FLUORESCENCE OF LUCENSOMYCIN UPON BINDING TO ERYTHROCYTE GHOSTS

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1. Introduction

Lucensomycin is a macrolide polyenic antibiotic formed by a mycosamine moiety linked by a glycosidic bond to a C_{25} lactone ring which contains a tetraene sequence [1]. Like other related polyenes, it is known to affect the permeability of cholesterol-containing biological or artificial membranes [2–5]. It has recently been shown that addition of this compound to tumour cells *in vitro* results in a severe inhibition of nucleic acid biosynthesis [6, 7]. This effect is strictly correlated to the alterations of the plasma membrane of the cells [7].

We report here a spectrofluorimetric investigation of the binding of lucensomycin to erythrocyte ghosts.

2. Methods

Erythrocyte ghosts were prepared according to Dodge et al. [8], with minor modifications, and resuspended in isotonic NaCl + isotonic phosphate buffer pH 6.8 (9:1, v/v). Lucensomycin and *N*-acetyl-lucensomycin were a kind gift of Prof. F. Arcamone and Prof. M. Ghione of Farmitalia, Milano, Italy. They were kept as dry powders under nitrogen at +4° until use, then dissolved at 5 mg/ml in dimethylsulfoxide, and this stock solution diluted in the appropriate solvent.

The fluorescence spectra were recorded in an Aminco-Bowman spectrophotofluorometer using 1 cm cuvettes, excitation slit of 0.1 mm and emission slit between 0.1 and 0.5 mm. Temperature was constantly kept at 24°. All spectra were recorded 2 to 3 hr after preparation of the sample, so as to allow complete equilibration.

Absorption spectra were recorded in a Beckman DB spectrophotometer.

Proteins were determined according to Gornall et al. [9] and verified by use of a Coleman Nitrogen Analyser. Cholesterol was determined with the 15949 TCAA Colorimetric test of Boehringer, Mannheim, Germany.

In all experiments reported, the same membrane preparation, containing 1.3 mg protein/ml and 1.9 mg cholesterol/ml was used. Other preparations, anyhow, were found to behave in an identical manner.

3. Results

Fig. 1 shows the fluorescence characteristics of lucensomycin in saline-phosphate buffer, in the absence or in the presence of erythrocyte ghosts, and in a dioxane–water mixture (11:1, v/v). It may be noted that the fluorescence intensity, which was extremely low in aqueous medium, increased considerably upon addition of membranes. The excitation and emission spectra, apart from the differences in intensity, had very similar shapes in the three cases, except for a 20 nm blue shift of the low intensity emission maxima of lucensomycin alone in saline-phosphate buffer. No clearcut difference in

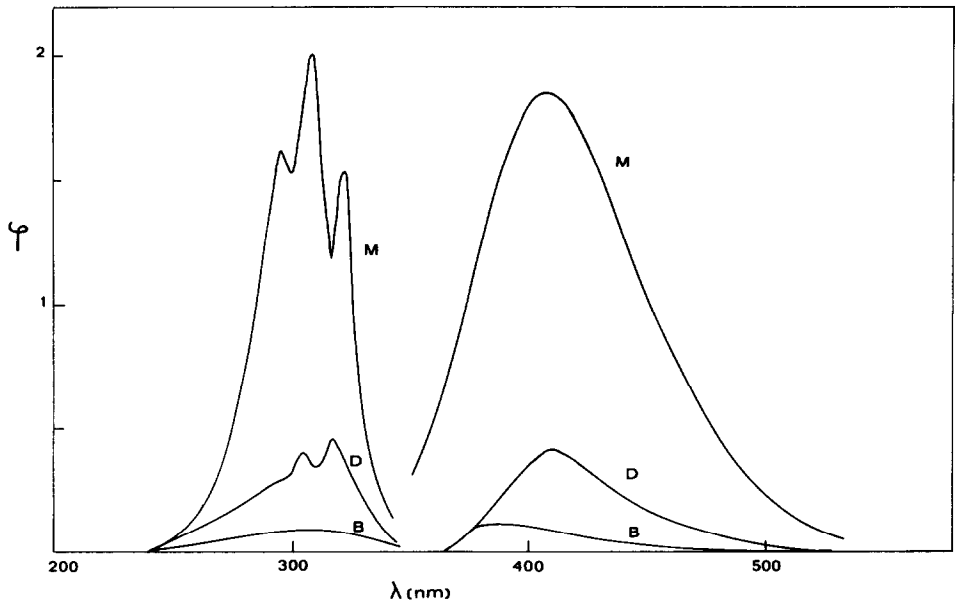

Fig. 1. Excitation and emission fluorescence spectra of lucensomycin. The spectra were recorded with excitation and emission slits of 0.1 mm. Fluorescence is expressed as conventional units (1 unit = 1.6 μV) Lucensomycin concentration: 3 μg/ml in dioxane (curve D); in saline-phosphate buffer (curve B), or in saline-phosphate buffer containing, per ml, 50μl of erythrocyte membrane preparation (curve M). The curves were uncorrected for background fluorescence, for scattering or for different absorption.

