Biochemical Pharmacology, Vol. 28, pp. 2427–2432.

0006-2952/79/0815-2427 $02.00/0

A COMPARATIVE STUDY OF THE HEMOLYTIC EFFECT OF POLYENIC ANTIBIOTICS AND OF OTHER CHOLESTEROL-BINDING AGENTS

R. STROM, C. CRIFÒ and A. BOZZI

Institutes of Biological Chemistry, Universities of Rome and of Cagliari and School of Medicine, L'Aquila; Center for Molecular Biology, C.N.R., Rome, Italy

(*Received* 29 *December* 1978; *accepted* 25 *January* 1979)

Abstract—Macrocyclic polyenic antibiotics were compared, on the basis of their hemolytic efficiency (expressed as critical occupancy level and as mean intrinsic association constant) with each other and with other cholesterol-specific hemolytic agents, such as digitonin and streptolysin O.

In all cases, except for the larger polyenic antibiotics (amphotericin B and nystatin), the experimental results were compatible with the existence in the membrane of a large number of identical binding sites which are independent from each other.

Simultaneous addition of two different agents gave either synergistic or antagonistic effects, indicating that digitonin, streptolysin O and filipin have different mechanisms of action from each other and from the mycosamine-containing polyenes.

Polyenic antibiotics are known to increase the permeability of sterol-containing membranes towards water-soluble substances [1]. The most plausible hypothesis for their mechanism of action is the formation, upon interaction of these polyenes with membrane sterols, of aqueous pores, several antibiotic molecules being involved in the formation of each pore [2–5].

In previous work [6] we showed that, in phospholipid–cholesterol vesicles, the polyenic antibiotic lucensomycin increased membrane permeability only after having occupied approximately one-half of the total number of binding sites.

Such a study could also be extended to bovine erythrocytes; in this system, since direct spectroscopic evaluation of the binding of the polyenes to the membrane was obscured by the presence of hemoglobin, the minimal level of occupancy necessary for hemolysis could be derived by measuring the concentrations of polyene which caused 50% hemolysis at different concentrations of erythrocytes [6]. A comparative study using this method, of various hemolytic agents which are known to have a specific affinity for membrane cholesterol, is presented in this paper.

MATERIALS AND METHODS

Lucensomycin (trade name: Etruscomycin®) and *N*-acetyllucensomycin were kind gifts of Prof. M. Ghione, Farmitalia, Milan, Italy. Filipin (containing 96% filipin III) was donated by Dr. G. B. Whitfield Jr., Upjohn Co., Kalamazoo, MI, USA). Amphotericin B and nystatin were gifts of E. R. Squibb & Sons Inc., Princeton, NJ, USA and pimaricin (trade name: Natamicin®) was from Gist Brocades N.V., The Netherlands.

The methyl ester of *N*-acetyllucensomycin was prepared by reaction, at 0°, of *N*-acetyllucensomycin dissolved in methanol–diazomethane, and subsequent purification by silica gel chromatography, using ethyl acetate–methanol (7:3) as a solvent.

Digitonin was from Merck, Darmstadt, W. Germany. Streptolysin O was a lyophilized preparation from the Istituto Sieroterapico Sclavo, Siena, Italy, and was used without further purification immediately after reconstitution with 77 mM NaCl in 79 mM Na–K phosphate buffer, pH 6.5.

All polyenes were dissolved immediately before use in a minimal amount of dimethylsulfoxide, and then diluted in 140 mM NaCl–15 mM phosphate buffer, pH 7.0. Digitonin was similarly dissolved in a minimal amount of methanol, and then diluted in the same buffer. In both cases, the organic solvent had been diluted to such an extent (over 200-fold) that it had no effect by itself. For the experiments with streptolysin O, the NaCl phosphate buffer, pH 6.5, was used.

Egg lecithin was prepared according to Pangborn [7]. Cholesterol was recrystallized from hot acetic acid. Liposomes were formed by evaporating chloroform solutions of lecithin with or without cholesterol under reduced pressure and allowing the solid residue to swell in NaCl–phosphate buffer for 15 hr at room temperature under constant agitation. Quantitative determination of phospholipids was performed by the ammonium molybdate–ammonium vanadate colorimetric method (no. 15920) of Boehringer, Mannheim, W. Germany. Cholesterol was determined by the Liebermann–Burchard reaction and/or by the Boehringer 15732 cholesterol oxidase colorimetric test.

