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APPROXIMATE DIMENSIONS OF MEMBRANE LESIONS PRODUCED BY STREPTOLYSIN S AND STREPTOLYSIN O

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Membrane lesions produced by the streptococcal membranolysins streptolysin S and streptolysin O were investigated. Escape of labeled marker molecules of various sizes from resealed sheep erythrocyte ghosts treated with the toxins for 30 min allowed estimation of the sizes of the primary channels formed. Streptolysin S formed lesions ranging in size up to 45 Å in diameter, and even high toxin concentrations did not result in larger channels. The lesions produced by streptolysin O exceeded 128 Å in diameter. Kinetics experiments demonstrated that the primary streptolysin O lesions were formed rapidly (1–2 min), but release of marker molecules from streptolysin S-treated vesicles began only after a 5–15-min lag period. Label release from large unilamellar liposomes treated with streptolysin S suggested that membrane fluidity does not affect the size of the streptolysin S lesions.

Introduction

In efforts to define the role of the cytolytic toxins, streptolysin O and streptolysin S, in the pathogenicity of *Streptococcus pyogenes*, the mechanism of action of these toxins is under investigation. Both toxins act on eukaryotic cell membranes, but apparently in different ways. Studies with erythrocytes have shown that streptolysin S lyses cells by a colloid-osmotic process [1,2], while streptolysin O is thought to produce larger primary lesions resulting in noncolloid-osmotic lysis [3].

The specific characteristics of the membrane lesions produced by these cytolysins are still unknown. Direct electron microscopic observations have demonstrated ring-shaped structures formed by streptolysin O on erythrocyte membranes and cholesterol-containing liposomes [4–6]. Other thiol-activated toxins, which are considered closely related to streptolysin O, also form such membrane alterations [7–10]. The relationship of these structures to the lytic process is uncertain, but freeze-fracture studies have suggested that the morphological structures produced by the toxins did not penetrate both membrane leaflets [9,10]. There have been comparatively few direct observation of the streptolysin S-mediated lesions. Bangham et al. [11] observed 'channel' formation in artificial phospholipid membranes, but attempts to demonstrate structural changes in natural membranes have not been successful [5].

The monitoring of the escape of markers of various sizes from internal spaces has been a useful method to approximate functional dimensions of membrane lesions, both in living cells and with artificial membrane systems [12–17]. These experiments are based on the assumption that, given similar diffusion environments, molecules up to a certain size will pass through membrane lesions with given diameter. The size or effective diffusion radii of the passed and retained markers can then be used to approximate the functional 'pore' size in the membrane.

Thelestam and Mollby [12] determined 'leakage

patterns' from fibroblasts by various cytolysins using α-aminoisobutyric acid, uridine and ribosomal RNA as cytoplasmic markers. Levels of streptolysin O used in these experiments allowed escape of the two smaller molecules, but the RNA (M_r 200000) was somewhat retained. Comparable amounts of streptolysin S released α-aminoisobutyric acid only.

A closer determination of the functional size of the lesions produced by streptolysin O and streptolysin S has not been reported. In the experiments described here, we used resealed sheep erythrocyte vesicles containing labeled molecules of various sizes to estimate the dimensions and the kinetics of formation of the primary lesions produced by streptolysin O and streptolysin S. Liposomes prepared by reverse-phase evaporation from purified phospholipids allowed comparison of lesion formation in synthetic and natural membranes.

Materials and Methods

Marker molecules. [^{14}C]Sucrose (480 mCi/mmol) was purchased from Schwarz-Mann, Orangeburg, NY and [^{3}H]methoxy inulin (114 mCi/g) from ICN, Irvine, CA. Cytochrome *c*, chymotrypsinogen A, ovalbumin, catalase and ferritin were obtained in kit form from Boehringer-Mannheim, Indianapolis, IN. Bovine serum albumin, thyroglobulin and urease were from Sigma Chemical Co., St. Louis, MO. The proteins were labeled by the chloramine T method [18] with Na^{125}I (Amersham Corp., Arlington Heights, IL). After labeling, the proteins were dialyzed against three changes of buffer, the first dialysis containing 5 mM KI.