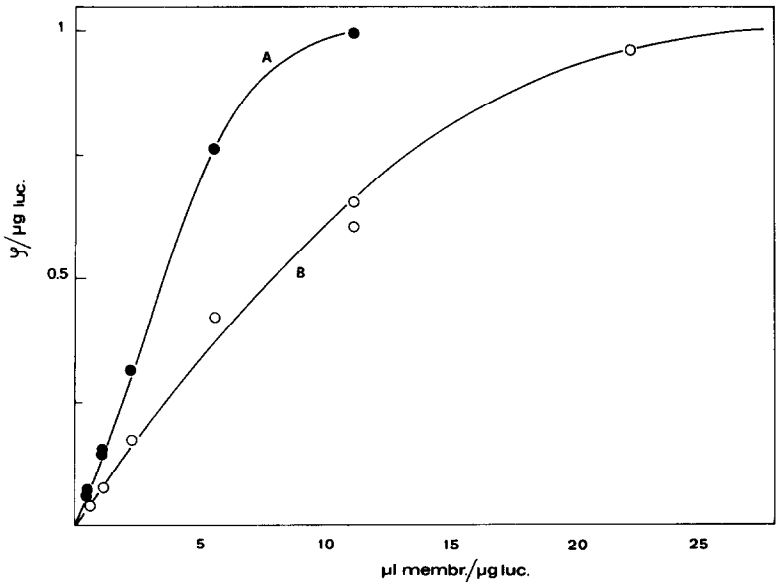

Fig. 2. Dependence of fluorescence intensity upon addition of membranes. Excitation at 308 nm, slit 0.1 mm; emission at 410 nm, slit 0.5 mm. Curve A (•): 9 μg lucensomycin/ml; Curve B (○): 0.45 μg lucensomycin/ml, in saline-phosphate buffer. Fluorescence intensity, expressed as conventional units (1 unit = 1.6 μV), was brought to comparable values by referring to the amount of lucensomycin per ml. The low fluorescence of lucensomycin in buffer without membranes was substracted from all the data.

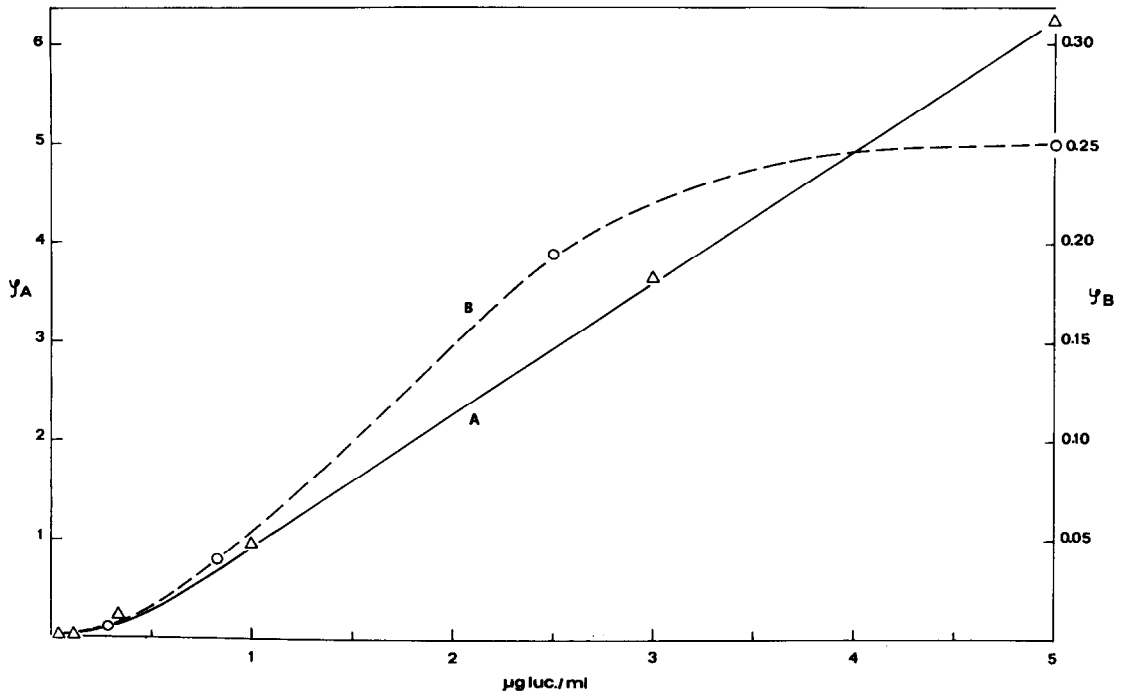

Fig. 3. Dependence of fluorescence intensity upon dilution of lucensomycin–membrane mixtures. Conditions as in fig. 2. Fluorescence expressed in conventional units as indicated in figs. 1 and 2. Curve A (△——△): 11.1 μl membranes/μg lucensomycin; Curve B (○——○): 0.22 μl membranes/μg lucensomycin.

the absorption spectra, either in shape or in intensity could be evidenced.

