Biochimica et Biophysica Acta, 600 (1980) 91—102

BBA 78854

EFFECT OF STREPTOLYSIN O AND DIGITONIN ON EGG LECITHIN/CHOLESTEROL VESICLES

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(Received November 6th, 1979)

Key words: Streptolysin O; Digitonin; Cholesterol vesicle; ESR

Summary

Artificial membrane vesicles (liposomes) have been used to study the lytic mechanism of the bacterial toxin, streptolysin O, compared to that of the well-known plant glycoside, digitonin. Two types of vesicle were prepared: large unilamellar vesicles and multilamellar liposomes. The vesicles were prepared with varying molar ratios of egg lecithin and cholesterol and loaded with the water-soluble spin label, TEMPO-choline chloride. Lysis of the vesicles was registered as release of spin label and monitored by change in the electron spin resonance (ESR) spectrum. In this system digitonin was able to lyse both large unilamellar vesicles and multilamellar liposomes. The effectiveness of lysis increased by increasing the percentage of cholesterol, but even at 0% cholesterol a significant level of lysis was observed by addition of a large enough concentration of digitonin. In contrast, no lysis was detected from multilamellar liposomes after exposure to streptolysin O, even when they consisted of 50 mol% cholesterol. On the other hand, large unilamellar vesicles could be lysed by streptolysin O, provided the cholesterol content was greater than 33%. At 67 mol% cholesterol in the membranes, the degree of lysis was diminished compared to 50%, which appeared to be optimal. This is the first demonstration of liposome lysis by streptolysin O and demonstrates the cholesterol specificity which has previously been shown by inhibition studies.

Introduction

Streptolysin O is a cytotoxic exocellular protein produced by most strains of group A and some of group C and G haemolytic streptococci. Streptolysin O is

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Abbreviation: TEMPO-choline: 4-[*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl]ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl.

oxygen-labile and requires reducing conditions in order to be activated [1]. Streptolysin O is a good immunogen and, therefore, antibodies are regularly formed in patients having streptococci infection [1,2]. Such antibody quantitation has great diagnostic value. Streptolysin O lyses erythrocytes and many other eucaryotic cells by disrupting the cytoplasmic membrane [3]. It has long been known that cholesterol and related sterols possessing a 3-β-hydroxy group and a hydrophobic group at position 17 inhibit this lytic activity of streptolysin O [4,5]. Such sterols can probably bind directly to streptolysin O, thus, [^{14}C]cholesterol binds to streptolysin O [6] and ^{125}I-labeled streptolysin O co-elute by gel filtration with sterol-containing liposomes [7]. Most earlier studies of the lytic effect of streptolysin O have been performed by using erythrocyte membranes as an indicator system. However, in recent years, model membrane systems (liposomes) have been developed and these are particularly useful in studying lytic effects [14].

Previous attempts to lyse cholesterol-containing liposomes with streptolysin O have unexpectedly failed, although ring and arc formations on the membrane surface have been detected by electron microscopy [8]. Similar phenomena have been observed on erythrocyte membranes treated with streptolysin O [8,9,14]. The failure of streptolysin O to disrupt cholesterol-containing liposomes is surprising, since saponins, which are lytic plant glycosides known to react with membrane cholesterol, are very effective in releasing internal markers from liposomes and also produce very similar, but smaller, rings in the membrane [10,11]. Two recent reports [12,13] have also demonstrated cholesterol-dependent glucose release from liposomes after addition of high concentrations of the closely related bacterial toxins, tetanolysin and cereolysin. Because of these conflicting results, and the new possibilities to make large oligo- and monolamellar lipid vesicles [15], we wanted to gain additional data on the action of streptolysin O and compare it with that of digitonin by using different types of liposome.

Materials and Methods

Lipids. Cholesterol and egg lecithin (phosphatidylcholine) were purchased from Sigma Chem. Co., St. Louis, MO, and digitonin from Th. Schuchardt Chem. Fabr., Görlitz, F.R.G. The reagents were used without further purification.

Spin label. TEMPO-choline chloride was prepared as described by Kornberg and McConnell [18].

Streptolysin O. Streptolysin O was isolated from supernatants of *Streptococcus pyrogenes* cultures by precipitations at 75% saturation of $(NH_4)_2SO_4$. The precipitate was dissolved in a small volume of water, dialyzed against 0.05 M Tris buffer, pH 8.5, and further separated on a DEAE-cellulose (Whatman DE-52) ion-exchange column. A low level of streptolysin O activity was detected in the material eluted with 0.05 M Tris, pH 8.5, whereas the main activity was eluted by a linear gradient (0—0.3 M NaCl) in the same Tris buffer. The fractions with haemolytic activity were pooled, lyophilized and further purified by gel filtration on Sephadex G-200 [16]. The haemolytic activity was eluted, corresponding to a molecular weight of about 70 000. A stock solution

of streptolysin O in phosphate-buffered saline, pH 7.2, was prepared and aliquots of 100 μl were kept frozen at −20°C. The protein concentration was estimated to be 1.4 mg/ml by measuring the absorbance at 280 nm.