The diameter of the markers was calculated from the Stokes radii [19]; sucrose, 8.8 Å, inulin, 29.6 Å; cytochrome *c*, 32.8 Å; chymotrypsinogen A, 44.8 Å; ovalbumin, 54.2 Å; bovine serum albumin, 72.2 Å; catalase, 108 Å; ferritin, 120 Å; urease, 128 Å.

Toxins. Streptolysin O was prepared as previously described [20]. The specific activity of a typical preparation was $1.7 \cdot 10^5$ hemolytic units (HU) per mg protein. One hemolytic units is defined as the amount of streptolysin O which causes 50% hemolysis of a 0.9% erythrocyte suspension in phosphate-buffered saline after 30 min at 37°C.

Streptolysin S was prepared as described [21]. Lyophilized, resuspended toxin was dialyzed (45 min; 4°C) against distilled water before use. Streptolysin S activity was measured in HU by the same criteria specified above for streptolysin O. The streptolysin S specific activity was approx. $5 \cdot 10^5$ hemolytic units per mg protein.

Resealed erythrocyte vesicles. Resealed vesicles were prepared according to the procedure of Giavedoni et al. [14]. Fresh sheep red blood cells (obtained weekly from a local veterinarian) were washed three times in veronal buffer (145 mM NaCl/5 mM sodium barbital (Fisher Scientific Co., Fairlawn, NJ)/0.15 mM $CaCl_2$/5 mM $MgCl_2$ (pH 7.3)). One volume of washed cells was added to 9 vol. of cold veronal hypotonic buffer (veronal buffer without NaCl) containing $(1–4) \cdot 10^6$ cpm ^{125}I-labeled protein or 4.0 μCi/ml [^{3}H]inulin or [^{14}C]sucrose and incubated 10 min on ice. After addition of 0.5 vol. of 2.9 M NaCl, the vesicles resealed during a second 10-min incubation on ice. The vesicles were stabilized at 37°C for one hour.

Three washes in cold veronal buffer (23000 × *g*, 15 min) were sufficient to separate vesicles from untrapped markers. The pellet from the final centrifugation was resuspended in 5–7 vol. veronal buffer and 0.5 ml of this suspensions was used for each experimental sample. The resealed vesicles prepared in this way contained 230–300 μg membrane protein/sample as determined by protein assays of freeze-thawed vesicles.

Marker-release assays. Resuspended vesicles were incubated with 0–4000 hemolytic units streptolysin S or streptolysin 0, 0.1 ml Triton X-100 (Sigma), or no addition, in a total volume of 1 ml veronal buffer for 30 min at 37°C. The incubation period was varied from 0 to 120 min for kinetic experiments. The samples were centrifuged at 23000 × *g* for 15 min at 4°C and the amount of radioactivity in the supernatant was determined in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, IL) or a Beckman automatic gamma counter (Beckman Instruments, Inc., Levine, CA). The results were expressed as percent release, the amount of radioactivity in a given sample supernatant divided by the amount of radioactivity released by 0.1 ml Triton times 100. All

sample counts were corrected for spontaneous release measured in control samples. Spontaneous release after 30 min was usually 5–10% of the total radioactivity present in the vesicles. Control experiments were performed with streptolysin O inhibited by 0.5 mM cholesterol or streptolysin S inhibited by 100 μg Trypan blue.

The toxins were tested by hemolytic assay immediately prior to each experiment. Since streptolysin O activity is decreased by divalent cations [22], the assays were done on sheep red blood cells suspended in veronal buffer. Streptolysin S was tested under the same conditions, although streptolysin S activity is not affected by divalent cations.

Liposomes. Large, unilamellar liposomes were prepared and assayed as previously described [21]. The radioactive molecules added to serve as internal markers were [^{14}C]sucrose (10 μCi), [^{3}H]inulin (10 μCi), or ^{125}I-labeled chymotrypsinogen A or ovalbumin ($5 \cdot 10^5$ cpm).

