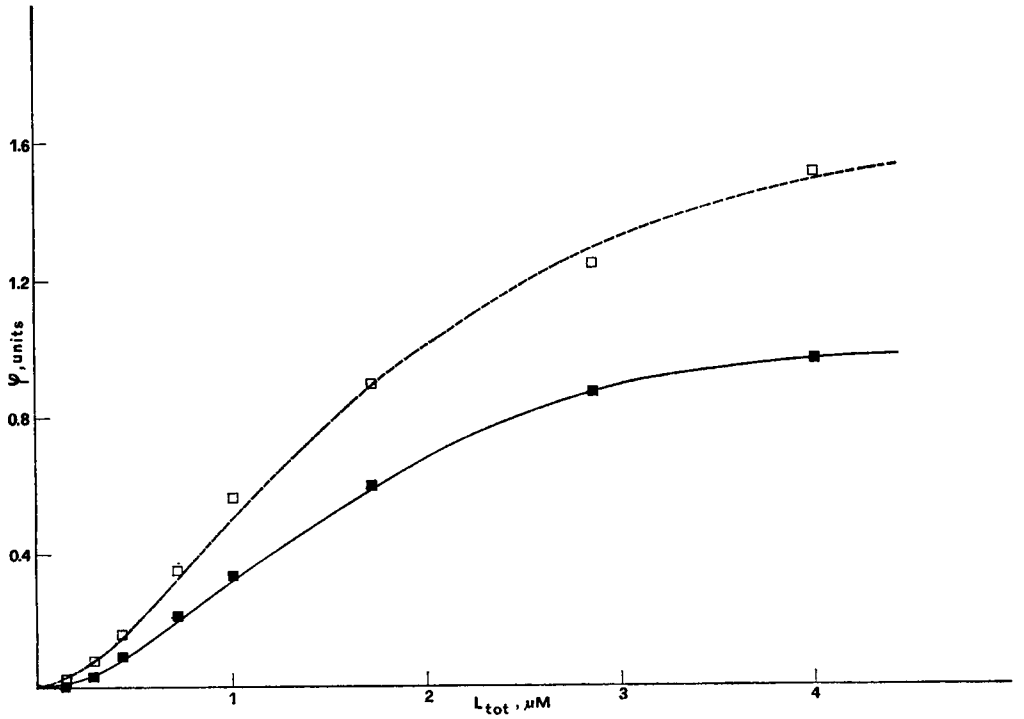

FIGURE 5 Fluorometric titration of erythrocyte ghosts in the absence (□----□) or presence (■——■) of 0.06% sodium dodecylsulfate; membrane concentration (as cholesterol): 3.6 μM.

had, as shown in Fig. 3, a sigmoidal shape, which, though more apparent when low amounts of membranes were used, was present in all cases. The slopes of the initial region of the curves had, in a double logarithmic plot, values between 2.8 and 3.1. The maximal fluorescence reached, at "infinite" lucensomycin concentrations, was strictly proportional to the membrane concentration; only if the polyene was > 4 μM could a concentration quenching be detected.

If the increasing amounts of lucensomycin were added to a given membrane concentration in the presence of *N*-acetyl-lucensomycin, which, as mentioned in the accompanying paper (1), did not fluoresce at neutral pH, the sigmoid character of the titration curve decreased, reaching finally a hyperbolic shape (Fig. 4).

Addition of nonionic or anionic detergents (Triton X 100 [Rohm and Haas Co., Philadelphia, Pa.], Nonidet P40, sodium dodecylsulfate) caused the fluorescence of the polyene-membrane complex to be quenched. As shown in Fig. 5, the whole titration curve became flatter, but the sigmoid character remained unaffected.

