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Effects of Temperature, Time, and Toxin Concentration on Lesion Formation by the *Escherichia coli* Hemolysin

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We performed osmotic protection experiments to test the hypothesis that the *Escherichia coli* hemolysin forms a discrete-size pore in erythrocyte membranes. The effects of toxin concentration, assay time, temperature, and protectant concentrations were examined. The results we present here raise doubts about the existing model of pore formation by hemolysin. We demonstrate that osmotic protection by various sugars of different sizes is a function of hemolysin concentration and assay time. The data indicate that under various conditions, lesion sizes with a diameter ranging from <0.6 to >1.2 nm can be inferred. Quantification of hemolysin permitted the estimation of the number of HlyA structural protein molecules required per erythrocyte for lysis in the presence of each protectant. It appears that hemolysin induces heterogenous erythrocyte lesions which increase in size over time. Influx experiments utilizing radioactive sugar markers indicated that time-dependent osmotic protection patterns are independent of the diffusion rates of individual protectants. We demonstrate that the rate of the putative growth in the size of hemolysin-mediated lesions is temperature dependent. The erythrocyte membrane lesions formed at 37°C can be stabilized in size when shifted to 4°C. On the basis of these data, new models for the nature of the hemolysin-mediated erythrocyte membrane lesions are presented.

The *Escherichia coli* hemolysin is the best-characterized member of a family of widely disseminated cytolytic toxins known as the RTX toxins, named for a series of tandem repeats in their structural proteins. Other members of this family include the *Pasteurella haemolytica* leukotoxin, exotoxins from *Actinobacillus pleuropneumoniae*, the *Actinobacillus actinomycetemcomitans* leukotoxin, hemolysins from *Proteus vulgaris* and *Morganella morganii*, and the *Bordetella pertussis* adenylate cyclase/hemolysin. The RTX toxins exhibit a wide range of target cell specificities (11, 46). Pore formation is postulated to be the mechanism of cytolytic activity for these toxins (6).

The evidence for pore formation by these toxins comes primarily from osmotic protection experiments. The theoretical premise for osmotic protection is that solutes with molecular diameters larger than the size of a pore formed in the target cell membrane can prevent target cell lysis. Smaller solute molecules can pass through pores, resulting in an osmotic gradient; this is followed by influx of water into cells, cell swelling, and colloid osmotic lysis. In osmotic protection experiments, the transition from a state of little protection against toxin-induced lysis by a small solute to full protection by a larger solute is used to define discrete, finite pore sizes lying between the diameters of the two solutes. These discrete-sized pores are presumed to be protein-lined hollow structures formed by one or more molecules of the particular pore-forming agent.

An earlier report of osmotic protection of erythrocytes by dextran 4 yielded a predicted pore diameter of 2 to 3 nm induced by the *E. coli* hemolysin (6). Different pore sizes were obtained for other RTX toxins. The *P. haemolytica* leukotoxin and the *A. actinomycetemcomitans* leukotoxin were estimated to form 0.9-nm pores protected by sucrose in cell swelling, lactate dehydrogenase leakage, and chromium release assay systems (10, 24). The *M. morganii* hemolysin, *A. pleuropneumoniae* hemolysin, and *B. pertussis* adenylate cyclase/hemolysin pore sizes of 1.5 to 3 nm, 2 nm, and 0.6 nm, respectively, were determined by osmotic protection of erythrocytes (16, 17, 27).

Other experiments that support the formation of a static-size pore by hemolysin involve the use of planar lipid bilayers and small unilamellar lipid vesicles. These experiments yielded conflicting results (4, 5, 27, 30, 31, 39). One group of investigators found a linear relationship between membrane conductance and hemolysin concentration and concluded that the toxin acts as a monomer (30, 31), while another group found a nonlinear, biphasic relationship that suggests cooperativity and oligomer involvement (4, 5). The susceptibility of pure lipid membranes to channel formation by hemolysin, the lifetime of the channel and its stability, the dependence of single-channel conductance on salt concentration, and the predicted pore size (1 versus 2 nm) are other areas of disagreement among the investigators performing bilayer studies (5, 31).