Bovine erythrocytes were freshly collected in the presence of citrate from the slaughterhouse and kept at 4° until used (not more than 4 days later). Just before use they were washed free of plasma and resuspended in the NaCl–phosphate buffer, except when streptolysin O was used subsequently, when the NaCl phosphate buffer, pH 6.5, was used instead. For lysis experiments, known numbers of erythrocytes were added in a final volume of 4 ml to varying concentrations of the hemolytic agent(s). Experiments with streptolysin O were then incubated at 37°; all other experiments were per-

formed at room temperature. After an interval which was sufficient for completion of hemolysis (4 hr in most cases), the degree of hemolysis was evaluated from the concentration of hemoglobin in the supernatant after low-speed centrifugation.

Fluorescence measurements were performed with an Aminco–Bowman spectrophotofluorometer, with an excitation wavelength of 308 nm and an emission wavelength of 410 nm [8].

Analytical treatment of the experimental data. The binding of a membrane-specific hemolytic agent, L, to a population of erythrocytes can be described by a generation function, Φ, where each i-th term indicates which fraction of that population has a number, i, of sites occupied:

$$\Phi = \frac{E_0}{E} = 1 + \binom{t}{1} K_1[L] + \binom{t}{2} K_2^2[L]^2 + \ldots \binom{t}{i} K_i^i[L]^i + \ldots K_t^t[L]^t \quad (1)$$

where $[L]$ is the concentration of the free hemolytic agent, $[E_0]$ is the total concentration of erythrocytes, $[E]$ is the concentration of completely unoccupied erythrocytes, t is the total number of sites per cell, and K_1, K_2, K_i and K_t are the mean association constants for occupancy of 1, 2, i and t sites. In the most general case, K_i can be considered equal to $\bar{K} \times f(i)$, where $\bar{K}$ is the intrinsic association constant of the isolated site and $f(i)$ is a function of the level of site occupancy denoting how occupancy of a certain number of sites affects further binding of the antibiotic to other sites in the membrane. The total concentration of the lytic agent, $[L_0]$, is then given by:

$$[L_0] = [L] + [E] \times \sum_{i=1}^{t} \binom{t}{i} \times i \times \bar{K}^i \times \{f(i)\}^i \times [L]^i$$

$$= [L] + [E_0] \times \frac{\sum_{i=1}^{t} \binom{t}{i} \times i \times \{\bar{K} \times f(i) \times [L]\}^i}{1 + \sum_{i=1}^{t} \binom{t}{i} \times \{\bar{K} \times f(i) \times [L]\}^i} \quad (2)$$

Within this framework, lysis of a single erythrocyte can be visualized as occurring only when a number of sites equal to or greater than $(n+1)$ has been occupied. The fraction of non-lysed cells, F_N, is then:

$$F_N = \frac{1 + \sum_{i=1}^{n} \binom{t}{i} \times \{\bar{K} \times f(i) \times [L^*]\}^i}{1 + \sum_{i=1}^{t} \binom{t}{i} \{\bar{K} \times f(i) \times [L^*]\}^i} \quad (3)$$

where $[L^*]$ is the concentration of free hemolytic agent giving $(1 - F_N)$ lysis.

If all the sites are identical and independent, $f(i) = 1$. Equations 1–3 then become:

$$\Phi = (1 + \bar{K} \times [L])^t \quad (1')$$

$$[L_0] = [L] + [E_0] \times \frac{t\bar{K} \times [L]}{1 + [\bar{K}][L]} \quad (2')$$

$$F_N = \sum_{i=0}^{n} \binom{t}{i} \times \left\{\frac{\bar{K} \times [L^*]}{1 + \bar{K} \times [L^*]}\right\}^i \times \left\{\frac{1}{1 + \bar{K} \times [L^*]}\right\}^{(t-i)} \quad (3')$$