Assay of haemolytic activity. Streptolysin O was activated by mixing 100 μl of the stock solution of streptolysin O with 25 μl 0.04 M dithiothreitol for 30 min. Dilution series of the toxin in $8 \cdot 10^{-3}$ M dithiothreitol in phosphate-buffered saline were made. 50 μl of the toxin solution were added to 250 μl 1% sheep erythrocyte suspension. The tubes were then incubated for 30 min at 37°C. 1 haemolytic unit (1 HU) is defined as the amount of streptolysin O required to produce complete lysis of 1 ml of the erythrocyte suspension. The haemolytic activity of the activated streptolysin O solution was about 10^3 HU/ml. A similar procedure, but without dithiothreitol, was used for digitonin.

Antistreptolysin O. A human serum (EA), having a monoclonal IgG component with very high antistreptolysin O titre (166 000 IU/ml), was used without further purification. To avoid possible interaction with the complement system, the serum was heat-inactivated for 30 min at 56°C.

Multilamellar liposomes. Multilamellar liposomes were prepared as described by Kinsky et al. [17]. 2 μmol of lipids with a varying cholesterol : egg lecithin molar ratio were dissolved in chloroform in a 10 ml conical flask. The solvent was removed by distillation under reduced pressure, after which the lipid formed a thin uniform film inside the flask. The dried lipids were swollen for 2 h at 40°C in 0.5 ml phosphate-buffered saline (pH 7.2) containing 0.077 M TEMPO-choline chloride (spin label solution). For removal of extraliposomal spin label, the solution was passed through a small Sephadex G-25 column. The concentration of washed liposomes was approx. 0.5 μmol lipid/ml.

Large unilamellar vesicles. Large unilamellar vesicles were prepared by the reverse-phase evaporation method as described by Szoka and Papahadjopoulos [15]. 30 μmol lipids, dissolved in chloroform with a cholesterol : egg lecithin molar ratio varying from 0 : 1 to 2 : 1, were added to a round-bottomed flask and the solvent removed under reduced pressure. The lipids were redissolved in 1.5 ml diethyl ether. Then a 0.5 ml spin label solution was added and the resulting two-phase system was sonicated at +4°C for 5 min in a bath-type sonicator until the mixture became a homogeneous turbid dispersion. The mixture was again placed on the rotary evaporator and the ether removed under reduced pressure (2—300 mmHg) at 20°C.

During the evaporation the material first formed a viscous gel and then it became an aqueous suspension. The mixture was evaporated for an additional 15 min at 20°C to remove traces of ether. For removal of the extraliposomal spin label, samples of 50 μl were diluted in phosphate-buffered saline and dialyzed against three changes of 1000 vols. of phosphate-buffered saline. The sample was now diluted to 3 ml. The concentration of lipids is then 1 μmol/ml.

Assay of spin label release. For monitoring the degree of membrane lysis we started to use the technique described by Rosenqvist and Vistnes [19]. This method is based on the observation that if a high concentration of nitroxide free radicals are trapped inside liposomes, only low amplitude ESR signals are observed due to spin exchange. Upon the release of spin label due to membrane damage, it is possible to observe a many-fold increase in the ESR line amplitude. The technique needs no additional reagent, no separation of lysed