Fig. 2 shows how the increase of fluorescence emission depends upon the amount of membrane added. The quantum yield, at infinite membrane concentration from the area of the emission curve by use of a quinine disulfate standard [10], was about 0.25, while in the absence of membranes it was as low as 0.3×10^{-3}.

Although formaldehyde treatment according to Butler [11] makes erythrocytes resistant to lucensomycin-induced lysis [12], formaldehyde-treated erythrocyte membranes were still able to cause the increase of lucensomycin fluorescence. Other treatment, such as sonication (3 × 5 sec in a MSE 100 W sonifier), or exposure to 70° for 30 min, or shaking with 3 volumes of chloroform–methanol 3:1 (without separation of the phases, but followed by evaporation and resuspension by sonication in water), were also unable to modify the capacity of the membranes to enhance lucensomycin fluorescence.

Table 1

Solvent	Dielectric constant	φ
n-Hexane	1.9	0.024
Dioxane	2.2	0.413
Chloroform	4.8	0.118
2-Chloroethanol	25	0.085
Ethylene glycol	37	0.080
Dimethylsulfoxide	45	0.173
Water	80	0.010
Formamide	109	0.054
Membranes, 50 μl/ml in water	–	3.59

Effects of different solvents on fluorescence of lucensomycin solutions. Lucensomycin was 3 μg/ml. Fluorescence intensity was measured with identical settings of the spectrophotofluorometer, with excitation at 308 nm and emission at 410 nm. All values were corrected for background fluorescence of solvent. For comparison, the fluorescence of lucensomycin after addition of a quasi-saturating amount of membranes is reported.

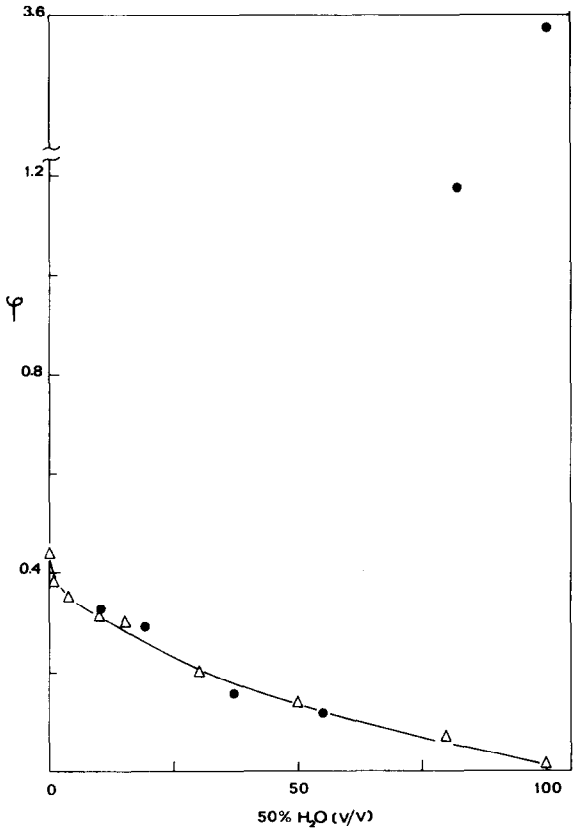

Fig. 4. Fluorescence of lucenosmycin in dioxane–water mixtures. Lucensomycin concentration: 3 μg/ml. Other conditions as in fig. 2. △——△: fluorescence of dioxane–water mixtures; •: fluorescence after addition of 50 μl membranes/ml.

Fig. 3 shows the variation of fluorescence intensity upon dilution of lucensomycin–membrane mixtures. Apart from the expected quenching at high lucensomycin and/or membrane concentrations, due to self-absorption and, even more, to light scattering, it was found that dilution caused fluorescence to decrease more than would be expected by simple proportionality. This phenomenon may possibly be attributed to a dissociation of the fluorescent lucensomycin–membrane complex.