In this case, when $F_N = 1/2$, since both t and n have very large values, the solution of equation 3′ is:

$$n = t \times \frac{\bar{K} \times [L^*]}{1 + \bar{K} \times [L^*]}$$

Substituting in equation 2′, we find that the total concentration, $[L_0^*]$, of the lytic agent which causes 50% lysis at an erythrocytes concentration $[E_0]$ is given by:

$$[L_0^*] = \frac{n}{t - n} \times \frac{1}{\bar{K}} + n \times [E_0] \quad (4)$$

In a double logarithmic plot of $[L_0^*]$ vs $[E_0]$, the curve interpolating the experimental data will then have a horizontal asymptote with ordinate

$$\log \frac{n}{(t-n) \times \bar{K}},$$

and an oblique asymptote crossing the ordinate axis at $\log n$.

RESULTS

Dependence upon erythrocytes concentration of the 50% lytic concentration of the various polyenes. Figure 1a shows a comparison, in the double logarithmic plot of $[L_0^*]$ vs $[E_0]$, of the lytic efficiencies of filipin, lucensomycin, pimaricin, and the methyl ester of *N*-acetyllucensomycin.

From the positions of the oblique asymptotes it appears that filipin, as compared to lucensomycin, requires a much lower level of site occupancy (i.e. a lower value of n) in order to cause hemolysis. Since the

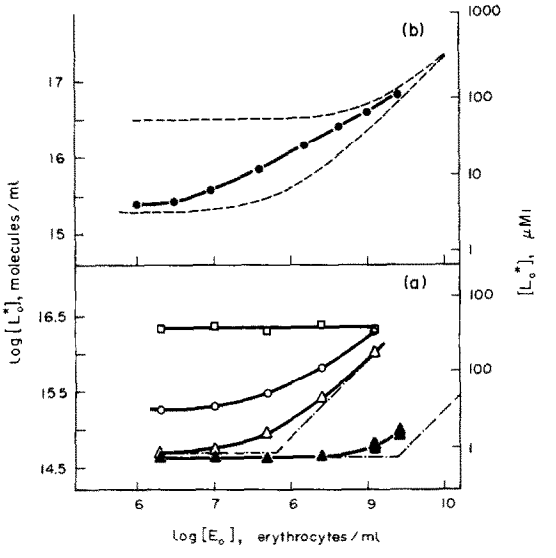

Fig. 1. Dependence of the 50% lytic concentration, $[L_0^*]$, of various small-ring polyenes on erythrocytes concentration $[E_0]$. Both scales are logarithmic. (a): □——□, Pimaricin; ○——○, *N*-acetyllucensomycin methyl ester; △——△, lucensomycin; ▲——▲, filipin; the asymptotes of the lucensomycin and filipin curves are indicated by dash-dotted lines. (b): ●——●, *N*-acetyllucensomycin; the two broken lines illustrate how the experimental curve can be reconduced, in the framework of the proposed model (see text), to the combination of two simpler binding curves (dashed lines).

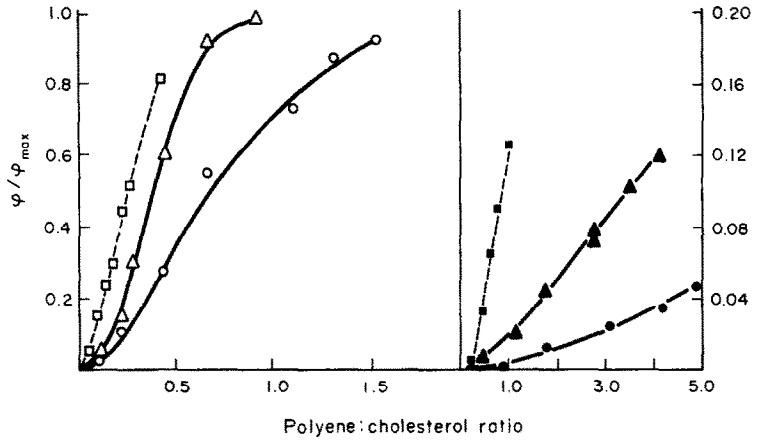

Fig. 2. Fluorometric titration of egg lecithin–cholesterol vesicles with lucensomycin (open symbols) and pimaricin (closed symbols). Final cholesterol concentrations were 5 μM (○,△,●,▲) or 20 μM (□,■). The lecithin–cholesterol ratio (w:w) was either 2:1 (□,△,■,▲) or 4:1 (○,●). Open symbols represent lucensomycin, closed symbols represent pimaricin. Fluorescence is expressed in both cases as a fraction of the maximal fluorescence which can be obtained with lucensomycin. The difference in the ordinate scales should be noted.