Results

Release of markers from resealed vesicles

Vesicles containing markers of various sizes were incubated with 0–2000 hemolytic units of streptolysin S or 0–4000 hemolytic units of streptolysin O for 30 min at 37°C. Fig. 1 shows the release of marker molecules from streptolysin S-treated vesicles. It can be seen that release was a function of toxin concentration; increasing amounts of the markers were released with higher concentrations of streptolysin S until a plateau was reached, in most instances at 100–300 hemolytic units. In addition, the maximum amount of each marker that could be released was related to the size of the marker. After 30 min incubation with 300 hemolytic units, for example, all of the vesicles contained lesions large enough to release sucrose, but only about 30% of the vesicles contained lesions sufficiently large to allow chymotrypsinogen A to diffuse out of the vesicles. There was little or no release of ovalbumin or bovine serum albumin in 30 min. These results suggest that the size of the primary membrane lesions produced by streptolysin S is heterogeneous. The release of markers by streptolysin S was completely abolished by Trypan blue, an inhibitor of this toxin.

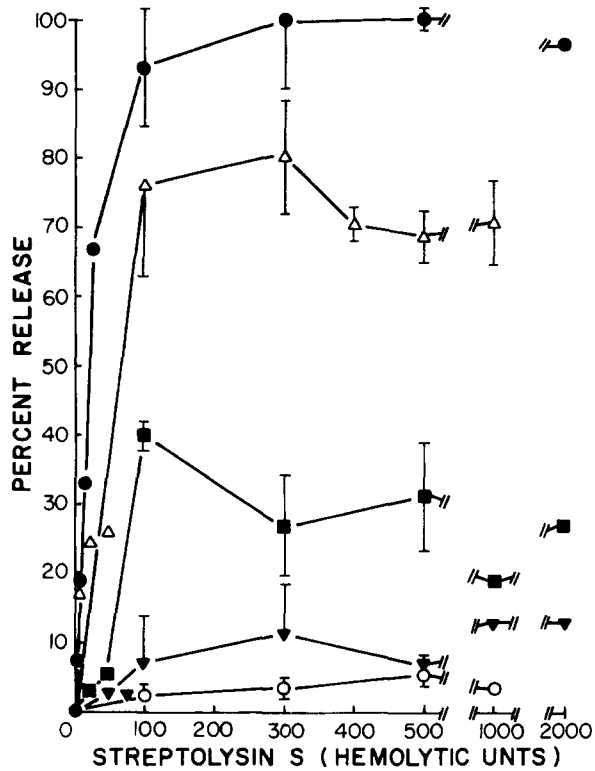

Fig. 1. Release of labeled markers from sheep erythrocyte vesicles by streptolysin S. Sheep erythrocytes were lysed and resealed as described in Materials and Methods trapping the indicated markers. The vesicles were incubated with 0–2000 hemolytic units streptolysin S for 30 min at 37°C. After centrifugation of the samples, the amount of radioactivity in the supernatants was determined and expressed as a percent of the supernatant radioactivity of Triton X-100-treated samples. All values were corrected for release from control samples. Each point is the average of three or more experiments. ●, [^{14}C]sucrose; △, [^{3}H]inulin; ■, ^{125}I-labeled chymotrypsinogen A; ▼, ^{125}I-labeled ovalbumin; ○, ^{125}I-labeled bovine serum albumin.

To determine whether membrane lesions were produced and accessible to the diffusion of small markers under conditions where larger markers were only partially released, vesicles were prepared that contained sucrose, cytochrome *c* or both markers. The vesicles were treated with various concentrations of streptolysin S and the release of sucrose in the presence or absence of cytochrome *c* (and vice versa) was determined. A typical experiment, shown in Table I, demonstrates the release of the small marker was virtually unaffected by the presence of a larger marker molecule that was only

TABLE I

EFFECT OF STREPTOLYSIN S (SLS) ON MARKER RELEASE FROM VESICLES CONTAINING BOTH [^{14}C]SUCROSE AND ^{125}I-LABELED CYTOCHROME *c*

HU, hemolytic units; Cyt *c*, cytochrome *c*.

SLS (HU)	Release (%)			
	Sucrose		Cytochrome *c*	
	Alone	+ Cyt *c*	Alone	+ sucrose
100	75	100	19	13
300	100	100	21	23
500	100	100	30	22

partially released from the same vesicles.