Fluorescence upon Binding to Phospholipid-Cholesterol Micelles

As previously mentioned (1), addition of micelles prepared with the total lipid fraction from beef erythrocytes to lucensomycin in aqueous solution gave results superimposable, on a cholesterol basis, on those obtained with whole erythrocyte ghosts. In this case too, the titration of known amounts of lipids with varying

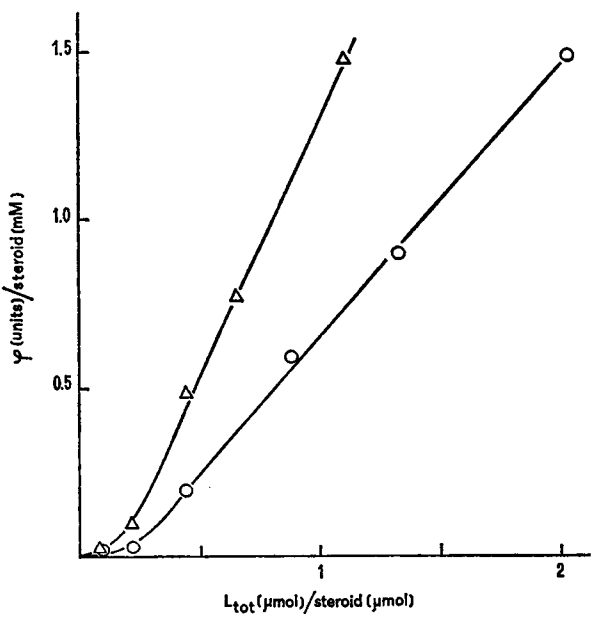

FIGURE 6 Fluorometric titration of 10:1 (w/w) lecithin-cholesterol micelles; cholesterol concentration equals 3.25 μM (○——○) or 6.5 μM (△——△).

lucensomycin had a sigmoidal shape. Curves with pronounced sigmoidicity were also found upon titration with lucensomycin of micelles formed by egg lecithin and cholesterol (Fig. 6).

Effect of Acetic Acid on Titration Curves of Colloidal Cholesterol and of Erythrocyte Membranes

In the former paper of this series (1), we showed that titration curves of colloidal cholesterol with increasing polyene did not show any sigmoidicity. A "cooperative" pattern was instead present if cholesterol was included in a phospholipid micelle, formed either by crude egg lecithin or by whole erythrocyte lipids. Preliminary experiments with purified phospholipids have not so far led to definite conclusions about the nature of the groups responsible for this effect. It was, however, found that a pronounced sigmoidicity could be induced in titration curves of colloidal

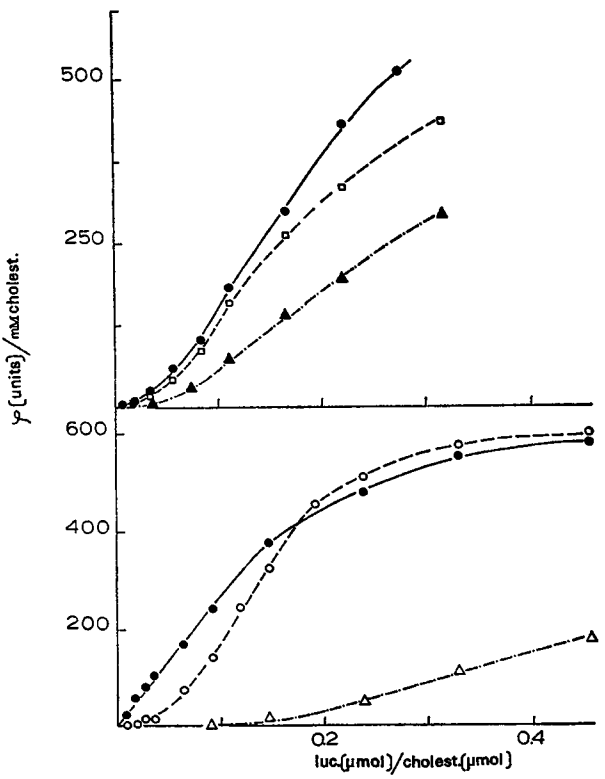

FIGURE 7 Above: Fluorometric titration of beef erythrocyte membranes (cholesterol concentration: 7 μM) with lucensomycin, in the absence (●——●) or presence of 0.43 M (□----□) or 1.30 M (▲·····▲) acetic acid. Below: Fluorometric titration of 5.2 μM colloidal cholesterol with lucensomycin, in the absence (●——●) or presence of 2.17 M (○—○) or 4.34 M (△·····△) acetic acid.

cholesterol by addition of relatively small amounts of acetic acid (Fig. 7, below). Even smaller amounts of acetic acid could increase the sigmoidicity of erythrocyte membranes titration curves (Fig. 7, above). The maximal fluorescence obtainable (at "infinite" lucensomycin) was not modified by such addition.