position of the horizontal asymptote is, however, practically the same for filipin and lucensomycin, if we further assume that the total number of binding sites per cell, t, is the same for both polyenes, the intrinsic association constant $\overline{K}$ would be about 40 times lower for filipin than for lucensomycin.

Pimaricin and *N*-acetyllucensomycin methyl ester appear to have, as far as lysis is concerned, a value of n very close to that of lucensomycin (which has a chemical structure closely related to theirs). They differ from lycensomycin by having a much lower value of $\overline{K}$. (This is especially true of pimaricin.) By taking advantage of the fact that the fluorescence intensity of macrocyclic tetraenes, such as pimaricin and lucensomycin, undergoes a conspicuous increase upon interaction with cholesterol, the different affinity of these two polyenes for cholesterol can be verified by fluorometric titration (Fig. 2); the interaction of pimaricin with cholesterol is found to be, compared with that of lucensomycin, more closely dependent on the absolute concentration of the sterol and on the sterol–lecithin ratio.

A somewhat different behaviour is exhibited by *N*-acetyllucensomycin, a compound which has a net negative charge at neutral pH. As shown in Fig. 1b, only in the extreme regions is the polyene concentration needed for 50% hemolysis either fully independent or strictly proportional to the number of erythrocytes. This behaviour can be explained by the existence of more than one value of $\overline{K}$; as shown in Fig. 1b, and as it has been demonstrated for similar plots in a different system [9], such an experimental curve may be seen to be a combination of at least two populations (indicated by the dotted curves of Fig. 1b) having different values of $\overline{K}$, the low-affinity population being the most abundant one. Since *N*-acetyllucensomycin is known to interact poorly with cholesterol when in the ionized state [8], it can be assumed that the two populations correspond to the existence of the polyene in the ionized (low affinity) and non-ionized (high affinity) forms, rather than to a variety of sites.

A study of the hemolytic efficiency of the larger polyenes (nystatin and amphotericin B) shows (Fig. 3) that the dependence of the 50% lytic concentration of the polyene on the concentration of the erythrocytes

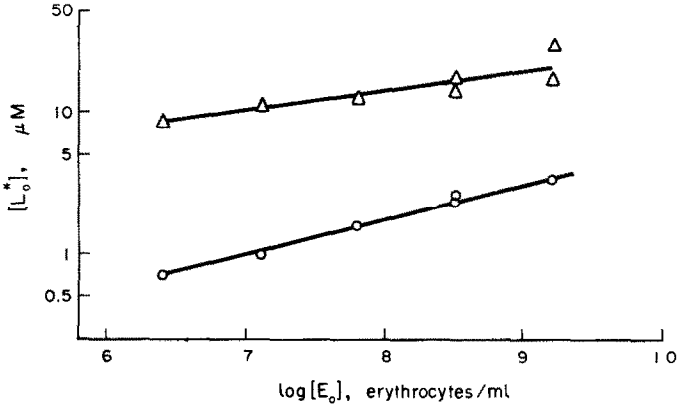

Fig. 3. Dependence of the 50% lytic concentration $[L_0^*]$, of amphotericin B (○——○) or nystatin (△——△) on erythrocyte concentration $[E_0]$. Both scales are logarithmic.