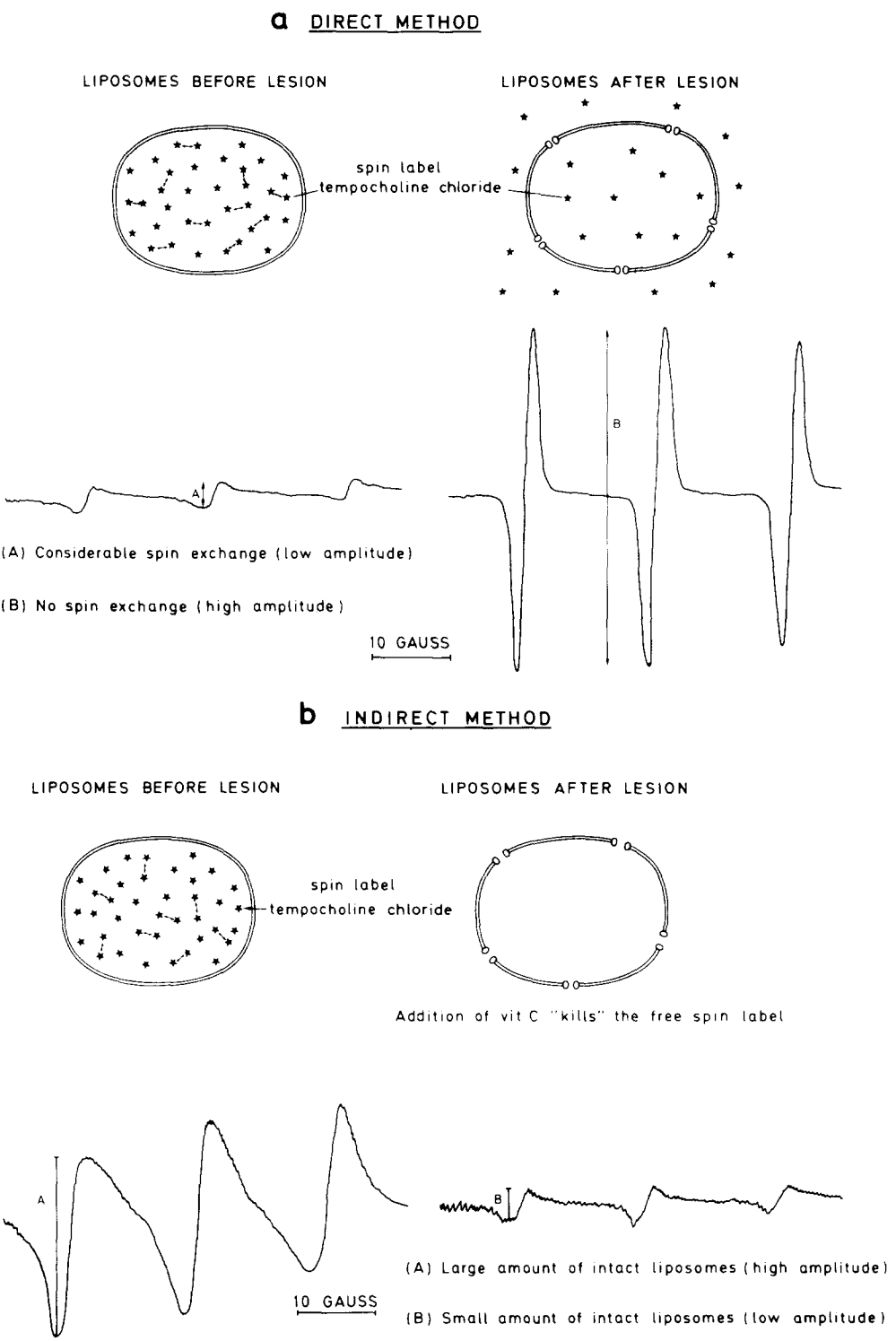

Fig. 1. Schematic representation of the direct (a) and the indirect method (b). Comparison of the amplitudes of the ESR spectra before (A) and after (B) lysis provides a measure of the degree of lysis.

and unlysed liposomes, and is very simple to perform. We call this the direct method (Fig. 1a).

The reagents to be tested were added to the liposome suspension (25 μl multilamellar liposomes or 10 μl large unilamellar vesicle) and the total volume adjusted to 60 μl with phosphate-buffered saline. The mixture was incubated for varying times and temperatures and then transferred to a glass capillary, sealed, and placed in the ESR cavity. The ESR spectra were recorded with a JEOL X-band spectrometer (type JES-ME-1X) equipped with a cylindrical cavity. The sample holder was a cylindrical quartz tube, 3 mm inner diameter. The parameter used for quantitative measurements was the peak-to-peak amplitude of the mid-field line in the first-derivative absorption spectrum.

A troublesome problem associated with this technique was the loss of ESR signal due to reduction of the spin label by dithiothreitol and reduced streptolysin O. Our experiments at 20°C showed a half-life time, $t_{1/2}$, of approx. 7 h for $1 \cdot 10^{-4}$ M TEMPO-choline in $8 \cdot 10^{-3}$ M dithiothreitol and $t_{1/2} = 2$ h for $1 \cdot 10^{-4}$ M TEMPO-choline in $8 \cdot 10^{-3}$ M dithiothreitol plus streptolysin O. This means that incubation for longer periods will give substantial error. To overcome this problem a modified method was developed. After the liposomes and streptolysin O solution had been incubated, 10 μl of $1 \cdot 10^{-2}$ M ascorbic acid were added, the sample was cooled down with ice, and the ESR spectrum was immediately recorded. To avoid interaction with the ascorbic acid signal, the amplitude of the low-field line was measured. The ascorbic acid will give a complete reduction and loss of signal to all the free spin label which has leaked out, but will not affect the spin label inside the undamaged liposomes. We will therefore observe a decrease in amplitude with an increasing degree of lysis. 100% lysis was measured by addition of 25 μl 10% Triton X-100. We call this the indirect method (Fig. 1b). With both methods, the ESR amplitude ratios for Triton X-100 : untreated liposomes, or the reverse, were about 10—15.