Estimate of Binding Characteristics by Nonfluorometric Means

The modifications of optical density which occur upon binding of lucensomycin to cholesterol and to erythrocyte membranes can be utilized to obtain titration curves based on measurements independent from fluorescence characteristics. In Fig. 8 is illustrated such a titration, corresponding approximately to the lowest one (●——●) of Fig. 3. No sigmoidicity is apparent, though the shape of the curve seems to indicate the presence of secondary phenomena.

Direct investigation of the binding of lucensomycin to erythrocyte membranes could also be performed by equilibrium gel filtration experiments, according to Fairclough and Fruton (2). Unfortunately, only polyene concentrations above

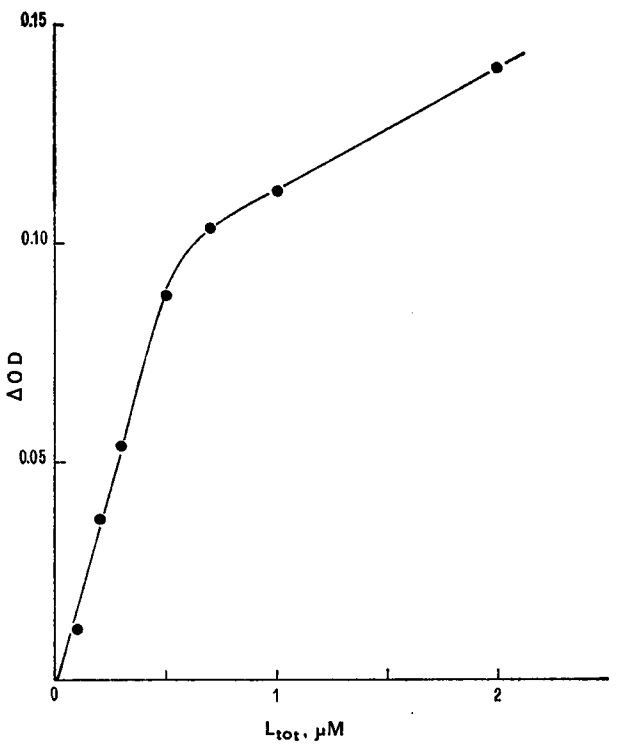

FIGURE 8 Spectrophotometirc titration of erythrocyte membranes (final cholesterol concentration: 1.1 μM) with increasing amounts of lucensomycin. On the ordinates is reported the difference of optical density at 319 nm, between the combined sample and the sum of the absorbancies of membranes and polyene separately.

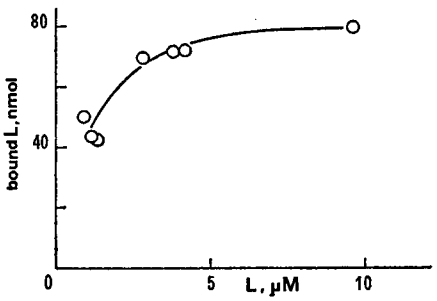

FIGURE 9 Ordinates: area, expressed as nanomoles of lucensomycin, of the trough after the membrane peak, when 150 μl of erythrocyte membranes, corresponding to 213 nmol of cholesterol, are passed through a 0.5 × 100 cm Sephadex G25 column equilibrated with lucensomycin. Abscissas: lucensomycin concentration.

1.3 μM could be evaluated quantitatively, on the basis of their extinction coefficient at 304 nm. A saturating value of approximately 0.38 μmol lucensomycin per μmol of membrane cholesterol was obtained (Fig. 9), while an estimate of the dissociation constant was beyond experimental reach.