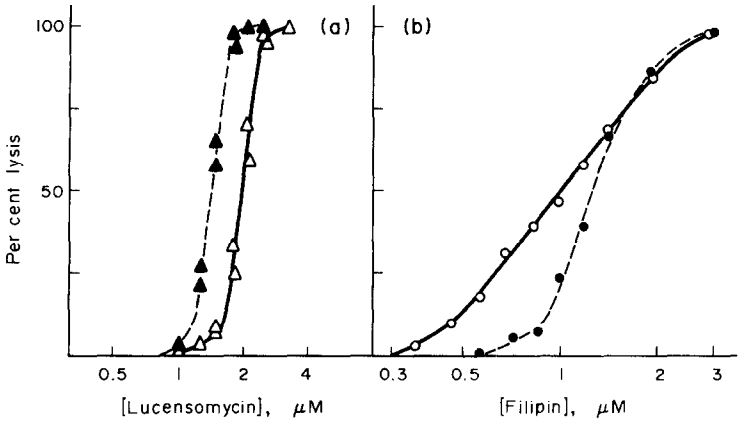

Fig. 4. Effect of the association between small ring polyenic antibiotics. (a) Synergistic effect of pimaricin on hemolysis by lucensomycin. Open symbols, lucensomycin alone; closed symbols, lucensomycin + 13 μM pimaricin. Final erythrocyte concentration was 10^6/ml. (b) Antagonistic effect of lucensomycin on hemolysis by filipin. Open symbols, filipin alone; closed symbols, filipin + 8.6 μM lucensomycin. Erythrocyte concentration was 2.5×10^6/ml.

can be described, in the double logarithmic plot, by a straight line of slope 0.14 (nystatin) or 0.25 (amphotericin B), no region with a slope of either 0 or 1 being detectable. This pattern can hardly be compared to that obtained with the small polyenes, and appears to indicate an extremely heterogeneous distribution of $\overline{K}$ values.

Hemolysis by association of two different polyenic antibiotics. As mentioned in the introduction, several polyene molecules are supposedly involved in the formation of a single aqueous pore, and such pores are responsible for the increase in membrane permeability.

If we assume that pore formation occurs upon occupancy of an $[(n + 1)/t]$ fraction of the cholesterol sites in the membrane, the simultaneous addition of two different polyenes to a limited number of erythrocyte membranes may then be expected to result in a clear cut synergism if the two antibiotics share a common mechanism of action to the point of forming mixed polyene pores, or in antagonism if they compete for cholesterol sites but are unable to cooperate in the formation of the same pore.

Figure 4 shows that if we perform such experiments by adding a known number of erythrocytes to preformed mixtures of two different polyenes, synergism can be seen upon association of lucensomycin with its derivatives (*N*-acetyllucensomycin or *N*-acetyllucensomycin methyl ester) or with pimaricin (Fig. 4a), i.e. with compounds which have a chemical structure very similar to that of lucensomycin itself.

Association of filipin and lucensomycin fails to produce any shift of the lysis curves, except when, as in Fig. 4b, hemolysis by filipin in the presence or absence of sublytic amounts of lucensomycin is examined at very high erythrocyte concentrations. In this case, it can be shown that occupancy by lucensomycin of a certain number of membrane sites results in an increased resistance to filipin.

The large polyenes (nystatin and amphotericin B), act synergistically with lucensomycin. In this case, however, as shown in Fig. 5, this does not affect all the erythrocytes: out of the whole population of red blood cells, a fraction is lysed by the large polyene alone (i.e. occupancy is $>n$), another behaves as if all polyene-binding sites were completely free, while a third one, which has an intermediate behaviour and is lysed by a smaller concentration of lucensomycin, seems to exist as a well-defined subpopulation (possibly with occupancy $\simeq n/2$).

Hemolysis by non-polyenic cholesterol-specific agents. Other substances, completely unrelated to the polyenic antibiotics, are also known to increase membrane permeability through interaction with cholesterol. Figure 6 shows that with both digitonin, which is a spirostane glycoside [10], and with streptolysin O, which is a protein [11], the 50% lytic concentration depends on the erythrocyte concentration in a manner similar to that found with lucensomycin. A most curi-