Fig. 2 shows marker release from streptolysin O-treated vesicles after 30 min. Again, the release of the markers increased with higher concentrations of toxin, but in sharp contrast to the situation with streptolysin S, substantial release of all markers was produced by 300 hemolytic units streptolysin O. At higher toxin concentrations, at least 75%, and in most cases greater than 90% of the markers tested had diffused from the vesicles. No marker was found that was not substantially released by streptolysin O. In control experiments, no release was observed when cholesterol, an inhibitor of streptolysin O and other thiol-activated cytolytic toxins, was included.

Kinetics of marker release

To determine how rapidly the toxins brought about marker release, kinetics experiments were carried out over a 30-min period. The release of markers from vesicles incubated with 300 hemolytic units of streptolysin S was characterized by a 5–15-min lag period prior to marker release (Fig. 3A). No release of sucrose occurred during the first five minutes of incubation, and with a larger molecule, chymotrypsinogen, the lag period extended for 15 min. In contrast to the situation with streptolysin S, the release of markers from vesicles treated with 100 hemolytic units of streptolysin O began immediately after toxin addition (Fig. 3B). Complete release of sucrose and bovine serum albumin was seen within 5 min, and even with the largest marker, urease, 75% of the total amount

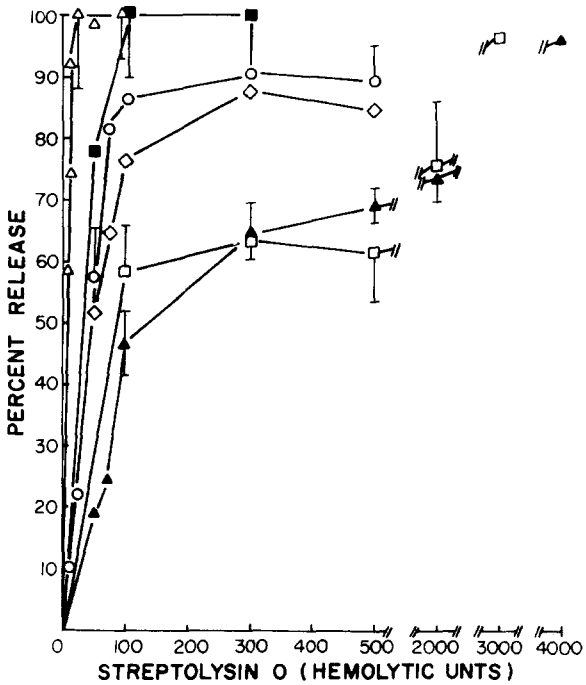

Fig. 2. Release of labeled markers from sheep erythrocyte vesicles by streptolysin O. Conditions were as in Fig. 1, except 0–4000 hemolytic units of streptolysin O was used in place of streptolysin S. △, [^{3}H]inulin; ■, ^{125}I-labeled chymotrypsinogen A; ○, ^{125}I-labeled bovine serum albumin; ◇, ^{125}I-labeled catalase; ▲, ^{125}I-labeled ferritin; □, ^{125}I-labeled urease.

released occurred within 5 min.

To see whether prolonged incubation periods would result in greater release of those markers that were only partially released by streptolysin S in 30 min, marker release from vesicles treated with 300 hemolytic units of streptolysin S was followed for 120 min. The results showed a significant increase in the amount of chymotrypsinogen lost (Fig. 4). The release of ovalbumin also increased, but there was little or no loss of markers ≥ 72 Å in diameter, even after 120 min. With streptolysin O, there was no significant increase in marker release over 120 min (data not shown).

Determination of the size of the membrane lesions

To facilitate the estimation and comparison of the primary size of the membrane lesions produced by the streptococcal toxins, the greatest marker release obtained within 30 min by toxin concentrations as high as 1000 hemolytic units of

