Characteristics of Streptolysin O Hemolysis: Kinetics of Hemoglobin and \(^{86}\)Rubidium Release

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The characteristics of hemolysis produced by streptolysin O (SLO) were investigated in rabbit, human, and rat erythrocytes. Kinetic studies of hemoglobin (Hb) release showed that rabbit and human erythrocytes exhibited typical “multi-hit” survival curves. Extrapolation of these curves to the ordinate indicated that approximately two molecules of SLO may be sufficient to produce lysis of a single cell. In contrast, exponential (“single-hit”) survival curves were observed when rat erythrocytes were treated with SLO. At 0 C, high concentrations of SLO rapidly lysed rabbit erythrocytes; low concentrations had no effect on the cells at this temperature. The release of intracellular \(^{86}\)rubidium (\(^{86}\)Rb\(^+\)) and Hb in rabbit erythrocytes exposed to SLO was investigated. In the presence of phosphate-buffered saline, rubidium and Hb were lost at the same rate from toxin-treated cells. The addition of bovine serum albumin to the suspending medium did not retard the escape of Hb, and the efflux of \(^{86}\)Rb only slightly preceded Hb loss. Addition of sucrose to the cells delayed both Hb and rubidium release. These results are interpreted as indicating that the “colloid-osmotic” lytic process is not involved in the hemolysis of erythrocytes by streptolysin O.

Streptolysin O (SLO) is a bacterial toxin produced by most group A streptococci and by a few strains of streptococcal groups C and G. SLO is one of the oxygen-labile cytolytic toxins which are produced by several different gram-positive bacteria and are characterized by their antigenic relatedness and inhibition by cholesterol, as well as by their activation by sulfhydryl-compounds (5). All of these toxins produce hemolysis of erythrocytes in a matter of several minutes, suggesting that their initial effect involves an alteration of the cell membrane. Virtually nothing is known about the nature of this alteration or of the events which culminate in lysis of the cell. Despite the similarities seen in this group of toxins, some experiments suggest that their mode of action on susceptible cells may be different (4).

Electron micrographs of SLO-treated erythrocytes indicate that the toxin produces what have been described as 50-nm “holes” in the cell membrane (6). Complement, and a number of other cytolytic agents, produced somewhat similar lesions on erythrocyte cell membranes. A number of studies in recent years have shown that complement brings about immune hemolysis by a “colloid-osmotic” process (7, 8, 14). Functional holes are produced in the membrane which abolish the ability of the cell to prevent the free exchange of ions; because of the Donnan effect there is a net flow of ions (and consequently water) into the cell. This process leads to swelling and eventual lysis of the cell. If the osmotic pressure of the external medium is increased by the addition of nonpenetrating molecules such as albumin or dextran, swelling is prevented and hemoglobin (Hb) does not escape from the cell. However, the loss of an intracellular electrolyte such as K\(^+\) from these damaged, stabilized erythrocytes can still be observed.

In this paper the kinetics of Hb release from SLO-treated cells were studied and, because of the similarity of the morphological lesions produced by SLO and complement on erythrocyte membranes, the possibility that SLO-induced hemolysis also involves a colloid-osmotic mechanism was examined. The results suggest that the events which lead to hemolysis in SLO-treated cells are fundamentally different.

MATERIALS AND METHODS

SLO preparation (13, 16). SLO was prepared from 14- to 20-h cultures of Streptococcus pyogenes, type 3, Richards strain, grown in brain heart infusion broth (Difco) supplemented with 2% proteose peptone no. 3 (Difco), 1% glucose, and 1% Na\(_2\)HPO\(_4\) (13). After about 12 h of growth, the pH of the culture was maintained between 6.5 and 7.0 by periodically adding NaOH. The cells were removed by centrifugation, ethylenediaminetetraacetic acid was added to a con-
centration of 0.01 M (16), and the supernatant was frozen at −20 C. The material was then placed at 4 C, and the first 30% to thaw was collected and brought to 80% saturation with solid (NH₄)₂SO₄. The precipitate was collected by centrifugation, resuspended in and dialyzed overnight against phosphate-buffered saline (PBS: NaCl, 137 mM; KH₂PO₄, 2 mM; Na₂HPO₄, 8 mM; pH 7.2), and applied to a 2.5 by 4 cm Sephadex G-100 column equilibrated with PBS at 4 C. Fractions (5 ml) containing the highest titters of toxin were pooled. The pooled material was concentrated with coarse Sephadex G-25, and the partially purified toxin was activated by the addition of 0.01 M cysteine, then stored frozen in 1-ml samples at −70 C.