Electron microscopy. Negative staining was performed by placing a drop of the appropriate suspension of liposomes, with or without streptolysin O, on carbon-coated formvar grids. After 1 or 2 min the excess fluid was removed with a small piece of filter paper. The grids were stained with 1% phosphotungstic acid (pH 7.2) or uranyl acetate and immediately examined in a JEOL 100B electron microscope operated at 80 kV.

Results

Effect of digitonin and streptolysin O on multilamellar liposomes

By using the direct method, significant lysis was observed after addition of digitonin to the multilamellar liposomes (Fig. 2). The lytic effect of digitonin increased with increasing cholesterol content in the liposomes, but even without cholesterol we observed a significant lysis with the highest concentrations of digitonin.

In contrast to the effect of digitonin, streptolysin O did not lead to any detectable loss of spin label from multilamellar liposomes by the direct method, even after exposure to 50 haemolytic units streptolysin O (10^3 HU/μmol lipid), and with liposome preparations having a cholesterol content of up to 50%.

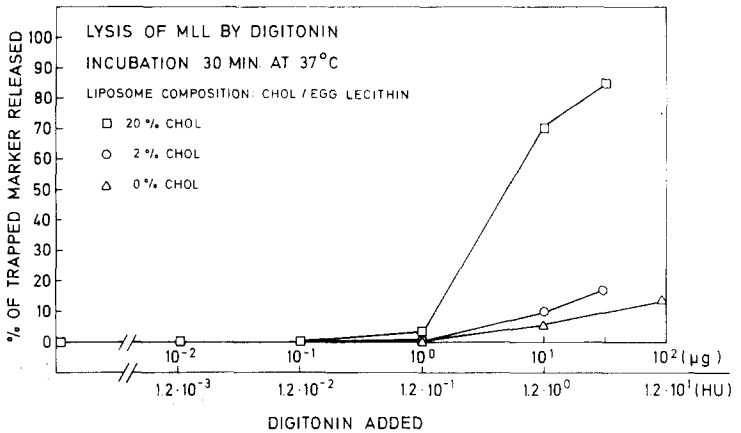

Fig. 2. Effect of digitonin addition on multilamellar liposomes (MLL) measured by the direct method. Liposomes (50 μl = 25 nmol lipid) with the indicated lipid composition were incubated with 10 μl digitonin/phosphate-buffered saline solutions of varying concentration. CHOL, cholesterol.

Effect of digitonin and streptolysin O on large unilamellar vesicles

As for multilamellar liposomes, significant lysis was observed after addition of digitonin to large unilamellar vesicles, even when they were prepared from only egg lecithin, providing the digitonin was added in large enough concentrations (Fig. 3). The lytic susceptibility to digitonin was clearly higher for large unilamellar vesicles than for multilamellar liposomes.

In contrast to multilamellar liposomes which were not lysed under any conditions by streptolysin O, large unilamellar vesicles were indeed lysed by streptolysin O measured by the indirect method. The lysis was very fast and completed within a few minutes, even at room temperature. This gave us also the possibility of using the direct method to measure lysis. After the mixing of

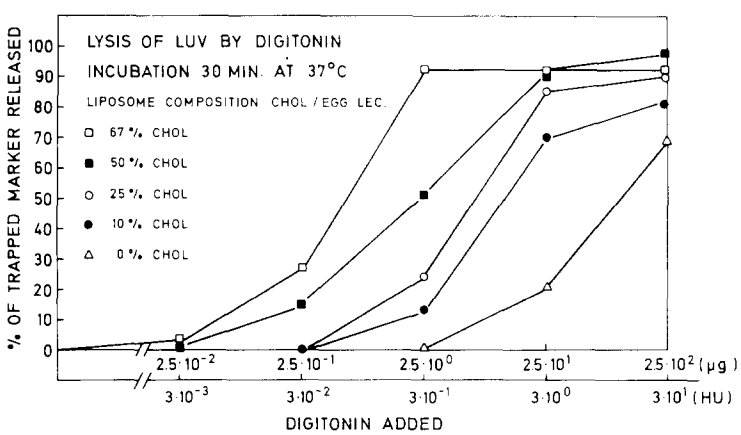

Fig. 3. Effect of digitonin addition on large unilamellar vesicles (LUV) measured by the direct method. Lipid vesicles (10 μl = 10 nmol lipid) with the indicated lipid composition were incubated with 25 μl digitonin/phosphate-buffered saline solutions of varying concentration in a total volume of 60 μl. CHOL, cholesterol; EGG LEC., egg lecithin.

