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STOICHIOMETRY OF HEMOLYSIS BY THE POLYENE ANTIBIOTIC LUCENSOMYCIN

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SUMMARY

The stoichiometry of hemolysis by the polyene antibiotic lucensomycin was investigated. It appears that hemolysis occurs only when a relatively high fraction (probably between 15 and 40 %) of the cholesterol sites in the erythrocyte membrane have combined with the polyene. Also in phospholipid-cholesterol vesicles the increase of permeability requires occupancy of 40–50 % of the existing cholesterol sites.

As for the possible cooperative effect in the hemolytic process, it is probable that several (at least 9–10) lucensomycin-cholesterol adducts must interact on each side of the membrane to form an aqueous channel; the distribution of these adducts in the erythrocyte membrane occurs, however, apparently at random.

INTRODUCTION

Polyenic antibiotics are known to cause red blood cell lysis by combining with the cholesterol of the cellular membranes. The extent of lysis appears to be dependent on, though not proportional to, the polyene/cell ratio, the effectiveness of a given concentration of the polyene depending on the erythrocyte concentration [1]. The increase of permeability is probably related, at least when filipin is used, to the formation of large pits with an inner diameter of approx. 100–200 Å [2–4]. With some polyenes other than filipin, instead, smaller aqueous pores ($\approx$4 Å) have been described [5, 6], which lead to a more graded and selective increase of permeability. From the molecular point of view, these aqueous channels have been postulated to be formed by assembly of several molecules of polyene held together by their interaction with cholesterol [4].

On the other hand, it is a common finding [1] that lysis of a given red blood cell suspension does not start to occur until a critical polyene concentration has been reached, but that thereafter it reaches completion upon further slight addition of the

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polyene. This phenomenon, which results in an extermely sharp hemolysis curve, should allow, besides an estimate of the mean number of lucensomycin molecules needed to cause hemolysis of a red blood cell, also the analysis of the extent of cooperation between lucensomycin molecules in the formation of the aqueous channel.

The experimental results thus obtained with erythrocytes shall be correlated to data obtained with model membrane systems, and compared to the effects brought about by digitonin, another cholesterol-specific hemolytic agent chemically unrelated to lucensomycin.

MATERIAL AND METHODS

Aqueous solutions of lucensomycin (trade name: etruscomycin^R, Farmitalia, Milan, Italy) were obtained by dissolving the polyene in a minimal amount of dimethylsulfoxide, and diluting the solution thus prepared in a large volume (100–500 ×) of isotonic NaCl. Digitonin aqueous solution were prepared in a similar manner, except that methanol was used as the first solvent.

For lysis experiments, either of these solutions was added to freshly collected bovine erythrocytes, which had previously been washed and resuspended in isotonic NaCl; lysis was evaluated after 6–7 h (a time which had been found to be sufficient for the systems to reach equilibrium) by low-speed centrifugation (1000 × g) and measurement of the absorbance of the supernatant in the absorption regions of hemoglobin. The absorption band and the optical pathlength were chosen so as to minimize instrumental error.

Dichromate-loaded liposomes were prepared according to Weissmann and Sessa [7], by letting 30 mg of a dried mixture of phosphatidylcholine (either synthetic dipalmitoylphosphatidylcholine or egg yolk phosphatidylcholine purified according to Pangborn [8])/dicetylphosphate/cholesterol (in a molar ratio of 7 : 2 : 1) swell in 10 ml of 0.154 M sodium dichromate in H_2O. The suspension was then submitted to 3 min sonication with a 100 watt MSE ultrasonic disintegrator under a nitrogen stream, keeping the samples above the transition temperature of the phospholipid fatty acyl chains (i.e. at room temperature for egg phosphatidylcholine, at about 50 °C for dipalmitoyl phosphatidylcholine). The multilayered liposomes were by this procedure predominantly transformed into single-shelled vesicles, as monitored by a more translucent aspect of the suspension, and occasionally confirmed by negative stain electron microscopy and/or by high resolution NMR spectrometry. These vesciles were, like the liposomes, practically impermeable to dichromate ions, and were preferred because their use avoided the problem of differential accessibility to the polyene of the various bilayers and simplified the kinetics of dichromate release.