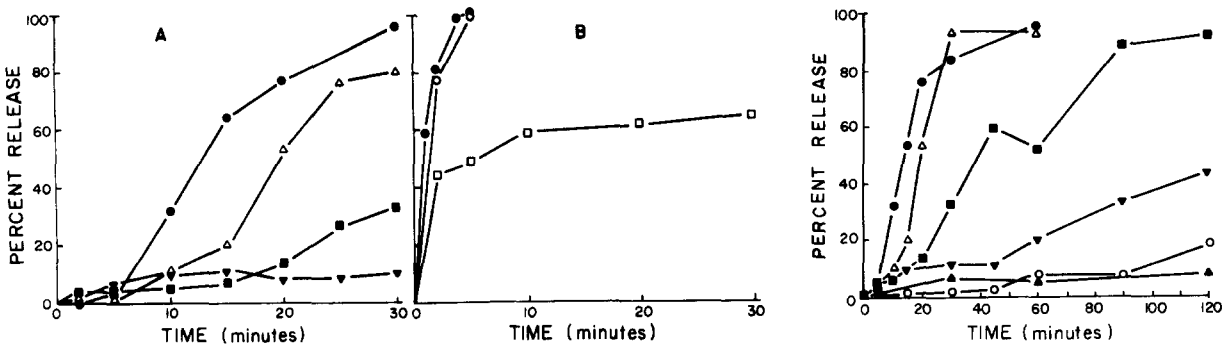

Fig. 3. Kinetics of marker release by the streptolysins. Samples were incubated with 300 hemolytic units streptolysin S (A) or 100 hemolytic units streptolysin O (B) for 0–30 min at 37°C. Total release was determined by Triton X-100 incubation for 30 min. Symbols are as in Figs. 1 and 2.

Fig. 4. Marker release upon extended incubation with streptolysin S. Resealed vesicles containing the indicated markers were incubated with 300 hemolytic units streptolysin S for 0–120 min at 37°C. Total release was determined by detergent treatment for 30–60 min. Control samples were examined in parallel with experimental samples for each time period to monitor the stability of the vesicles under the conditions used in the experiment. Symbols are as in Fig. 1.

streptolysin S or 4000 hemolytic units of streptolysin O was plotted against the diameter of each marker molecule. Fig. 5 illustrates again the heterogeneous nature of the membrane lesions produced by streptolysin S: although all the vesicles contain lesions having a diameter of at least 8.8 Å,

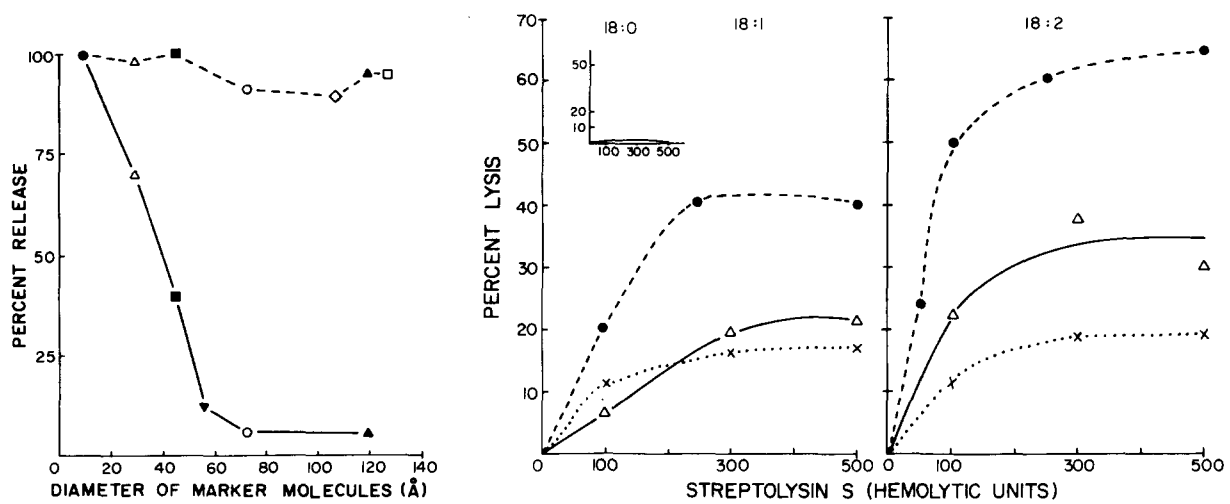

Fig. 5. Summary of marker release by streptolysin S and streptolysin O. The percent total release by 1000 hemolytic units of streptolysin S (dashed line) or up to 4000 hemolytic units of streptolysin O (solid line) was replotted as a function of the diameters of the entrapped markers. Symbols are as in Figs. 1 and 2.