Data presented here, from experiments utilizing the *E. coli* hemolysin as a model for the RTX toxin family, indicate that formation of erythrocyte lesions by RTX toxins is possibly a more complicated event than the creation of a simple, static, protein-lined pore. Our results indicate that osmotic protection experiments as a means of lesion size determination are sensitive to factors of time, concentration, and temperature. These variables were not considered in previous studies of the RTX toxins. Our data suggest that hemolysin creates a lesion with a very small initial size which increases in apparent diameter over time.

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MATERIALS AND METHODS

Bacterial strains. Strains used as source of hemolysin in most experiments were WAM589 and WAM582 (harboring

TABLE 1. Osmotic protectants used

Osmotic protectant	Mol wt	Molecular radius/ diam (nm)
Arabinose (monosaccharide)	150.4	0.62
Inositol (monosaccharide)	180.2	0.72
Cellobiose (disaccharide)	342.3	0.92
Sucrose (disaccharide)	342.3	0.9
Melezitose (trisaccharide)	504.4	1.14
Raffinose (trisaccharide)	594.5	1.20
Dextran 4[a]	4,000–6,000	3.50(?)

[a] See text for discussion of dextran 4.

the *hly* operon cloned in pUC19 [pWAM04] and pACYC184 [pSF4000], respectively) (47). WAM839, a human clinical isolate with a single chromosomal copy of the *hly* operon, was the gift of D. Low. WAM1824, a high-hemolysin-producing strain used in influx experiments, is pSF4000 in the JM15 background. Other strains used as sources of hemolysin included WAM238, WAM664, WAM665, and WAM742. WAM238 (P49) is a hemolytic isolate of porcine origin kindly provided by H. W. Smith. WAM664 and WAM665 (harboring, respectively, pSU233 and pSU316) were gifts from F. de la Cruz (14, 15). They produce plasmid-mediated hemolysins from pig and human isolates, respectively. WAM742, harboring the hemolysin recombinant pANN202-312, was a gift from Werner Goebel (22). pANN202-312 encodes a plasmid-mediated hemolysin originally isolated from a mouse fecal strain of *E. coli*. Strain WAM1127 secretes a hybrid toxin containing the 383 N-terminal amino acids of *P. haemolytica* leukotoxin structural protein (LktA) and the 632 C-terminal amino acids of HlyA (19). The hybrid gene was constructed in pUC18, and the matching *hlyA* clone is WAM1015 (19). Both strains are transcomplemented with *hlyCBD* for activation and secretion of the toxin. The strain used as a negative control in influx experiments was WAM675 (pUC19 in the DH1 background).

Preparation of hemolysin. Bacteria were grown at 37°C in Luria broth supplemented with appropriate antibiotics (100 μg of ampicillin per ml and 20 μg of chloramphenicol per ml). Culture supernatants were used as the source of toxin in all experiments and were harvested by centrifugation and filtration (0.45-μm Acrodisc [Gelman Sciences]). Further dilutions of toxin (supernatants) were made in Luria broth. For some influx experiments, a polyethylene glycol (PEG)-precipitated hemolysin preparation was made as described previously (6). Culture supernatant from WAM589 grown to an A_{600} of 0.8 was made 3% in glycerol (30 ml of 100% glycerol per liter of supernatant) and precipitated by addition of 200 g of PEG 3350 (Sigma Chemical Co.) per liter of supernatant. The solution was stirred for 1 h at 4°C. The PEG precipitate was collected by centrifugation and resuspended in 10 ml of saline per liter of culture supernatant precipitated. Aliquots were frozen at −70°C.

Sugar solutions. All sugars used as osmotic protectants were purchased from Sigma Chemical Co., except dextran 4 (Serva Chemicals). Sugar solutions used in most assays were 290 mM and were supplemented with 10 mM $CaCl_2$, except dextran 4, which was 30 mM in 90 mM NaCl–45 mM KCl–12.5 mM phosphate buffer (pH 7.2). For comparative experiments, all other sugars were also 30 mM in the salt solution. Table 1 lists the molecular weights and molecular diameters of the osmotic protectants used.

HlyA quantification. HlyA present in culture supernatants was precipitated by 10% trichloroacetic acid at 4°C for 1 h and then subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis as described previously (45). Bio-Rad high-range molecular weight standards (Bio-Rad, Richmond, Calif.) were used in serial dilutions on each gel. The protein bands were visualized by Coomassie blue staining. Densitometry tracing of the β-galactosidase (116-kDa) band was used to construct standard curves to which densitometry readings for HlyA bands (110 kDa) were fitted.