After sonication, excess dichromate was removed by extensive dialysis. Given amounts of lucensomycin were then added to equal fractions (0.5–1 ml) of the vesicle suspension, and the release of the previously trapped dichromate evaluated by further 12 h dialysis at room temperature. Fluorometric measurements could be directly performed, after suitable dilution, on the same lucensomycin-containing vesicle suspensions; to this purpose, an Aminco-Bowman spectrofluorometer was used, the intensity of emission at 410 nm being recorded upon excitation at 308 nm [9, 10].

The binding of lucensomycin to whole erythrocytes could not be estimated by

fluorometric techniques, due to the presence of hemoglobin. The concentration of the polyene in supernatants containing different concentrations of hemoglobin could be evaluated after transformation of hemoglobin to acid hemichromogen by addition of acetic acid (4.4 M, final concentration), and by measuring the absorbances at 305 and 392 nm. Calibration curves showed that lucensomycin concentration, L, could be derived by using the formula

$$L(\text{mM}) = \frac{A_{305} - 0.420 \times A_{392}}{70}$$

Black lipid membranes were prepared essentially according to Mueller et al. [11], by spreading an egg lecithin/cholesterol/*n* decane/chloroform/methanol solution (2 : 2 : 18 : 48 : 32, w : w : v : v :v) on a 1.5 mm diameter hole connecting two teflon vessels. Both vessels were filled with 10^{-2} M KCl, pH 7.0, kept at 37 ± 1 °C. Formation of the bimolecular film were followed by direct observation with a stereomicroscope at 40×; the same instrument was used to measure the area of the black membrane, which was $7.8 \pm 1.5 \cdot 10^{-3}$ cm^2. Two Ag/AgCl electrodes, upon application of 40–100 mV potential differences produced by a d.c. generator (mod. S 8 Stimulator, Grass Instruments, Quincy, Mass. U.S.A.) was used to measure, through a high-impedance electrometer (mod. M4A, W. P. Instruments, Inc., Hamden, Conn. U.S.A.), the electrical resistance of the black membrane.

RESULTS AND DISCUSSION

1. *Evaluation of the relation between lysis and site occupancy, by titration at different cell concentrations*

The mean critical number of lucensomycin molecules which, upon binding to an erythrocyte, make it undergo hemolysis can be derived by measuring the lucensomycin concentrations which give 50 % lysis at different concentrations of erythrocytes. The rationale of this approach is the following: we may assume that the membrane has a number t of sites, each having the same intrinsic association constant K for lucensomycin and that lysis does not occur until a number n of these sites is occupied by the polyene. Indicating by L_0 the total concentration of lucensomycin, by L the concentration of the free polyene and by E_0 and E respectively the concentrations of all erythrocytes and of those to which no poylene molecules are bound, we have, by application of the mass action law:

$$E_0 = E(1+KL)^t \tag{1.1}$$

$$L_0 = L + tKLE(1+KL)^{(t-1)} \tag{1.2}$$

and therefore

$$L_0 = L + \frac{tKLE_0}{1+KL} \tag{1.3}$$

On the other hand, if up to n sites can be occupied on a same erythrocyte without causing lysis, and if we call by L^* the concentration of free lucensomycin corresponding to 50 % lysis

$$\sum_{i=0}^{n} \binom{t}{i} K^i L^{*i} = \frac{(1+KL^*)^t}{2} \tag{1.4}$$

This equation can be written in the form of a binomial distribution:

$$\sum_{i=0}^{n} \binom{t}{i} \left[\frac{KL^*}{1+KL^*}\right]^i \cdot \left[\frac{1}{1+KL^*}\right]^{(t-i)} = \frac{1}{2} \tag{1.5}$$

If t is a large value (as it is in the case of polyenes, since there are about $134 \cdot 10^6$ cholesterol molecules/cell), the solution n of Eqn. 1.5 is the mean value of the binomial distribution, i.e.