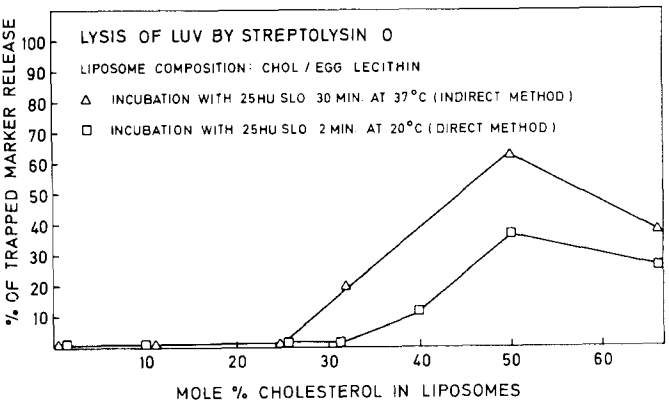

Fig. 4. Effect of cholesterol (CHOL) concentration on streptolysin O (SLO) damage to large unilamellar vesicles (LUV). Lipid vesicles (10 μl = 10 nmol lipid) with the indicated lipid composition were incubated with (25 μl = 25 HU) activated streptolysin O solution in a total volume of 60 μl. □, ESR signal measured by the direct method; △, ESR signal measured by the indirect method.

liposomes and streptolysin O, the amplitude of the ESR signal was followed for a period of 10 min. Following the first rapid increase of the signal, a slow decrease in amplitude, probably due to reduction of the spin label, was observed. For calculation of percent lysis, the amplitude was taken as the highest observed signal. With both methods similar effects were observed.

The lytic effect of streptolysin O was highly cholesterol-dependent and, thus, liposome damage could only be detected with liposomes having 33% or more cholesterol (Fig. 4). At 67% cholesterol in the membrane, the spin label release was diminished compared to 50%. The human antistreptolysin O serum

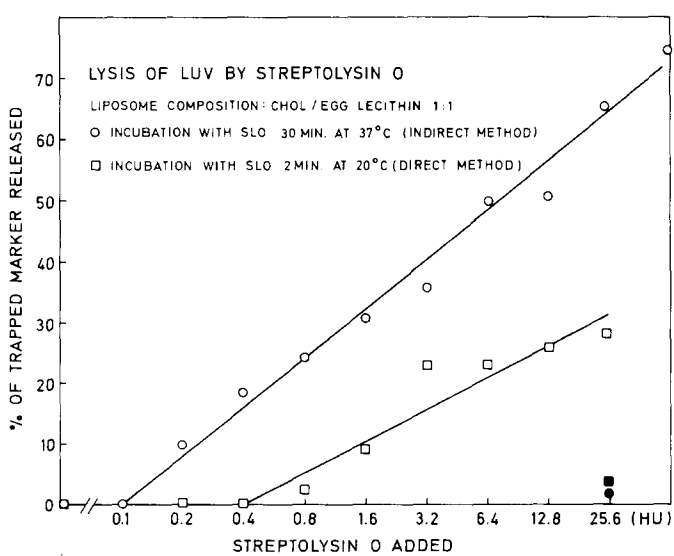

Fig. 5. Dose-response curve for streptolysin O (SLO) addition to large unilamellar vesicles (LUV). Lipid vesicles (10 μl = 10 nmol lipid) with 50% cholesterol (CHOL) were incubated with 25 μl activated streptolysin O solution of varying concentration. The effect of adding 41.5 IU antistreptolysin O is shown by ■ (direct method), and ● (indirect method).