**Erythrocytes.** Rabbit blood was collected from New Zealand White rabbits or purchased locally from a veterinarian. Rat blood was obtained from Lewis inbred rats, and human blood was obtained from local donors. The blood was centrifuged at 2,000 × g, the plasma and Buffy coat were removed by aspiration, and the erythrocytes were washed three times in PBS. After the final wash, the erythrocytes were resuspended in PBS to a concentration of 0.7%.

**Assay of SLO.** Toxin was diluted in PBS containing 0.01 M cysteine and added to tubes containing 2 ml of the 0.7% erythrocyte suspension; the total volume of the mixture was always 4 ml. The tubes were incubated at 37 C, and lysis was measured by determining Hb release colorimetrically at 540 nm in a Coleman model 44 spectrophotometer. Complete hemolysis was determined by including 0.2 ml of 1% saponin in the suspension. One hemolytic unit (HU) is defined as that amount of toxin which produces 50% hemolysis after a 30-min incubation at 37 C.

**Measurement of *rubidium efflux.** Fresh rabbit cells were washed three times in potassium-free PBS buffer (NaCl, 137 mM; Na₂HPO₄, 10 mM; NaH₂PO₄, 3 mM; pH 7.4) and resuspended in potassium-free PBS buffer containing 0.5% glucose (PBSG-K). For each experiment, a 30-ml portion was removed, centrifuged at 2,000 × g for 3 to 5 min, and resuspended in 1.8 ml of PBSG-K. A 0.1- or 0.2-ml sample (100 or 200 μCi) of **Rb**⁺ (New England Nuclear Corp., Boston, Mass.) was added, and the cell suspension was incubated at 37 C for 90 to 150 min. The cells were centrifuged, washed one time in PBSG-K, and resuspended in 30 ml of the same buffer. Two-milliliter portions were distributed to a series of tubes which were incubated at 37 C. At various times, 2-ml samples of SLO diluted in PBSG-K or control solution (PBSG-K and 0.01 M cysteine) were added. At the appropriate time, the tubes were removed and centrifuged for 60 s (120 s in some experiments), and a 0.1-ml sample of each supernatant was quickly removed and placed in scintillation vials. The tubes were then read at 540 nm to determine Hb release. The beta-emitting energy of the **Rb**⁺ samples was counted in a Packard Tri-Carb liquid scintillation spectrometer. The total intracellular amount of Hb and **Rb**⁺ was determined in cell samples lysed by saponin. Control experiments showed that less than 1% of the intracellular **Rb**⁺ leaked out of untreated cells during the 10 to 12 min required to carry out each experiment. Virtually no **Rb**⁺ or Hb appeared in the supernatant once the cells had been pelleted by centrifugation.

**RESULTS**

**Kinetics of SLO hemolysis.** Although several investigators have studied the kinetics of Hb release from SLO-treated cells, this phenomenon was re-examined by using three different cell types, and the results were plotted in the form of survival curves. Cell suspensions were incubated at 37 C and, at various times, a toxin dilution in 37 C PBS was added. At the end of the timed period, all tubes were removed, centrifuged, and the percent hemolysis was determined as described in Materials and Methods. The results (Fig. 1 and 2) are shown as the ratio of unlysed cells (S) at each time period to the number of cells at “zero” time (S₀). Rabbit and human erythrocytes were almost equally susceptible to SLO and both exhibited “multi-hit” survival kinetics. Extrapolation of the linear descending part of such curves to the ordinate provides the value of n, the number of effective units (molecules) required to produce hemolysis of a single cell; for rabbit and human cells this value appears to fall between 1.5 and 2. Rat cells were found to be 2 to 3 times more resistant to SLO than rabbit cells, and the kinetics of hemolysis were characteristic of exponential (“one-hit”) survival curves. The results shown in Fig. 1 and 2 were observed consistently using different erythrocyte and SLO preparations.

The ability of SLO to lyse rabbit erythrocytes at low temperatures was studied in similar experiments carried out in an ice bath at 0 C. At extremely high concentrations of SLO, lysis
toxin concentrations. There was no difference in the rate of loss of \textsuperscript{86}Rb\textsuperscript{+} and Hb under these experimental conditions. If SLO hemolysis involves a colloid-osmotic process, the addition of a large, nonpenetrating molecule to the extracellular medium should retard Hb release, but \textsuperscript{86}Rb\textsuperscript{+} efflux from the damaged cells should not be hindered. When the same experiment was carried out in the presence of 2.5% bovine serum albumin (BSA) in PBSG-K, Hb release was not retarded, and in fact, with 2 HU of toxin, hemolysis occurred much more rapidly. We therefore treated the cells with approximately 0.8 HU of SLO in 2.5% BSA. There was little difference in the rate of efflux of rubidium and Hb from the cells, although \textsuperscript{86}Rb\textsuperscript{+} loss slightly preceded Hb loss (Fig. 5A). A similar experiment was carried out in the presence of 5.0% BSA (Fig. 5B). Hb release was slightly slower in this experiment, but rubidium efflux was also slower than in 2.5% BSA.