$$n = t \frac{KL^*}{1+KL^*} \tag{1.6}$$

Combining Eqns. 1.3 and 1.6, the $L_0{}^*$ value of L_0 which gives 50 % lysis is:

$$L_0^* = \frac{n}{K(t-n)} + nE_0 \tag{1.7}$$

In a plot of $\log L_0{}^*$ vs. $\log E_0$, as in Fig. 1, the horizontal asymptote shall therefore yield the value of $n/K(t-n)$, and the value of the oblique asymptote when $E_0 = 1$ erythrocyte/ml shall give the value of n. The validity of this approach is shown by the fact that, as required by Eqn. 1.7, the maximal distance of the experimentally determined curve from its asymptotes corresponds to the intersection of the two asympto-

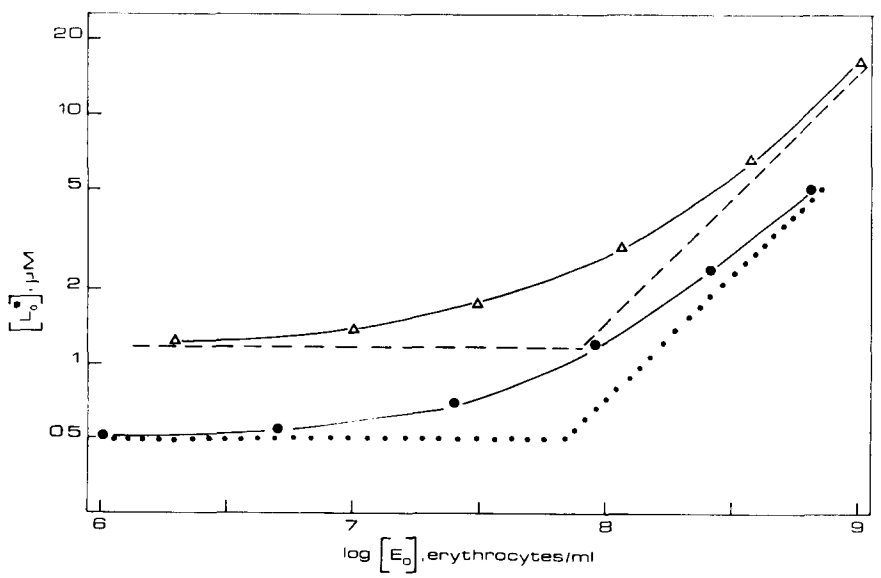

Fig. 1. Relation between number of erythrocytes/ml, E_0, and lucensomycin (△ — △) or digitonin (● — ●) concentrations giving 50 % hemolysis. Both scales are logarithmic, but in a 1 : 2 ratio. The broken and dotted lines are the asymptotes.

tes, and is equal to log 2. The value of n, which can be obtained from the experimental points shown in Fig. 1 is, for lucensomycin, between 8 and $12 \cdot 10^6$ binding sites per cell. The same approach can be used to evaluate the mean critical number of digitonin molecules needed for hemolysis (Fig. 1, broken line), the value thus obtained being around $4–5 \cdot 10^6$ binding sites per cell.

2. *Evaluation of the relation between lysis and site occupancy by use of Poisson distribution*

Similar information about the mechanism of lucensomycin-induced lysis can be obtained by analysis of single hemolysis vs. polyene concentration curves in terms of a Poisson distribution, i.e. in the way classically used for the evaluation of cell infection by viral particles [12].

Let m indicate the multiplicity of binding, i.e. the mean number of lucensomycin molecules bound per cell at each point of the titration curve, N the number of non-lysed cells/ml, E_0 the total number of cells/ml, n the number of sites which, in a single cell, can be occupied without lysis to occur, and L_N the number of lucensomycin molecules bound by the non-lysed cell (i.e. those molecules which remain bound in a low-speed precipitate).