Fig. 6. Release of markers from liposomes by streptolysin S. Liposomes containing the indicated markers were incubated with 0–500 hemolytic units streptolysin S for 30 min at 37°C. The samples were applied to 1 × 10 cm Sepharose 4B columun to separate liberated marker from retained radioactivity. Release was expressed as the percent radioactivity in the second peak of the total entrapped cpm (determined by detergent treatment of the liposomes). All values were corrected for spontaneous release in the absence of toxin. 18:0, distearoylphosphatidylcholine; 18:1, dioleoylphosphatidylcholine; 18:2, dilinoleoylphosphatidylcholine; ●, [^{14}C]sucrose; △ [^{3}H]inulin; ×, ^{125}I-labeled chymotrypsinogen A.

only about 40% have lesions with a diameter of 44.8 Å. and around 10% have lesions 54.2 Å in diameter. In contrast to streptolysin S, the vesicles treated with high concentrations of streptolysin O for 30 min all have lesions large enough to allow the escape of urease, a molecule having a diameter of 128 Å.

Marker release from streptolysin S-treated liposomes

We have previously shown that the sensitivity of large, unilamellar liposomes to streptolysin S (using [^{14}C]sucrose as an internal marker) depends on the fluidity of the liposomal membranes [21]. To examine the nature of the streptolysin S lesions in more detail, liposomes composed of distearoylphosphatidylcholine, dioleoylphosphatidylcholine or dilinoleoylphosphatidylcholine were prepared, using [^{14}C]sucrose, [^{3}H]inulin, or ^{125}I-labeled chymotrypsinogen A as internal markers. After incubation for 30 min with various concentrations of streptolysin S, the amount of marker release from each type of liposome preparation was determined.

The results (Fig. 6) show a significantly greater release of sucrose from dilinoleoylphosphatidylcholine liposomes than from dioleoylphosphatidylcholine liposomes. Virtually no sucrose was released from liposomes composed of distearoylphosphatidylcholine. A similar pattern of release was observed when inulin loss was measured, although the maximum amount of this marker released was considerably less than that observed with sucrose. Similar amounts of chymotrypsinogen were released from dilinoleoylphosphatidylcholine and dioleoylphosphatidylcholine, but less than 20% of the liposomes contained channels large enough to release this marker. There was virtually no release of a larger molecule, ovalbumin, from the liposomes (data not shown). The differences in marker release from dilinoleoylphosphatidylcholine and dioleoylphosphatidylcholine liposomes seem to reflect differences in the proportion of liposomes having lesions of a given diameter, rather than differences in the sizes of the lesions produced in the two types of liposome.

Discussion

The data presented were used to estimate the dimensions and the kinetics of formation of membrane lesions formed by the two hemolysins of *S. pyogenes*, streptolysin S and streptolysin O. Certain assumptions are made in the interpretation of these experiments. That resealed erythrocyte vesicles are adequate models of the natural membrane has been demonstrated [14,23]. It is further assumed that functional 'pore' size can be estimated by passage of molecules of given diffusion radii. Numerous studies have been carried out using this concept [13–17,23,25] and it has provided a direct approach for measuring membrane lesion size. The 'pores' discussed may not be geometrically symmetrical structures in the dynamic membrane. Reference to pores, channels or lesions are meant to indicate functional entities with the potential to allow passage of molecules up to a certain size. Furthermore, repair and resealing phenomena, especially in the case of living systems, would affect the potential of these functional channels. Finally, the complexity of the diffusion process itself must be appreciated. Friction, charge, and polar interactions between solute and solvent as the marker molecules cross the phase boundaries of the membrane are presumed to act similarly in all cases and not to contribute to experimental differences.

Little prior information is available on the size of membrane lesions produced by streptolysin S. The osmotic mechanism of streptolysin S lysis [1,2] would require only a slight increase in membrane permeability to allow ion leakage. Experiments reported here demonstrate that after 30 min, lesions ranging in size from 8.8 to 45 Å but less than 55 Å are formed. The lesions appears to be distributed such that only a portion of the vesicle population contains lesions 30–45 Å in diameter after 30 min of exposure to streptolysin S, while virtually all of the vesicles have lesions at least 8.8 Å in diameter. Enhanced release of chymotrypsinogen A and partial release of ovalbumin in 2 h may reflect the formation of larger lesions in vesicles which originally contained only smaller channels. The preferential release of the smaller markers can be observed in doubly-labeled vesicles. Table I shows the complete release of sucrose with only

partial release of cytochrome *c* from vesicles containing both labels. These results also indicate that escape of smaller markers is not retarded by the presence of larger molecules trapped in the vesicles.