Determination of HU for influx experiments. Hemolytic units (HU) were defined and assessed exactly as described by Bhakdi et al. (6). Briefly, 50-μl samples of toxin were serially diluted twofold in saline in microtiter plates. A 50-μl volume of a 2.5% sheep erythrocyte suspension was added to each well, and titers were read visually after 60 min at 37°C. The HU were defined as the last dilution resulting in greater than 90% hemolysis.

Hemolysis assays and osmotic protection experiments. Hemolysis assays were set up with 800 μl of a 2.5% sheep erythrocyte suspension (approximately 5×10^8 cells) in an appropriate assay solution (0.85% NaCl or a sugar solution). Two hundred microliters of toxin was added to the erythrocytes for a total volume of 1 ml. Assays were performed at various temperatures and incubation times, as stated in Results. The intact erythrocytes were removed by centrifugation (8,000 × *g*, 1 min), and the amount of lysis was measured by quantification of hemoglobin release as determined by A_{540}. Results were expressed as percentages of 100% hemoglobin release obtained by lysing 200 μl of 10% sheep erythrocyte suspensions in 800 μl of water. Lysates with absorbance readings of >0.850 were diluted 1:10 and used to fit to a linear plot of absorbance readings. For experiments involving the binding of hemolysin to erythrocytes with the subsequent removal of unbound toxin, assays were set up as described above at 0 to 4°C, and mixtures were incubated for various periods of time. Unbound toxin was separated from the toxin-bound erythrocytes by centrifugation (8,000 × *g*, 4°C, 5 s), and the hemolysin-bound erythrocytes were resuspended in fresh solutions. Experiments involving the exchange of sugar solutions were performed similarly.

Influx experiments. Experiments were performed by methods previously described by Bhakdi et al. (6), which were based on modifications to the protocol of Jorgensen et al. (25). Sheep erythrocyte suspensions (33%) were prepared in 30 mM dextran 4 buffer. The radioactive markers [^{3}H]inositol (Amersham), [^{3}H]sucrose (New England Nuclear, Boston, Mass.), and [^{3}H]raffinose (New England Nuclear) were added to individual suspensions to give 5×10^5 to 2×10^6 cpm of radioactivity per ml of supernatant. Sheep erythrocytes were equilibrated with the radioactive marker at 37°C prior to addition of toxin. For each experiment and each protectant, three concentrations of hemolysin (128, 10, and 5 HU/ml) were tested. The negative control was PEG-precipitated WAM675 culture supernatant. Following addition of toxin, duplicate samples were removed at 1, 5, 10, 15, and 25 min and centrifuged, and radioactivity in the supernatants was determined. Results were expressed as percent change in the concentration of marker in supernatants (6).

RESULTS

Osmotic protection is a function of hemolysin concentration. The prevalent molecular model of hemolysin cytolysis involves formation of a protein-lined, aqueous pore by insertion of an HlyA monomer into the target cell membrane and disruption of normal membrane function (6). The model is principally based on osmotic protection experiments in which 30 mM raffinose affords no protection against hemolysin-