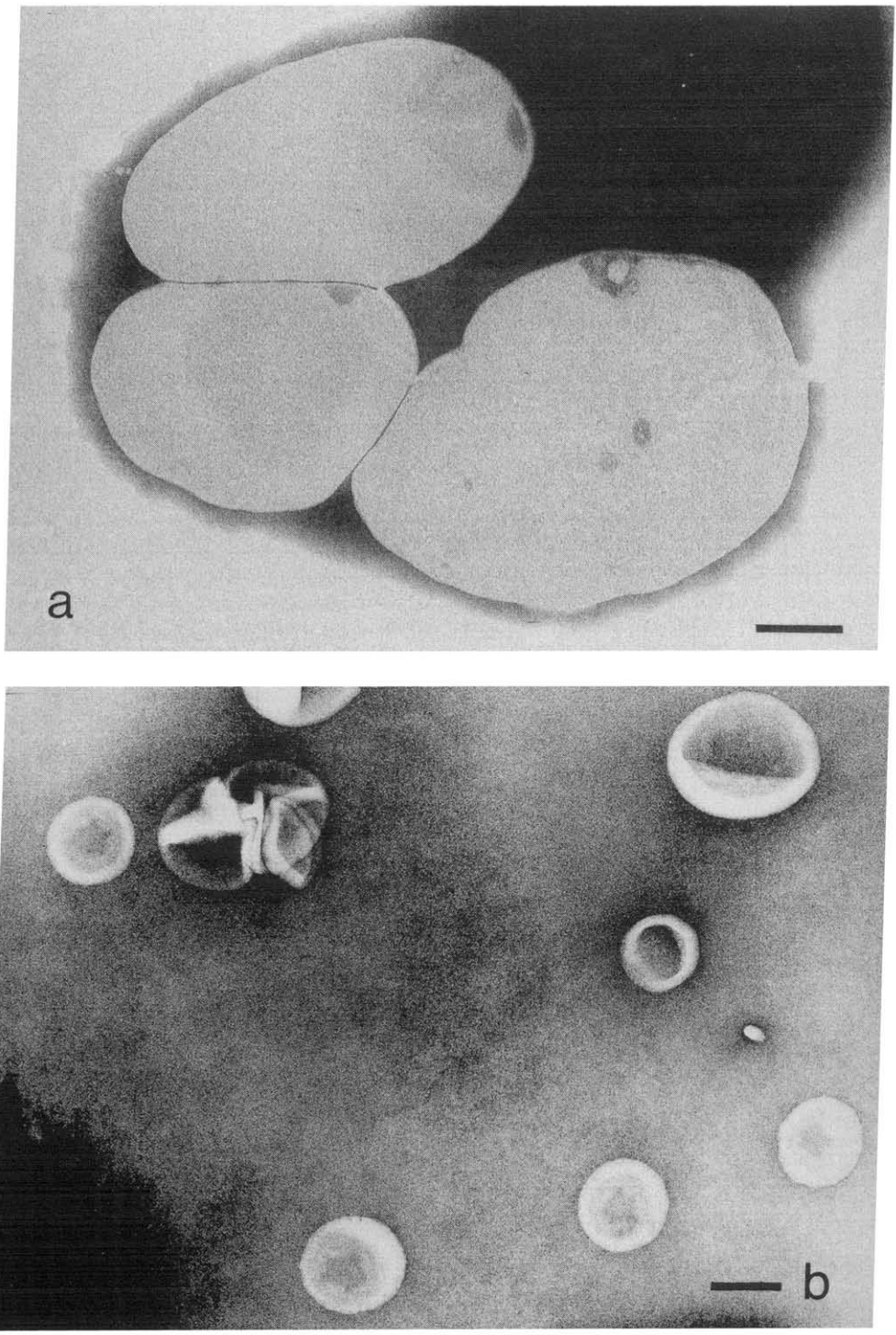

Fig. 6. Different preparations of large unilamellar vesicles consisting of egg lecithin/cholesterol (1 : 1 molar ratio). Negatively stained with (a) 1% phosphotungstic acid, (b) 1% uranyl acetate. The bar indicates 100 nm.