The probability $P(k)$ that a number k of sites be occupied is such that

$$\frac{N}{E_0} = \sum_{k=0}^{n} P(k) = e^{-m} \cdot \sum_{k=0}^{n} \frac{m^k}{k!} \tag{2.1}$$

we have then

$$\frac{L_N}{E_0} = m \cdot e^{-m} \cdot \sum_{k=0}^{(n-1)} \frac{m^k}{k!} \tag{2.2}$$

from which, by derivation

$$\frac{d\frac{L_N}{E_0}}{dm} = e^{-m} \cdot \sum_{k=0}^{(n-1)} \frac{m^k}{k!} - \frac{L_N}{E_0} + m \cdot e^{-m} \cdot \sum_{k=0}^{(n-2)} \frac{m^k}{k!} \tag{2.3}$$

Experimentally, L_N passes through a maximum $L_N{}^*$, corresponding to a multiplicity m^*; at this point

$$\frac{d\frac{L_N^*}{E_0}}{dm^*} = 0,$$

and therefore

$$\frac{L_N^*}{E_0} = e^{-m^*} \cdot \frac{m^{*(n+1)}}{(n-1)} \tag{2.4}$$

Since, from Eqn. 2.1 and 2.2,

$$\frac{L_N}{E_0} = m \cdot \frac{N}{E_0} - m \cdot e^{-m} \frac{m^n}{n!} = m \cdot \frac{N}{E_0} - \frac{1}{n} \cdot e^{-m} \cdot \frac{m^{(n+1)}}{(n-1)!}$$

$$\frac{L_N^*}{E_0} = m^* \cdot \frac{N^*}{E_0} - \frac{1}{n} \cdot \frac{L_N^*}{E_0} \tag{2.5}$$

$$\frac{1}{n} = m^* \cdot \frac{N^*}{L_N^*} - 1 \tag{2.6}$$

The experimental data obtained with $31 \cdot 10^6$ beef erythrocytes/ml are reported in Fig. 2. In the lower part the values are shown of percent lysis and of L_N, while in upper prat are shown the variations of L_b, (the total amount of bound poleyne), as estimated from the difference between the total lucensomycin concentration in the assay and the free lucensomycin remaining in the supernatant after high-speed (60 min at 80 000 $\times$ g) centrifugation.

The values of N^* and L_b^* corresponding to the maximum L_N^* of the L_N vs. L_0 curve of Fig. 2 can be easily obtained, though with a certain error (which in fact greatly limits the usefulness of this approach). For $L_N^* = 0.44(\pm 0.01)$ μM = $265(\pm 6) \cdot 10^{12}$ molecules/ml, and $E_0 = 31 \cdot 10^6$ cells/ml, we have $N^*/E_0 = (100 - \%$ lysis$)/100 = 0.620 \pm 0.007$ and $L_b^* = 781(\pm 5)$ pmol/ml; we can derive: $m^* = L_b^*/E_0 = (781 \pm 5)/(31 \cdot 10^6) = 25.2(\pm 0.16) \cdot 10^{-6}$ pmoles/cell = $15.2(\pm 0.1) \times 10^6$ molecules/cell.

Therefore, from Eqn. 2.6

$$1 + \frac{1}{n} = 15.2(\pm 0.1) \cdot \frac{620(\pm 7) \cdot 31 \cdot 10^3}{265(\pm 6) \cdot 10^6} = 1.106 \pm 0.11$$

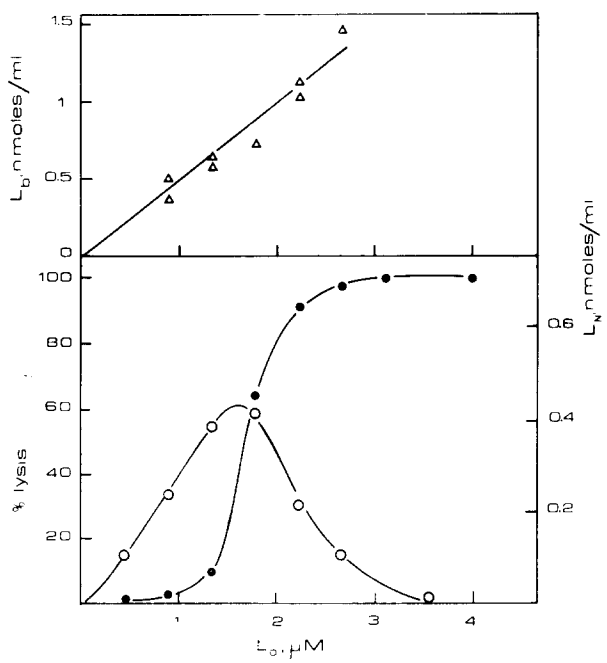

Fig. 2. Poisson distribution analysis of hemolysis. Lower graph: % lysis, (○—○) and concentration of polyene bound to non-lysed cells (L_N), (●—●). Upper graph: concentration of polyene bound to membranes of both lysed and non-lysed cells (L_b), (△—△). Erythrocytes concentration: $31 \cdot 10^6$ cells/ml.