Centrifugation of erythrocyte membranes-lucensomycin mixtures on 5–40% sucrose gradients showed that the polyene, in the presence of excess membranes, migrated in the fraction containing the membranes. The precision of this method was, however, not sufficiently high to allow obtention of titration curves.

DISCUSSION

The data obtained in the present work indicate that, because of the sigmoid character of fluorescence vs. polyene titration curves, the stoichiometry of the binding of lucensomycin (L) to erythrocyte membranes or to cholesterol-phospholipid micelles (M) is not amenable to the simple $L + M \leftrightharpoons LM$ equilibrium.

By analogy with results obtained in protein systems (3, 4), such a behavior can be accounted for by the existence in each micelle, or ghost, of several binding sites for lucensomycin. Evidence presented in the accompanying paper (1) indicates that polyenic antibiotics bind specifically to cholesterol. Some cholesterol molecules may not, however, be available for binding, or each polyene moiety may require more than a single cholesterol molecule, as shown by titration curves (Figs. 3 and 9) indicating a stoichiometric sterol-polyene ratio around 2.5. Nevertheless, in each ghost or micelle the number of cholesterol molecules may be envisaged to be high enough so as to allow for the existence of a multiplicity of binding sites.

The sigmoidal shape of the titration curves can be accounted for by two different mechanisms. The first one would be that of a cooperative interaction between binding sites, i.e., binding of the first lucensomycin molecule would favor binding of subsequent ones. In a micelle, or in a ghost, each site M, able to bind a single

polyene molecule, is surrounded by a number $(q - 1)$ of similar sites with which cooperative interaction occurs, this hypothesis shall be represented by the system:

$$\begin{aligned} L + M_q &\overset{K_1}{\rightleftharpoons} L_1 M_q \\ L + L_1 M_q &\overset{K_2}{\rightleftharpoons} L_2 M_q \\ &\cdots\cdots\cdots \\ L + L_{(q-1)} M_q &\overset{K_q}{\rightleftharpoons} L_q M_q \end{aligned} \qquad (1)$$

Fluorescence would, in this case, be proportional to the concentration of bound lucensomycin, i.e.

$$\varphi = \Phi \sum_{i=1}^{q} i(L_i M_q), \qquad (2)$$

so that

$$\varphi/(M_{\text{tot}}) = \Phi(M_{\text{liganded}})/(M_{\text{tot}}). \qquad (3)$$

$\varphi/(M_{\text{tot}})$ expresses therefore the fractional saturation $\bar{y}$ of membrane sites, when exposed to a given polyene concentration.

This model can also be, to a first approximation, treated by indicating the overall reaction as:

$$nL + M_n \overset{K'}{\rightleftharpoons} (LM)_n, \qquad (4)$$

where K' is the association constant of this nth order reaction. Simple application of mass law leads to the following equation (3, 4):

$$(L_{\text{tot}})/(M_{\text{tot}}) = \bar{y} + [1/(M_{\text{tot}})][\bar{y}/K'(1 - \bar{y})]^{1/n}. \qquad (5)$$

At sufficiently high M_{tot} concentrations the second term on the right side vanishes and $\bar{y} = 1$, i.e. $\varphi/(M_{\text{tot}})$ reaches its maximum value Φ, for $(L_{\text{tot}})/(M_{\text{tot}}) = 1$. In fact, this would be true if we had expressed (M_{tot}) as the concentration of binding sites; Fig. 3 shows that in erythrocyte ghosts, if (M_{tot}) is expressed as cholesterol concentration, this ratio is around 0.42. This would indicate that in ghosts each binding site is formed by approximately 2.5 cholesterol molecules. In colloidal cholesterol micelles (see Fig. 3 of reference 1) a ratio of about 0.5 had been found.

As for the sigmoid character of the titration curves, at low M_{tot} concentrations and at low fractional saturation $(\bar{y})$ values, Eq. 5 becomes, if $n > 1$,

$$(L_{\text{tot}})/(M_{\text{tot}}) \simeq 1/(M_{\text{tot}})[\bar{y}/K'(1 - \bar{y})]^{1/n} = 1/(M_{\text{tot}})[\varphi/K'\Phi(M_{\text{tot}})]^{1/n} \qquad (6)$$

and, in a double logarithmic plot,

$$\log [\varphi/(M_{tot})] = n\log (L_{tot}/M_{tot}) + \text{an additive term.} \quad (7)$$

The initial parts of the curves of Fig. 3, plotted in a double logarithmic plot, yield values of n around 3.