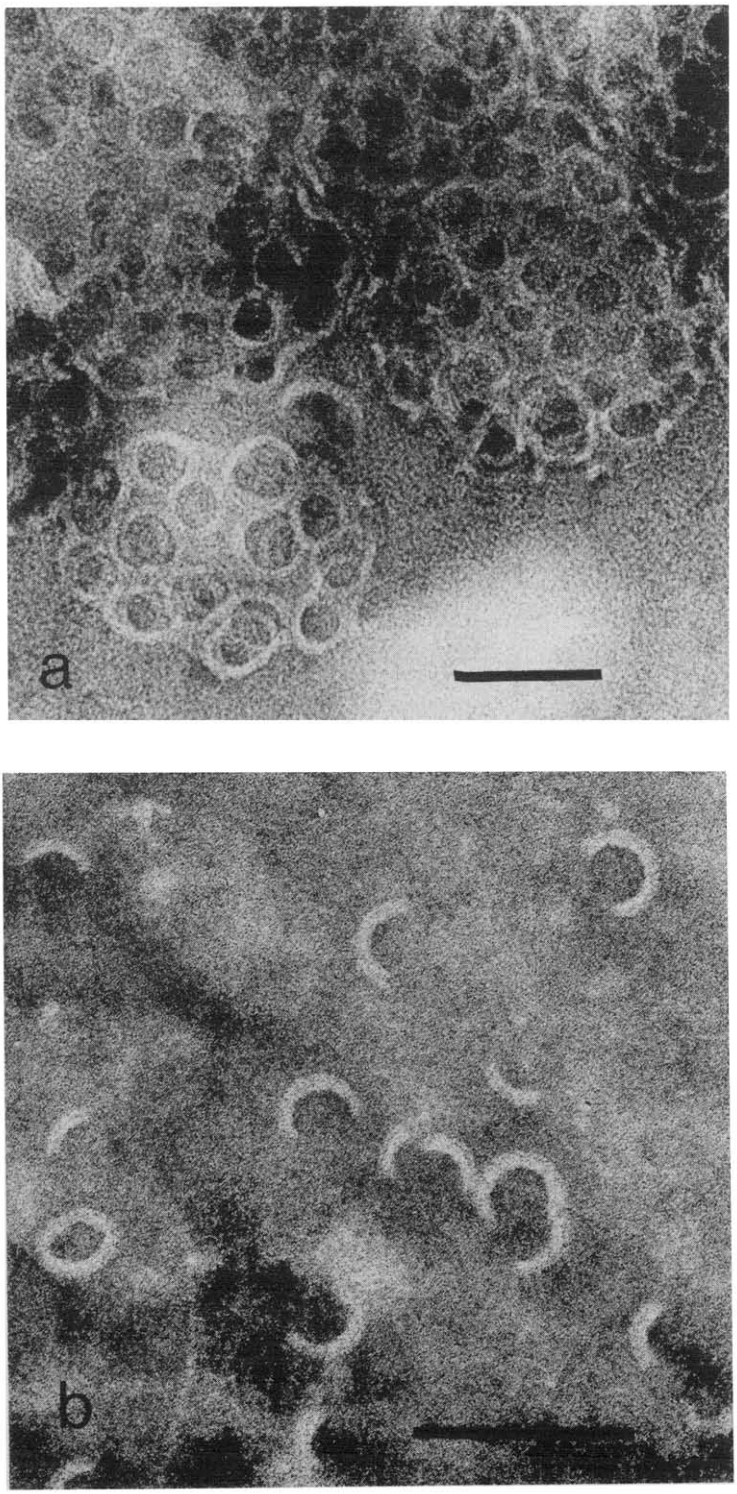

Fig. 7a and b.

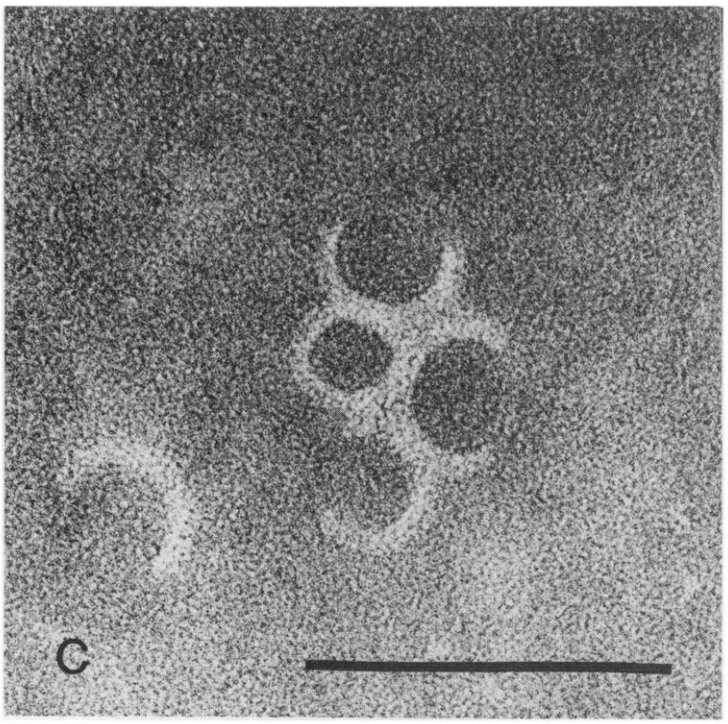

Fig. 7. Large unilamellar vesicles consisting of egg lecithin/cholesterol (1 : 1) after addition of streptolysin O, showing hole formations (a), isolated arcs (b) and rings (c). Negatively stained with 1% phosphotungstic acid. The bar indicates 100 nm.