From this calculation we would therefore derive a mean value of $n = 9.5$, possible values extending however from 5 to $+\infty$. This approach appears therefore suitable only to put a lower limit to the range of n, the upper limit remaining undefined.

On the other hand, the data of Fig. 2 can also be used to evaluate the L_N/N ratio, i.e. the mean number of molecules bound to each non-lysed cell. This ratio should, at high L_0 values, approach the value of n. From Fig. 2, at L_N^* (where the error is at its minimum) we have $L_N^*/N^* = [265(\pm 6) \cdot 10^{12}]/[620(\pm 7) \cdot 31 \cdot 10^3] = 13.77(\pm 0.03) \times 10^6$ molecules/cell; at higher concentrations of lucensomycin this ratio increases somewhat, reaching a value which can be estimated to be (though with a large error) around $20 \cdot 10^6$ molecules/cell.

3. *Direct comparison between binding of polyene and effect on membrane permeability*

As mentioned before, the presence of hemoglobin in erythrocytes strongly limits the use of optical techniques to evaluate the extent of binding of the polyene to the cell membrane. This difficulty was circumvented by using dichromate-loaded vesicles, since for fluorometric measurements this suspension had to be suitably diluted and interference by the colored $Cr_2O_4^{2-}$ ions was negligible.

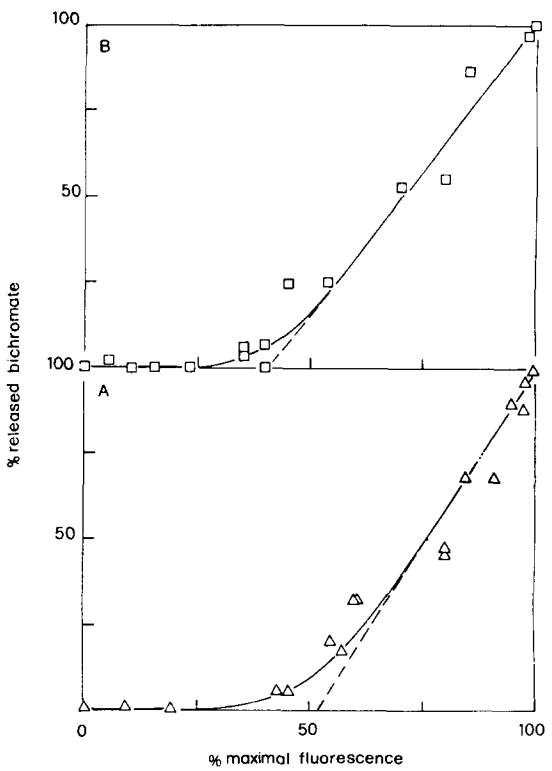

Fig. 3. Relation between binding of lucensomycin to cholesterol and efflux of bichromate ions from phospholipid-cholesterol vesciles. Vesciles were formed from either purified egg lecitin (A) or from synthetic dipalmitoyl phosphatidylcholine (B), to which dicetylphosphate (20 % of total lipid) and cholesterol (10 % of total lipid) had been added. Binding of lucensomycin to cholesterol was measured by the increase of fluorescence intensity [9], while efflux of $Cr_2O_4^{2-}$ was estimated by measuring, after extensive dialysis, the absorbance at 370 nm in the outer compartment.

It was found that, in the experimental conditions of Fig. 3, the presence of cholesterol in the vesicles was essential in order to obtain, upon addition of lucensomycin, both a measurable increase of fluorescence intensity and an efflux of dichromate ions. When instead cholesterol-containing vesicles were titrated with increasing lucensomycin, the binding curve, fluorometrically estimated, had a slightly sigmoid shape, thus confirming previous results [10, 13]. The increase of permeability, evaluated as amount of dichromate ions which could diffuse out of the vesicles, gave however, vs. lucensomycin concentration, a much more sigmoid curve. If, as in Fig. 3, we report against each other the two sets of data, expressed respectively as fraction of maximal fluorescence and as fraction of total dichromate contained in the vesicles, it can be seen that the increase of permeability starts only when 40–50 % of the sites have been occupied by the polyene. The question, whether all cholesterol molecules are uniformly accessible to the polyene (a problem which would assign great relevance to the possible persistence of some multi-layered liposomes in the vesicular preparation), is in fact circumvented by the direct fluorometric measurement of site occupancy and by the reference of the permeability increase to the level of this occupancy.