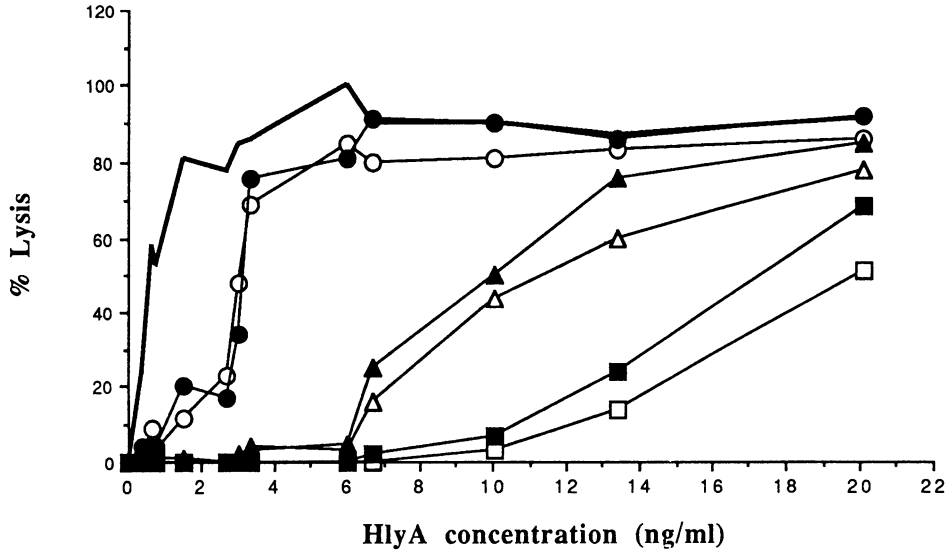

FIG. 1. Osmotic protection experiments and toxin concentration dependence. Hemolysis assays were performed with each protectant with various dilutions of WAM589 culture supernatant for 20 min at 37°C. All sugar solutions were used at 290 mM with 10 mM $CaCl_2$. The reported HlyA concentrations are for 1-ml hemolysis assay mixtures. Symbols: ——, saline; ○, arabinose; ●, inositol; △, cellobiose; ▲, sucrose; □, melezitose; ■, raffinose.

induced lysis in a 30-min hemolysis assay, whereas dextran 4, a larger molecule, prevents lysis (6). It was predicted that hemolysin forms a discrete pore with a diameter larger than the molecular diameter of raffinose (1.2 to 1.4 nm) but smaller than that of dextran 4 (2 to 3 nm). We tested this model by performing hemolysis assays with a range of toxin concentrations in 290 mM osmotic protectant solutions. Table 1 lists the osmotic protectants used in our experiments. We chose the 290 mM concentration of protectant as the osmotic equivalent of saline (0.85% NaCl = 145 mM NaCl). We obtained results different from those previously reported when we utilized this protectant concentration over a range of toxin concentrations. At lower HlyA concentrations (<30 ng/ml in culture supernatants), erythrocytes were protected from lysis with disaccharides and trisaccharides, while lysis remained close to 100% in saline- and monosaccharide-containing assay mixtures. Figure 1 shows the results of a sample protection experiment. In Fig. 1, we compare the patterns of protection afforded by arabinose and inositol (monosaccharides), cellobiose and sucrose (disaccharides), and melezitose and raffinose (trisaccharides) over a wide range of HlyA concentrations during a 20-min incubation. Erythrocytes are protected against lysis by all sugars except the monosaccharides at toxin concentrations of up to 6 ng/ml of assay mixture. At higher concentrations of toxin, the erythrocytes are no longer protected by any sugars. Concentrations of >40 ng/ml of assay mixture give 100% lysis for all sugars. Although Fig. 1 is representative, there were occasional differences in the behaviors of two disaccharides or two trisaccharides or in the calculated HlyA concentration at which lysis with disaccharides or trisaccharides began.

The quantification of HlyA used in these experiments correlates protection patterns to hemolysin protein concentrations. In Table 2 we summarize the approximate ranges of HlyA concentration necessary for lysis to begin in the presence of the different osmotic protectants. The lower value of each range represents the highest mean toxin concentration that gave full protection (<5% lysis), and the higher value is the lowest toxin concentration at which >25% lysis occurred (in a 20-min assay at 37°C). It should be noted that even the lowest HlyA concentrations used in these experimental conditions led to lysis in saline.

The approximate average number of input HlyA molecules required per erythrocyte for lysis in a 20-min assay at 37°C with each individual sugar solution can be calculated. The molecular size of monomeric HlyA is 110 kDa, and the 2% erythrocyte suspensions have an average of 5×10^8 cells per ml. In the case of the monosaccharides, we determined that the required range of 2 to 4 ng of HlyA per 1-ml assay mixture is equivalent to 20 to 40 input HlyA molecules per erythrocyte. For disaccharides, >25% lysis begins with approximately 100 to 150 molecules of input toxin present for each erythrocyte, while raffinose and melezitose require 200 to 300 HlyA monomers.

The osmotic protection by disaccharides and trisaccharides detected in these experiments is in contrast to data that indicated the lesion formed by hemolysin to be a discrete pore approximately 3 nm in diameter (6). To examine this incongruity, we performed parallel experiments using 30 and 290

TABLE 2. Comparison of ranges of HlyA concentrations required for lysis with different osmotic protectants

Osmotic protectant or control	HlyA concn range for >25% lysis (ng/ml)[a]
Arabinose	2–4
Inositol	2–4
Cellobiose	10–14
Sucrose	10–14
Melezitose	20–25
Raffinose	20–25
Saline	<1

[a] HlyA concentrations calculated by densitometry of Coomassie blue-stained gels electrophoresed with various dilutions of trichloroacetic acid-precipitated supernatant samples and fitted to the standard's densitometry curves. The values are for 1-ml assay mixtures in which 200 μl of undiluted supernatant was used and were calculated as one-fifth of the HlyA concentration (nanograms per milliliter) in the original culture supernatants. The assay time was 20 min.