Because the addition of even 5.0% BSA failed to significantly retard Hb release, the same type of experiment was carried out in the presence of

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**\textsuperscript{86}Rb\textsuperscript{+} release from SLO-treated cells.** If the lysis of SLO-damaged erythrocytes is the result of a colloid-osmotic process, it should be possible to detect the loss of the intracellular K\textsuperscript{+} at a time when very little Hb has escaped from the cell (7, 14). In these experiments, \textsuperscript{86}Rb\textsuperscript{+} was used to measure electrolyte efflux from toxin-treated cells. Rubidium and potassium are known to be transported and maintained intracellularly in a similar manner by erythrocytes (15, 17). Fresh rabbit cells were washed and labeled, and the experiments were carried out as described in Materials and Methods. The extracellular Hb and \textsuperscript{86}Rb\textsuperscript{+} counts in the control tubes at each sample time were subtracted from the appropriate experimental tubes, and the amount of \textsuperscript{86}Rb\textsuperscript{+} and Hb lost from toxin-treated cells at each time period was determined. The results are plotted in a graph of $1 - y$ versus time, where $y$ is defined as the proportion of zero-time intracellular material released from the cells at the time of sampling (8). Figure 4 shows the results of two separate experiments carried out in PBSG-K buffer with different

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**Fig. 2.** Kinetics of SLO hemolysis of rat erythrocytes. Approximate toxin concentrations: 6 HU, $\bigcirc$; 4 HU, $\bullet$; 2 HU, $\triangle$.

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**Fig. 3.** Kinetics of SLO hemolysis of rabbit erythrocytes at 0 C. Approximate toxin concentrations: 720 HU, $\bigcirc$; 280 HU, $\bullet$; 28 HU, $\triangle$. 
1-min experimental and control tubes, the cells were resuspended in 4 ml of PBSG-K, and Hb release was determined after incubation at 37 C for 10 min. The amount of hemolysis in the absence of sucrose for cells treated with 1.6 and 2.4 HU was 47 and 79%, respectively, indicating that sucrose had not inhibited toxin adsorption.

**DISCUSSION**

The results reported here show that the hemolysis of rabbit and human erythrocytes treated with SLO exhibit typical multi-hit survival kinetics. This observation suggests that the lysis of a single erythrocyte results from the cumulative action of more than one toxin molecule. When survival curves are plotted on a log scale, as has been done here, extrapolation of the descending part of the curve to the ordinate provides an estimate of the value of $n$, the number of molecules required to produce an effect (3). The curves depicted in Fig. 1 suggest that $n$ corresponds to between 1.5 and 2 for rabbit and human erythrocytes (the curves for higher SLO concentrations probably allow a more accurate estimate; SLO activity is progressively lost at 37 C). In contrast to the multi-hit curves obtained for rabbit and human erythrocyte hemolysis, rat erythrocytes exhibited exponential kinetics. These single-hit curves are obtained when a straight line with an initial slope proportional to the concentration describes the log proportion of surviving cells; according to classical target theory, this is

0.3 M sucrose. Sucrose has been shown to retard hemolysis, even when added after the adsorption of SLO to the cell (1; unpublished data). Labeled cells and toxin were suspended in PBSG-K containing 0.3 M sucrose, and the experiments were carried out by using approximately 2.4 and 1.6 HU of SLO (Fig. 6). Sucrose retarded Hb release in these experiments, but again there was little difference in the rate of loss of Hb and rubidium from the cells. To show that the retardation of hemolysis in the presence of sucrose was not simply due to slower adsorption of SLO molecules under these conditions, the supernatant was removed from the

![Fig. 4. Release of $^{86}$Rb$^+$ (open figures) and Hb (closed figures) from toxin-treated rabbit erythrocytes suspended in PBS. Approximate toxin concentrations: 2 HU, O, ●; 1.6 HU, △, ■.](image)

![Fig. 5. Release of $^{86}$Rb$^+$ (open figures) and Hb (closed figures) from rabbit erythrocytes exposed to 0.8 HU of toxin. (A) 2.5% BSA added to suspending medium. (B) 5.0% BSA added to suspending medium.](image)

![Fig. 6. Release of $^{86}$Rb$^+$ (open figures) and Hb (closed figures) from toxin-treated rabbit erythrocytes suspended in 0.3 M sucrose. Approximate toxin concentrations: 2.4 HU, O, ●; 1.6 HU, △, ■.](image)
interpreted as indicating that one SLO molecule is sufficient to lyse a single cell (12).