4. *Lucensomycin-cholesterol stoichiometry*

The experiments described in the previous paragraph show that there is a clearcut relationship between increase in membrane permeability and binding of lucensomycin to cholesterol. To this purpose, it should be recalled that the increase in lucensomycin fluorescence is directly dependent upon formation of a lucensomycin-cholesterol adduct [9, 10]. On the other hand, in previous work [9, 10, 13] we had

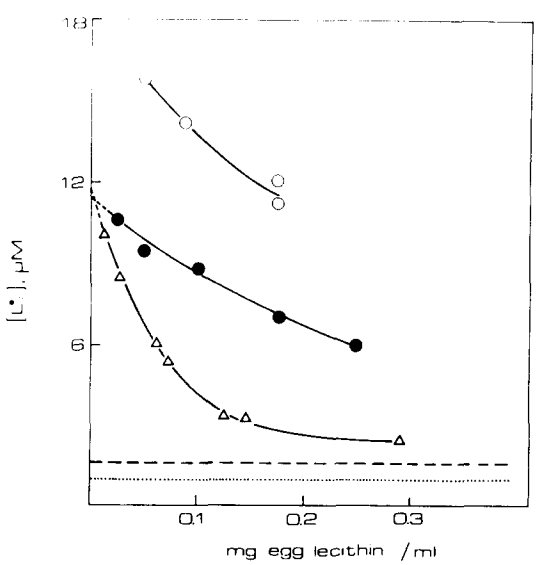

Fig. 4. Effect of egg lecithin on the ability of given amounts of cholesterol (24 nmol/ml, final concentration, open symbols; 12 nmol/ml, full circles) to shift the concentration of lucensomycin (triangles) or digitonin (circles) necessary for 50 % lysis of $8 \cdot 10^7$ erythrocytes/ml. The dashed and the dotted lines indicate respectively the lucensomycin and digitonin concentrations causing 50 % hemolysis in the absence of added cholesterol.

shown that fluorometric titrations supported the hypothesis that each lucensomycin-binding site be formed by two cholesterol molecules. According to other authors, there is instead experimental evidence in favor of a 1 : 1 polyene/cholesterol stoichiometry, especially as far as filipin is concerned [14–16]. The question was therefore approached again by thoroughly investigating the inhibitory effect of exogenous cholesterol on hemolysis by lucensomycin and by digitonin. At a fixed cholesterol-to-erythrocytes ratio, there was a shift to the left of the oblique asymptote of the curves of Fig. 1, the horizontal asymptote remaining unchanged; the extent of the shift depended on the above mentioned ratio. Due to the low solubility of cholesterol in water, a quantitation of the effect was not feasible if cholesterol alone was added; water-miscible organic solvents, such as ethanol, added in non-hemolytic amounts, were of some help, but even in this case a satisfactory reproducibility and reliability way not attained. Cholesterol could instead be easily solubilized if included in lecithin liposomes. However, the magnitude of the effects observed upon addition of a given amount of cholesterol was adversely affected by high phospholipid-to-cholesterol ratios (Fig. 4). This was particularly true for lucensomycin and for the other polyenes, less so for digitonin, possibly because the cholesterol-digitonin complex is practically insoluble. Upon extrapolation to zero phospholipid, the ability of cholesterol to interfere with hemolysis was about twice as intense in the case of digitonin than with lucensomycin. This, together with the fact that digitonin is known to form a 1 : 1 complex with cholesterol [17], supports our previous finding that the stoichiometry of the cholesterol-lucensomycin complex is 2 : 1.