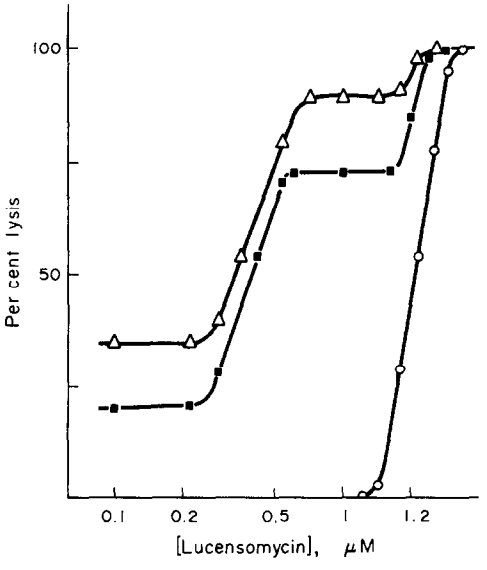

Fig. 5. Stepwise synergistic effect of nystatin on hemolysis by lucensomycin: O——O, lucensomycin alone; ■——■, lucensomycin + 6 μM nystatin; △——△, lucensomycin + 12 μM nystatin. Final erythrocyte concentration was 38×10^6/ml.

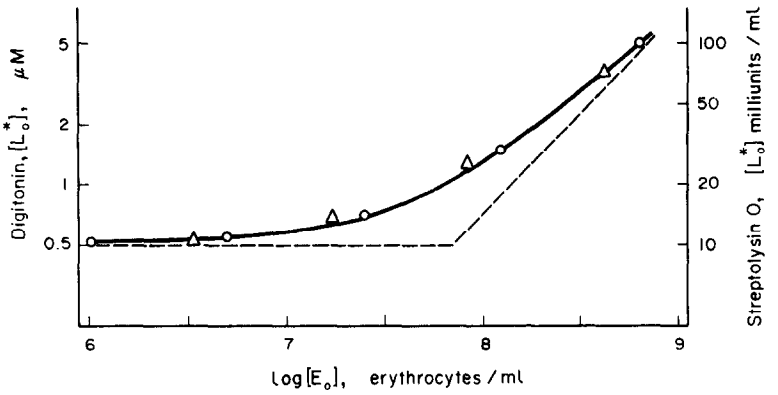

Fig. 6. Dependence of the 50% lytic concentration $[L_0^*]$ of digitonin (O——O) and streptolysin O (△——△) on erythrocyte concentration $[E_0]$. Both scales are logarithmic, the ordinates being chosen so as to superimpose the graphical interpolations of the two sets of data; the broken lines indicate the asymptotes.

ous finding is that digitonin and streptolysin O are equivalent in terms of lytic efficiency; the curves of Fig. 6 can be superimposed on each other if the "arbitrary unit" of streptolysin O is taken (although there is no theoretical justification for such an assumption) to be equivalent to 50 nmoles of digitonin.

Nevertheless, these two substances act as antagonists not only toward lucensomycin (Fig. 7), but also to each other, 10 milliunits of streptolysin O causing (Fig. 7a) a shift of 0.5 nmoles of digitonin.

DISCUSSION

The study of the dependence of $[L_0^*]$ on $[E_0]$ allows us to derive the critical level of occupancy, n, and the intrinsic association constant, $\overline{K}$, provided that the binding sites for the lytic agent are identical to and independent from each other.

Since all the hemolytic agents used in the present investigation are known to be fairly specific for cholesterol, the former condition (homogeneity of sites) is likely to hold, while the latter (independence of neighbouring sites) still needs to be verified. In fact, the results illustrated in Figs. 1a, 3 and 6 appear to indicate that both assumptions hold, within the limits of the experiment, for lucensomycin, *N*-acetyllucensomycin methyl ester, pimaricin, filipin, digitonin and streptolysin O. It is therefore possible, for this first group of compounds, to give an estimate of n and of $\overline{K}$, as shown in Table 1.

Since the mean erythrocyte area is 140 μm^2, the values of n thus obtained would correspond to a center-to-center "critical" mean distance between two adjacent occupied sites of approx. 40 Å for lucensomycin and approx. 300 Å for filipin. The corresponding values of $\overline{K}$, i.e. $1.8 \times 10^5\ M^{-1}$ for lucensomycin and $4.6 \times 10^4\ M^{-1}$ for filipin are similar to those obtained by spectroscopic techniques [12–14].