An alternative explanation for the nature of survival curves is based in part on the supposition that no fixed number of lesions ("hits") are necessary to kill a cell, but that any lesion has a certain probability of being lethal (10). According to this hypothesis, the shoulder seen in multi-hit curves is a reflection of repair processes available to the cell and of the accumulation of sublethal damage by the cell. When the number of hits exceeds the capacity of the repair system to cope with the lesions, the survival probability decreases exponentially. Similarly, an exponential (one-hit) survival curve would be obtained if no mechanism existed to repair the initial hits. Thus, one could propose that some process exists in rabbit and human cells whereby initial membrane lesions produced by SLO can be repaired; rat erythrocytes would be unable to repair this damage.

Alouf and Raynaud (2) calculated that 360 molecules of SLO were required to lyse a single erythrocyte, by simply determining the amount of protein in a SLO preparation which lysed 50% of the cells in a given erythrocyte suspension. This number must surely be incorrect, since it requires among other assumptions (i) that all the protein in the toxin preparation be active SLO molecules, (ii) that all toxin adsorption sites on the surface of a red cell be functional sites, and (iii) that all the toxin molecules in their system adsorb only to the cells which subsequently lyse. The theoretical determination reported here requires none of these assumptions.

It is also of interest that, although they exhibited single-hit kinetics, rat cells were more resistant to SLO than were rabbit or human erythrocytes, a result in agreement with the findings of Howard and Wallace (9). This difference in susceptibility could be a reflection of the affinity of SLO for membrane receptor sites on the different cell types. Alternatively, rat cells might contain a large number of nonfunctional receptor sites to which SLO could attach without producing any further effect.

The results showing that very high concentrations of SLO can produce hemolysis even at 0 C supports a similar observation made by Alouf and Raynaud (2). Although toxin concentrations in the range of 300 HU produced rapid hemolysis, cells treated with 28 HU of SLO were unaffected, even after several hours of incubation. At this temperature, a high threshold number of SLO "hits" may have to occur before any effect on the cell is seen.

The kinetics of **Rb** release closely paralleled Hb release from SLO-treated cells, suggesting that the colloid-osmotic process is not involved in hemolysis by this toxin. Madoff et al. (11) studied the hemolysis of rabbit erythrocytes by staphylococcal alpha-toxin, an oxygen-stable toxin unrelated to SLO. They found that even when the cells were suspended only in PBS, 50 to 75% of the intracellular K+ was lost from the toxin-treated cells during a prelytic period when no loss of Hb could be detected. When the experiment was carried out in the presence of sucrose or polyethylene glycol, Hb release was markedly inhibited (no loss for at least 10 min), but K+ efflux again proceeded at a rapid rate and was virtually complete after 5 min. These findings are compatible with the colloid-osmotic hemolytic process and suggest that staphylococcal alpha-toxin produces functional membrane "holes" with an effective radius less than 0.44 nm (from Table III of reference 14).

Hingson et al. (8) studied the kinetics of release of **Rb** from sheep erythrocytes treated with rabbit antibody and guinea pig complement in the presence of 2.5% BSA. They found that 75 to 80% of the intracellular rubidium was released into the suspending medium during a prelytic phase in which Hb remained within the cell. Sears et al. (14) examined immune hemolysis using human erythrocytes and complement and rabbit antihuman erythrocyte serum. When 3.9 mM BSA was added to the medium, only 19% of the Hb, but 91% of the cell potassium, was lost during a 2-h incubation period. The same cells, diluted 50-fold in isotonic saline showed 84% lysis. These investigators also examined the protection given by adding dextran fractions of various molecular weights to the cells. They found that the membrane defect produced by complement had approximately the same dimensions as 20,000 molecular weight dextran and estimated that the functional holes produced in the human erythrocytes had an effective radius of 3.2 nm.

Extracellular BSA at concentrations of 2.5 and 5.0% did not retard Hb release from SLO-treated cells, and, in one experiment (not shown) carried out in 20% BSA, 70% of the cell Hb was lost after 8 min. These results could be explained if the membrane lesions produced by SLO have an effective diffusion radius greater than that of Hb (3.25 nm) or albumin (3.55 nm) molecules. Thus, the addition of albumin to the suspending medium would not prevent the efflux of Hb from the cells, and Hb and **Rb** would be expected to escape at about the same rate. This explanation would not account for the stabilizing effect of sucrose, in which the loss of both Hb and rubidium from the cell was retarded. SLO might produce damage which
weakens large areas of the membrane, and the presence of high sucrose concentrations in the suspending medium might prevent the breaking away of these areas from the rest of the cell. Whatever the interpretation, it appears that the colloid-osmotic process does not play a role in the lysis of erythrocytes by SLO, a result in agreement with an observation made some years ago by Bernheimer (4) that SLO-treated erythrocytes did not undergo swelling prior to their disruption.

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LITERATURE CITED