Since chemical determination indicates the presence of about $134 \cdot 10^6$ cholesterol molecules/bovine erythrocyte, the value of t in equations 1.1–1.7 is therefore $134 \cdot 10^6$ for digitonin and $67 \cdot 10^6$ for lucensomycin. The corresponding values of the intrinsic association constant K are then around $6.4 \cdot 10^4$ M^{-1} for digitonin and $1.6 \cdot 10^5$ M^{-1} for lucensomycin.

5. *Are lucensomycin binding sites independent from each other?*

In their molecular model of pore formation, de Kruijff and Demel [4] show that, as far as amphotericin B is concerned, each aqueous channel through the membrane can be formed by association of 2×8 polyene-cholesterol adducts. A similar model would hold, according to these authors, also for lucensomycin. Since formation of such a channel obviously implicates some kind of spontaneous interaction between the various adducts, it would follow that the association constant of adduct formation depends upon its ability to take part in an aqueous pore, i.e. upon its proximity to other adducts. From the data of Fig. 1, and as it shall be discussed in a following paper, no such evidence can be found in the case of lucensomycin. This may, on the other hand, be due either to a low number of sites involved per pore as compared to the total number of sites which must be occupied before pore formation, or to a low value of the energy of interaction between the adducts as compared to the energy of adduct formation.

Experiments performed with cholesterol-containing black lipid membranes showed (Fig. 5) that, above a certain threshold, ionic conductance is dependent on the 9–10th power of lucensomycin concentration on both sides, thus extending to lucensomycin the results obtained with nystatin and amphotericin B by Finkelstein and Cass [5] and by Cass et al. [18]. As evidenced by Finkelstein and Holz [19], the

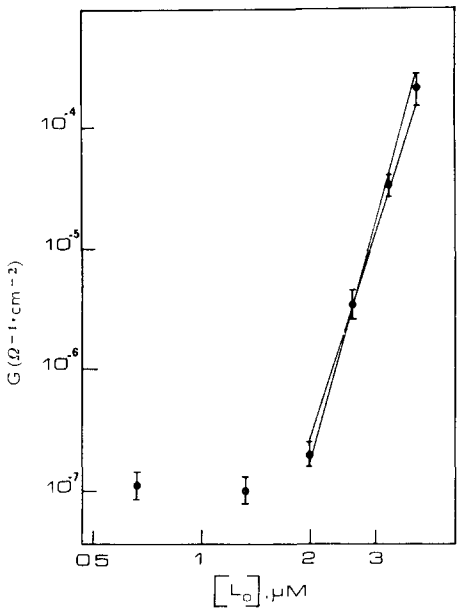

Fig. 5. Variations of black lipid membranes conductance as a function of lucensomycin concentration on both sides. Each point is the mean of six independent measurements; the resulting standard deviation being indicated. The two straight lines have been drawn assuming minimal and maximal slopes (9.2 and 10.7, respectively).

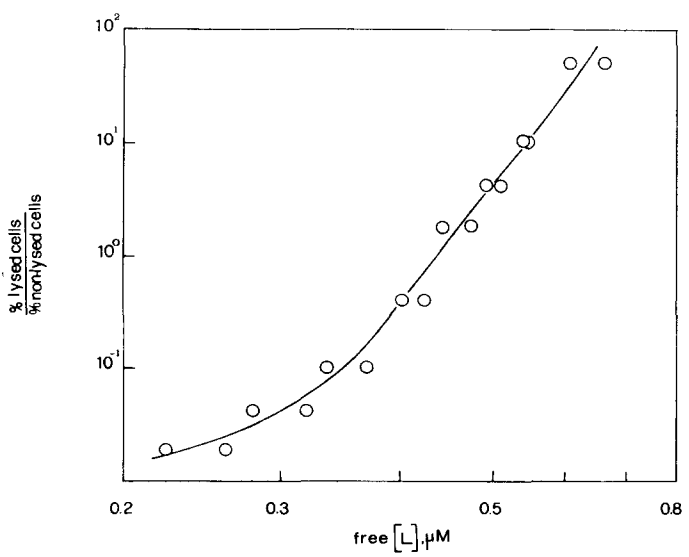

Fig. 6. Hill plot of hemolysis as a function of free lucensomycin. Erythrocytes concentration: $31 \cdot 10^6$ cells/ml. Both scales are logarithmic, but in a 1 : 8 ratio.

relation between conductance and antibiotic concentrations can be assumed to reflect the molecularity of the pore only if a low fraction of sites is occupied and if there is no preference for adjacent sites; the data obtained can therefore be interpreted as indicating a lower limit of 9–10 lucensomycin molecules involved in the formation of a half-pore.