The first proposed mechanism would therefore be that of a real cooperativity in the binding of lucensomycin to membranes. Since formation of a lipid micelle is due to cooperative interaction between its constituents, such a model, which is similar to that proposed by Changeux et al. (5) would be expected to hold whenever the binding of a ligand to its target is favored by inclusion of this target into the micelle. Cooperativity would not be present upon binding to a site not included in a micelle, and would be decreased if some of the binding sites were already occupied by nondetectable ligand analogues (e.g., *N*-acetyl-lucensomycin) or if the micelle was altered by disrupting agents. The first two conclusions are supported by experimental results (see reference 1, and Fig. 4). As for the last one, addition of detergents, though leading to decreased fluorescence, did not affect cooperativity (Fig. 5); this treatment, however, does not completely disrupt the micelles but rather alters their composition, so that this experiment cannot by itself be taken as disproving this first proposed mechanism.

An alternative hypothesis would be that, though binding itself is not necessarily cooperative, fluorescence is so: i.e. the equilibrium constants K_1, K_2, $K_3 \ldots K_q$ of Eq. 1 have a distribution which may (but also may not) be statistical (i.e., $K_i = (q - i + 1/i)\cdot \bar{K}$, where $\bar{K}$ is the intrinsic equilibrium constant), but only the L_iM complexes with several ($i \geq 3$) lucensomycin molecules have a relevant "weight" in the production of fluorescence. This mechanism can, in first approximation, be also expressed by Eq. 4, so that most of the experimental evidence is unable to distinguish between the two hypotheses. Fluorescence is, however, in this case, an index not of the fractional saturation $\bar{p}$, but only of some L_iM complexes.

The latter hypothesis is favored by the following facts:

(*a*) The maximal fluorescence reached upon addition, to given concentrations of lucensomycin, of increasing amounts of erythrocyte membranes (Figs. 1 and 2) is not strictly proportional to the concentration of the polyene. Moreover, fluorescence declines upon further addition of membranes. Such a behavior would be expected if fluorescence is related to the existence of L_iM_q species with $i > 1$ (or, even better, if only the L_qM_q species is fluorescent). In Fig. 10 is shown a theoretical titration curve in the simplest case where $q = 2$, assuming that the intrinsic equilibrium constant is $\bar{K} = 4\ \mu M^{-1}$ (and therefore $K_1 = 8\ \mu M^{-1}$ and $K_2 = 2\ \mu M^{-1}$) and that $\varphi = \Phi\cdot(L_2M_q)$: the results are qualitatively similar to those illustrated in Fig. 1; a 20–40-fold decrease in the value of K_1 (i.e. around 0.3 μM, with deviation from statistical distribution), but not of K_2, would give a better fit with the experimental data obtained with erythrocyte ghosts.