The high quantum yield of membrane-bound lucensomycin is not due to its location in a hydrophobic environment, as shown by experiments in which the polyene was dissolved in solvents of different dielectric constants (table 1): fluorescence

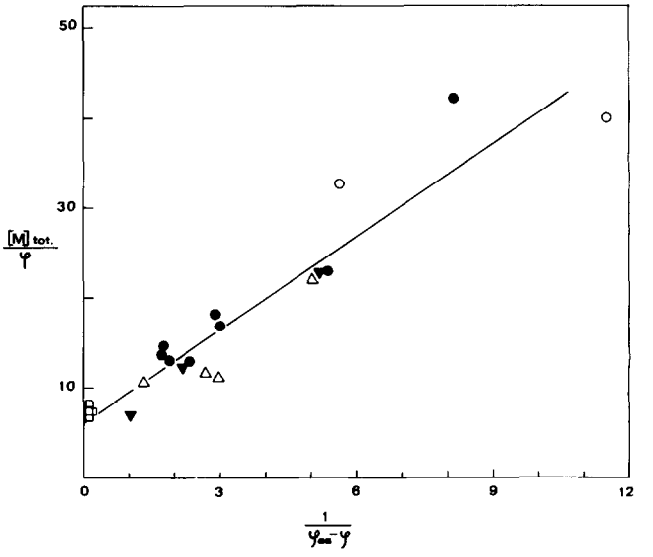

Fig. 5. Plot of $[M]_{tot}/\varphi$ vs $1/(\varphi_{as}-\varphi)$ (see text). Different symbols indicate different experiments with varying lucensomycin and/or membrane concentrations. $[M]_{tot}$ is expressed as μl membranes/ml of solution; φ and φ_{as} in conventional units (same as in figs. 1 and 2). Other experimental conditions as indicated in fig. 2.

intensity was stronger in dimethylsulfoxide or even in formamide than in n-hexane, and reached its highest values in dioxane, where the quantum yield was found to be 0.026. Addition of increasing amount of water to lucensomycin in dioxane caused a progressive decrease of fluorescence (fig. 4). Addition of erythrocyte membranes to lucensomycin in dioxane did not cause an increase of fluorescence, unless the percentage of water in the solution was above 70%.

N-Acetyllucensomycin, which is a slightly acidic derivative of lucensomycin obtained by acetylation of the mycosamine moiety, had fluorescence properties in organic solvents similar to those of the parent compound, but did not become fluorescent in aqueous solution upon addition of membranes. This behaviour is probably related to the lack of effectiveness of the *N*-acetyl derivative in increasing membrane permeability or in causing lysis of beef erythrocytes [5, 12].

4. Discussion

Fluorescence appears to be an extremely sensitive method for evaluating the binding of lucensomycin to erythrocyte membranes, the quantum yield increasing more than 500-fold.

The results shown in fig. 2, together with the non-dependence of fluorescence intensity on the polarity of the solvent, suggest a simple reaction of the type

$$L + M \rightleftharpoons LM$$

the fluorescence intensity φ being (apart from quenching effects due to high optical density or to high light scattering) proportional to the concentration of the LM complex, and reaching, at infinite membrane concentration, an asymptotical value φ_{as} which depends only on total lucensomycin concentration. Mathematical analysis of this type of reaction predicts that a plot of $[M]_{tot}/\varphi$ vs. $1/(\varphi_{as} - \varphi)$ would yield a straight line, the slope of which is the association constant, K_{ass}, of the LM complex. Fig. 5 shows such a plot for data obtained form several experiments.

The high fluorescence intensity of lucensomycin after addition of membranes is not correlated with a localization of the polyene in a hydrophobic environment, as has been suggested for fluorescence charges of vitamin A [13], of 8-anilino-1-naphthalene sulfonate [14–16] and of other fluorescent probes [17]. In fact, not only is the fluorescence intensity of lucensomycin in organic solvents rather independent of the dielectric constant of the solvent, but even in dioxane, where fluorescence is hightest, the quantum yield reaches 10.4% of the value obtained with saturating amount of erythrocyte membranes.

The ability to enhance lucensomycin fluorescence is modified neither by denaturation of membrane proteins by high temperatures, by formalinization or by organic solvents, nor by alteration of the phospholipid-protein association by sonication [18] or by chloroform–methanol treatment [19]. Lucensomycin can therefore be used as a fluorescent probe only to detect gross changes in membrane architecture and/or composition. On the other hand, the use of polyenic antibiotics as probes for fine membrane structure was a priori doubtful because of the conspicuous modifications of permeability they induce in membranes.

The use of lucensomycin can be expected to allow the comparison of different membranes on the basis of two, and probably three, distinct parameters: the extent of fluorescence enhancement (i.e. the maximum quantum yield which can be obtained at saturating membrane concentrations), the association constant between lucensomycin and membranes (as evaluated from experiments and plots analogous to those of figs. 3 and 5), and possibly the ability of membranes to bind lucensomycin also in the presence of various amount of dioxane or of other suitable organic solvents.

A comparison between different membranes appears of great interest in order to ascertain the structural background of fluorescence enhancement, and to verify whether physicochemical measurements confirm the observation [16] of a different susceptibility to this antibiotic of membranes from "normal" and neoplastic cells.

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