The possibility that cooperation between several polyene molecules be similarly involved in hemolysis of beef erythrocytes is supported by the sigmoid shape of the hemolysis vs. polyene concentration curves. As shown in Fig. 6, a plot, analogous to the Hill plot, of log % lysed cells/% non-lysed cells vs. log [free lucensomycin] gives, in its central part, a slope around 10. Although this finding may seem appealing, its significance and validity are however very doubtful. In fact, the large number of sites which must be occupied before lysis can occur would, even in the absence of any cooperative interaction, give an extremely sharp dependence of lysis upon lucensomycin concentration. This sharpness is indeed probably mitigated by a certain degree of heterogeneity in the erythrocytes population. The shape of 10 in the pseudo-Hill plot of Fig. 6 cannot therefore be interpreted as proving a cooperative binding, but reflects instead only the multiplicity of adducts needed for appearance of an all-or-none effect.

CONCLUSIONS

The effect of lucensomycin on erythrocytes permeability is most probably related to the formation of adducts between this polyene and membrane cholesterol. This assumption is justified by the high specificity of the polyene for cholesterol, by the inhibitory effect of exogenous cholesterol upon hemolysis, and by the relation, found in artificial vesicles, between presence to cholesterol and effect on permeability. It is also supported by the results obtained by several authors with different polyenes, all of which exhibit a specific requirement for the presence of sterols in membranes, in order to increase membrane permeability. As for the mechanism by which there is an increase of membrane permeability to aqueous solutes, the most likely scheme is, as proposed by Finkelstein and Holz [19] and by de Kruijff and Demel [4] for amphotericin B, that of 8–10 adjacent adducts, interacting together to form a half-channel on each side of the membrane. The question, as to how the polyene can reach not only the outer layer of the membrane but also the inner one, so as to form two half-channels, is still open. On the other hand, the anatomy of the aqueous channel thus formed is, in the case of lucensomycin, probably somewhat different from the model proposed by de Kruijff and Demel [4] for amphotericin B, the lucensomycin-cholesterol ratio being 1 : 2 rather than 1 : 1.

The results reported in the present paper indicate, however, that a large number of adducts must be formed in each erythrocyte membrane in order to obtain the increase of permeability. Out of the $67 \cdot 10^6$ binding sites, which correspond to the $134 \cdot 10^6$ cholesterol molecules present per erythrocyte membrane, $10–20 \cdot 10^6$ (i.e. 15–30 % of the total number) must be occupied for lysis to occur. The intrinsic association constant of each adduct can be estimated (from Fig. 1 and Eqn. 1.7) to be in the range $1.6–4.0 \cdot 10^5$ M^{-1}, corresponding to a standard free energy, $-\Delta G^\circ$, at 25 °C and at neutral pH, of 293–315 cal/mol of lucensomycin. A similar calculation gives, for digitonin, $134 \cdot 10^6$ available binding sites per erythrocyte membrane;

hemolysis occurs upon occupation of $4–5 \cdot 10^6$ sites, the intrinsic association constant of the single digitonin-cholesterol adduct being $6.4 \cdot 10^4$ M^{-1}, corresponding to $-\Delta G^\circ = 270$ cal/mol.

In this framework, pore formation requires some kind of interaction between adjacent adducts. The energy of interaction must however be relatively low, since there would otherwise be evidence for a preferential binding of lucensomycin (or digitonin) molecules to adjacent sites, with a strong dependence of the intrinsic association constant values on site occupancy. The possibility that lucensomycin be already in an aggregated form, prior to incorporation into the cholesterol-containing membrane, can be discarded, since the extinction coefficient of the polyene is, in the relatively dilute aqueous solutions which were used, concentration-independent and has a value very similar to that obtained in dimethylsulfoxide, dimethylformamide, aqueous methanol, or other good solvents of lucensomycin.

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