We have previously investigated the characteristics of streptolysin S-mediated release of sucrose from large unilamellar liposomes [21]. Similar experiments here using liposomes labeled with larger internal markers demonstrated that the characteristics of label release from these structures resembled those of release from erythrocyte vesicles (Fig. 6). The proportional decrease in the amount of release of larger markers from dioleoylphosphatidylcholine liposomes compared with dilinoleoylphosphatidylcholine liposomes suggests that the proportion of larger to smaller lesions resulting from toxin action remains relatively constant, even though the number of lesions may be decreased. The maximum lesion size after 30 min is less than 55 Å in liposomes as it is in resealed vesicles.

Kinetics studies showed a lag period for the formation of streptolysin S lesions of 5 to 15 min. This delay in channel formation might be a consequence of the time necessary for the streptolysin S peptides to insert and/or migrate in the membrane to form the functional 'pores'. The longer lag period observed for release of markers with diameters of 29.6–44.8 Å may indicate that additional time is needed for more toxin molecules to aggregate into the larger functional channels. Finally, experiments with 2-h incubation periods revealed the formation of a few functional pores larger than 44.8 Å but less than 72.2 Å in diameter, possibly due to coalescence of smaller lesions. It must be kept in mind that the colloid-osmotic hemolytic process which is initiated by primary lesions in erythrocytes is obviated here by the identical osmotic conditions inside and outside the resealed vesicles.

The size of lesions produced by streptolysin O is dependent on the amount of toxin presented to the membrane. Thus, after treatment with less than 25 hemolytic units of streptolysin O, little or no release of molecules larger than 72.2 Å in diameter (M_r 68000) was observed, but larger channels were formed with increased toxin concentrations. Substantial release of the largest marker molecules studied was seen after treatment of the vesicles with 300–500 hemolytic units of toxin, indicating that functional lesions > 128 Å are present under these conditions. Higher levels of hemolysin (4000 hemolytic units/ml) resulted in even greater release of the largest markers (Fig. 2).

In their studies of [^{3}H]RNA leakage from human fibroblasts, Thelestam and Mollby [25] found that this marker (M_r 200000) was retained after treatment of the cells with 15 hemolytic units of streptolysin O for 30 min, but 6 h of exposure allowed total marker release. Using ^{125}I-labeled urease, we found only a slight increase in tracer release between 30 and 120 min from vesicles treated with 100 hemolytic units of streptolysin O (Fig. 4B). Longer incubation times could not be used because of the increasing instability of the vesicles after 2 h. This discrepancy in the results, undoubtedly reflects differences in the two experimental systems, particularly the greater complexity of membrane structure, intracellular membrane networks, and the possibility for membrane repair found in living, nucleated, eukaryotic cells compared with resealed erythrocyte vesicles.

Experiments here with sucrose-loaded vesicles treated with 25–100 hemolytic units of streptolysin O indicate that small lesions are formed rapidly (1–2 min; Fig. 3B) provided sufficient toxin molecules are present. Comparable release of larger markers takes 5–10 min of exposure to 100 hemolytic units toxin. Since streptolysin O treatment at high concentrations results in total release of all markers, eventual loss of general vesicle membrane integrity cannot be ruled out. Electron microscope studies have revealed extensive damage to erythrocyte ghosts [4,5] and liposomes [5,6,26] after exposure to high levels of streptolysin O.

The passage of labeled molecules of various sizes is a useful way to measure functional channels in membranes. This general approach has been applied to the study of lesions produced by the complement system [14,27–29], staphylococcal α-toxin [13] and aereolysin [15]. These agents form ion-permeable channels as does streptolysin S in a colloid-osmotic hemolytic process and the range of 'pore' sizes for these factors compares with that of the channels formed by streptolysin S. In contrast, streptolysin O (which does not mediate lysis by an osmotic mechanism) produces much larger primary lesions (> 128 Å in diameter). Exposure to high

levels of streptolysin O appears to result in the loss of general membrane structural integrity.

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