The "anomalous" behaviour of *N*-acetyllucensomycin, nystatin and amphotericin B (Figs. 1b and 3) appears to indicate some kind of heterogeneity at the level of the binding sites. In the case of *N*-acetyllucensomycin, this behaviour can, as previously mentioned, be referred to the protonated and unprotonated forms of the antibiotic, the latter having a lower affinity for membrane cholesterol. With nystatin and amphotericin B, a most appealing explanation is that of some positive interaction between neighbouring sites, favouring the

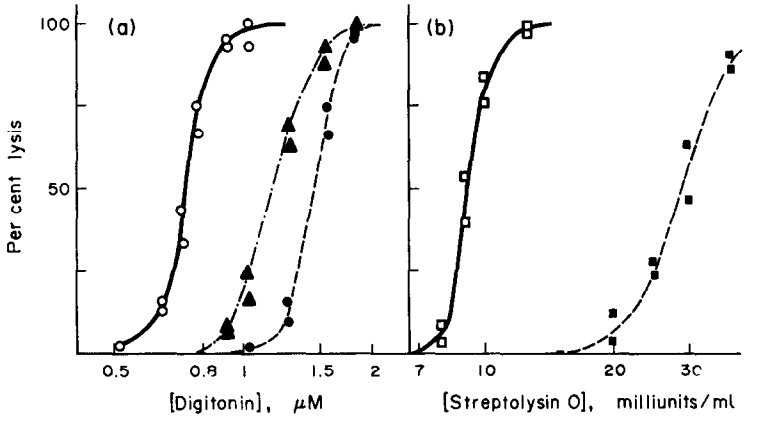

Fig. 7. Antagonism between the lytic effects of lucensomycin, digitonin and streptolysin O. (a) O——O, hemolysis of 4×10^7 erythrocytes/ml by digitonin alone; ▲——▲, hemolysis by digitonin in the presence of 10 milliunits/ml of streptolysin O; ●——●, hemolysis by digitonin in the presence of 0.4 μM lucensomycin. (b) Hemolysis of 10^7 erythrocytes/ml by streptolysin O alone (□——□) or in the presence of 0.5 μM lucensomycin (■——■).

Table 1. Comparison of lysis parameters of the various cholesterol-specific hemolytic agents

Hemolytic agent	$[L_{free}]$ for 50% lysis	n (molecules/cell)	Intrinsic K_{ass} (M^{-1})	Interaction with lucensomycin	Amount able to shift 1 nmole lucensomycin
Lucensomycin	0.8–1 μM	8–12 × 10^6	1.4–2.2 × 10^5		
N-Acetyllucensomycin	3.3 μM 50 μM	18 × 10^6	n.d.	Synergism	9 nmoles
N-Acetyllucensomycin methyl ester	3 μM	10–16 × 10^6	0.6–1.1 × 10^5	Synergism	3.1 nmoles
Pimaricin	37–40 μM	16 × 10^6	0.85 × 10^4	Synergism	80 nmoles
Amphotericin B	n.m.*	n.m. ($\leqslant 1 \times 10^5$?)	n.m.	Partial synergism	A fraction of RBC is lysed a 2.5 times lower concentration
Nystatin	n.m.	n.m. ($\leqslant 6 \times 10^6$?)	n.m.	Partial synergism	
Filipin	0.65 μM	0.2 × 10^6	0.46 × 10^5	Partial antagonism	0.04 nmoles
Digitonin	0.5 μM	4.2 × 10^6	0.65 × 10^5	Antagonism	2 nmoles
Streptolysin O	10 units/l	(1.4 × 10^{-10} units/cell)		Antagonism	38–42 × 10^{-3} units

* Not measurable.

For explanation of the symbols, see text. Concentrations are expressed in μmoles/liter^{-1}, except in the case of streptolysin for which the conventional "combining units" defined according to Hodge and Swift [18] were used. The intrinsic association constant $\bar{K}$ is calculated from equation 4, assuming that there are 134 × 10^6 cholesterol molecules per erythrocyte membrane, and that the cholesterol–polyene stoichiometry is 2:1 [6, 8, 12], while the cholesterol–digitonin stoichiometry is 1:1 [10].

formation of pores even under conditions of low overall occupancy. In such a case, the function $f(i)$ (equation 2) would assume different values according to the occupancy level i, so that no general derivation of n, $\bar{K}$ and $f(i)$ can be obtained from equations 1–3. This hypothesis is indeed supported by the results shown in Fig. 5, which suggest the existence, in the presence of sublytic amounts of nystatin or of amphotericin B, of well-defined erythrocyte subpopulations. These results may be tentatively related to the finding by Amati and Lago that different mammalian cell lines exhibit differential sensitivity to amphotericin B [15], but not to lucensomycin (P. Amati, personal communication).