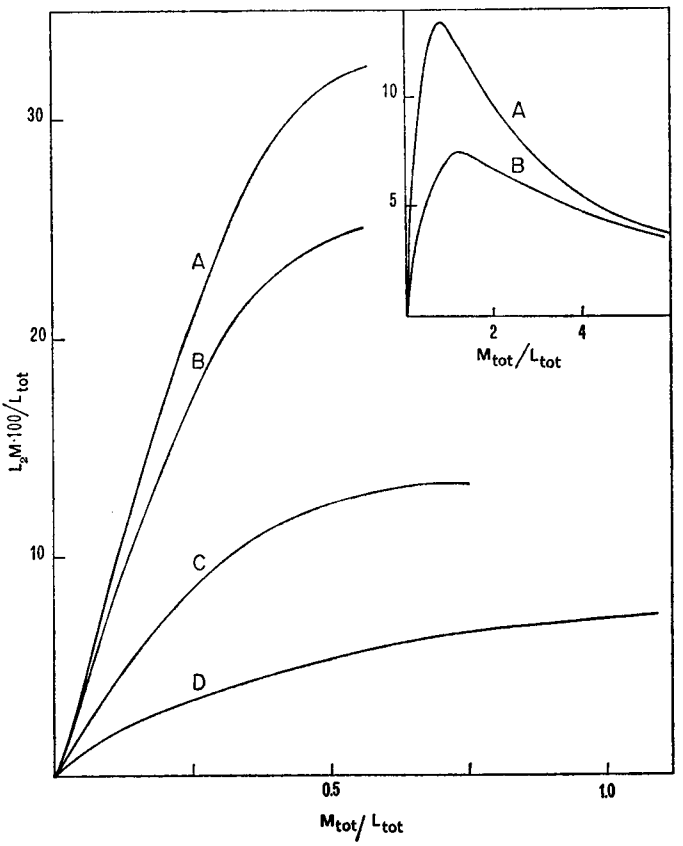

FIGURE 10 Theoretical titration curve of lucensomycin with cholesterol, assuming that each site M of the cholesterol-containing micelles can bind two lucensomycin molecules. The intrinsic equilibrium constant K was assumed 4 μM^{-1}, K_1 and K_2 after a statistical distribution ($K_1 = 8\ \mu M^{-1}$; $K_2 = 2\ \mu M^{-1}$) Lucensomycin concentrations: 5 μM (A); 2 μM (B); 0.5 μM (C); 0.2 μM (D). On the ordinate is reported the percentage of L_2M formed. In the insert, curves (C) and (D) are reported with different scales.

(*b*) Addition of acetic acid induces sigmoidicity in titration curves of colloidal cholesterol, and increases that of erythrocyte membranes. This would be explained, though it is not the only possible explanation, by a quenching effect of acetic acid on the L_iM_q species with lower values of i.

(*c*) Spectrophotometric evaluation of membrane-bound lucensomycin as a function of varying polyene failed (Fig. 8) to show a sigmoid shape. Assuming that absorbancy variations reflect the real binding of lucensomycin to cholesterol, cooperativity would appear to be related only to the ability of some molecular species to fluoresce, and not to reflect the characteristics of the binding itself. The validity of spectrophotometric and circular dichroism data as indices of binding is, however, far from being proven.

A further support to this hypothesis arises from the finding by Engelman and Rothman (6) that, in lecithin-cholesterol micelles, each cholesterol moiety is surrounded by phospholipids and is therefore well separated from other cholesterol molecules. More accurate evaluation of true binding parameters is, however, needed to choose between the two proposed mechanisms.

As for the possible topography of the polyene molecules in the membranes, in view of their effect on permeability a stacking of these large lactone rings on top of each other, leading to formation of an aqueous channel, can be envisaged: such a pattern has been shown to occur in crystals of polyenes (7). Fluorescence is, however, apparently not due to the formation of an excimer, i.e. of an excited state propagated to several polyene molecules, because in such a case the emission spectrum would be shifted to longer wavelengths (8). Lucensomycin can be visualized as becoming fluorescent only when, in a hydrophobic milieu, it is sandwiched between two similar molecules, so that its amino- and carboxyl groups are neutralized by interaction with adjacent polyenes.

From a functional point of view, lucensomycin, like other polyenic antibiotics, causes a severe increase of membrane permeability, leading to alterations of cell metabolism (9, 10). In the polyene range commonly used, this effect is strictly proportional to the lucensomycin:cell ratio (10), with no indication of cooperative effects. Other polyenes (e.g. amphotericin B, nystatin) have in fact been shown, with artificial bilayers, to exhibit a 4th–12th order cooperativity leading to increased conductance (11). The mechanism of formation of the aqueous pores, to which the increased conductance is due, is still unknown, being not amenable to a simple extraction of cholesterol from the membrane. Conspicuous rearrangements of the membrane architecture around the binding site of the polyene are likely to take place; cooperativity in the formation of pores would not, therefore, be necessarily related to the stoichiometry of binding.

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