(EA) at a dose of 41.5 IU completely inhibited the lytic effect of 25 HU streptolysin O on large unilamellar vesicles. Dose-response curves (Fig. 5) comparing the direct and indirect methods showed a higher percent of lysis by the indirect method (at 37° C) than by the direct method measured at 20° C.

Electron microscopy

Negatively stained large unilamellar vesicles, when inspected in the electron microscope showed that the vesicles varied in size, ranging from 100 to about 600 nm (Fig. 6).Most vesicles appeared to consist of only one or a few concentric bilayers with a large internal space. Smaller vesicles were often found within the interior aqueous space of the larger one. When liposomes composed of egg lecithin and cholesterol were treated with dithiothreitol-activated streptolysin O, very distinct rings in the membrane could be seen (Fig. 7). The outside diameter of the rings was approx. 40 nm, with a border thickness of 7 nm. In some areas, free rings or arc-shaped structures were observed. The arcs were of variable lengths. In some pictures fine ripples could be seen in the rings, indicating a periodic substructure.

Discussion

In agreement with the results of Duncan and Schlegel [8] we found that streptolysin O did not cause the release of marker from multilamellar liposomes

containing cholesterol and lecithin. However, by using unilamellar vesicles, we observed that streptolysin O caused rapid membrane damage and a release of internally trapped marker from the liposomes. The striking difference in the effect of streptolysin O on unilamellar as compared to multilamellar liposomes, might be explained if streptolysin O only damages an exposed membrane, whereafter it is inactivated by complex formation with cholesterol. Since streptolysin O therefore cannot cross a bilayer, the main content of multilamellar liposomes will be left intact, while all the content of the unilamellar vesicle rapidly leaks out through the relatively large pores formed (Fig. 7).

The lytic effect of streptolysin O was clearly cholesterol-dependent, showing no lysis at cholesterol : lecithin ratios lower than 1 : 2. Optimum lysis occurred at a molar ratio of 1 : 1, while a molar ratio of 2 : 1 resulted in decreased lysis. These results are very similar to those which Alving et al. [12] have found for tetanolysin and Cowell and Bernheimer [13] for cereolysin, and could indicate a common mechanism of action. The observed decrease in lytic effect at the highest cholesterol concentration in the membranes might indicate that, in this case, free excess of cholesterol could exist, which in turn could inactivate part of the available streptolysin O. This is in agreement with the observation that cholesterol/lecithin mixtures with more than 50% cholesterol are unstable, and free cholesterol forms a separate phase [20].

Why no lysis was observed below 33% cholesterol is uncertain, but it is well known that the influence of cholesterol on the structure and properties of bilayer membranes is markedly dependent on certain cholesterol : lipid ratios. A variety of experiments indicate that at about 33% cholesterol several properties of the bilayer are changed. For example the mobility of the polar head groups is altered [21], the phase transition disappears [22] and a stable complex forms at this molar ratio [23—25].

The lysis of liposomes by digitonin was also cholesterol-dependent, giving a higher degree of lysis with increasing cholesterol content. In this case, however, both multilamellar and unilamellar liposomes were affected, although the unilamellar liposomes were more sensitive to a low digitonin concentration. For digitonin lysis we observed no threshold value for the cholesterol concentration. Even liposomes without cholesterol were lysed when we used large enough concentrations of digitonin. This indicates also an unspecific lytic effect on phospholipid membranes in addition to the specific cholesterol-dependent lysis by digitonin. When using digitonin we did not observe an optimum cholesterol content in the liposomes. Vesicles prepared from 67% cholesterol showed apparently a higher degree of lysis after digitonin treatment than those prepared from 50% cholesterol.

It is also worth noting that we observed lysis of liposomes by streptolysin O treatment even at +1.3° C, while Duncan and Schlegel [8] reported that no rings were formed when cholesterol-containing liposomes and streptolysin O were incubated at 0° C. It is also known that erythrocytes bind streptolysin O but no lysis occurs at this temperature [26]. It has been suggested that streptolysin O acts via pore-formation by translocation of streptolysin O-cholesterol complexes laterally in the membrane. To explain the difference in sensitivity at low temperatures between cell membranes and liposome membranes, it might be that the fluidity of the erythrocyte membrane is so much lower than in

liposomes that this translocation is prohibited at 0° C in erythrocytes.

The dimensions of the rings we observed after streptolysin O treatment of the liposomes are in good agreement with the observations of Duncan and Schlegel [8] and with the study by Cowell and Bernheimer [13] on cereolysin-treated membranes. We find that the lengths of the arcs vary considerably. The periodic pattern in some of the arcs and the variability in length, we feel, is in favour of a model where the structures are composed of polymeric aggregates of streptolysin O-cholesterol complexes.

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