The possibility that the various antibiotic molecules in solution may interact with each other should also be considered. It is, for example, well known that amphotericin B and nystatin form large micelles in aqueous solutions [5, 16]. In fact, almost all the agents used are only sparingly soluble in water and tend to aggregate. The existence of such interactions may result either in a heterogeneous distribution of association constants (which however is the case only for the larger polyenes) or, when two different agents are used simultaneously, in the appearance of non-additive effects.

It is however worth noting that, from the results of the combination experiments, the various hemolytic agents fall into four different groups: streptolysin O, digitonin, filipin and mycosamine-containing polyenic antibiotics. Each agent is antagonistic towards agents from the other groups, while there is a synergism between the various compounds belonging to the last group (Table 1). Pimaricin behaves very similarly to lucensomycin, except for a much weaker association constant. A most interesting finding, which confirmed some conclusions by de Kruijff and Demel [17] but was nevertheless unexpected (because of the similarity in chemical structure), was the antagonism between filipin and the other polyenes.

These results seem to indicate that, although the agents all have membrane cholesterol as a common target, only some of them share a common mechanism of action at the molecular level.

Acknowledgements—We thank Dr. Alfredo Colosimo for most helpful discussions and for computer simulation of cooperative effects, and Mr. Vincenzo Peresempio for skilful technical assistance.

REFERENCES

1. J. O. Lampen, *Soc. Gen. Microbiol. Symp.* **16**, 111 (1966).
2. A. Finkelstein and A. Cass, *J. gen. Physiol.* **52**, 145S (1968).
3. A. K. Solomon and C. M. Gary-Bobo, *Biochim. biophys. Acta* **255**, 1019 (1972).
4. B. de Kruijff, W. J. Gerritsen, A. Oerlemans, R. A. Demel and L. L. M. van Deenen, *Biochim. biophys. Acta* **339**, 30 (1975).
5. P. van Hoogevest and B. de Kruijff, *Biochim. biophys. Acta* **511**, 397 (1978).
6. R. Strom, C. Crifò, F. Eusebi, F. Sabetta and A. Oratore, *Biochim. biophys. Acta* **455**, 961 (1976).
7. M. C. Pangborn, *J. biol. Chem.* **188**, 471 (1951).
8. R. Strom, C. Crifò and A. Bozzi, *Biophys. J.* **13**, 568 (1973).
9. C. Bruni, A. Germani, G. Koch and R. Strom, *J. theor. Biol.* **61**, 143 (1976).
10. M. H. Briggs and J. Brotherton, *Steroid Biochemistry and Pharmacology*, p. 265. Academic Press, New York (1970).
11. S. P. Halbert, in *Microbial Toxins* (Eds. T. C. Montie, S. Kadis and S. J. Ajl) Vol. 3, p. 69. Academic Press, New York (1970).
12. R. Strom, W. E. Blumberg, R. E. Dale and C. Crifò, *Biophys. J.* **16**, 1297 (1976).
13. A. W. Norman, R. A. Demel, B. de Kruijff and L. L. M. van Deenen, *J. biol. Chem.* **247**, 1918 (1972).
14. R. Bittman and S. A. Fischkoff, *Proc. natn. Acad. Sci., U.S.A.* **69**, 3795 (1972).
15. P. Amati and C. Lago, *Nature, Lond.* **247**, 466 (1974).
16. A. W. Norman, A. M. Spielvogel and R. G. Wong, *Adv. Lipid Res.* **14**, 127 (1976).
17. B. de Kruijff and R. A. Demel, *Biochim. biophys. Acta* **339**, 57 (1974).
18. B. E. Hodge and H. F. Swift, *J. exp. Med.* **58**, 277 (1933).