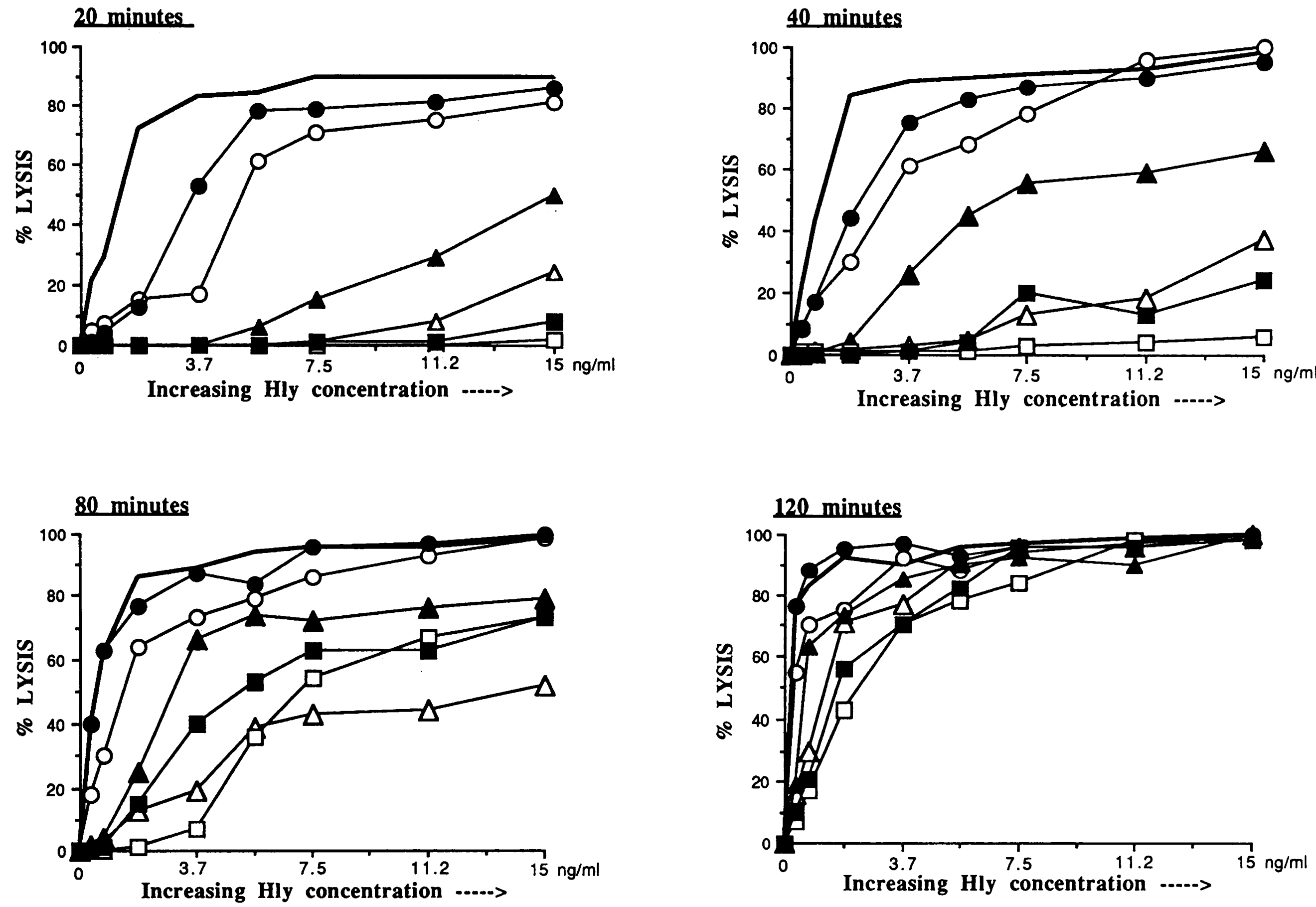

FIG. 2. Osmotic protection as a function of time. Hemolysis assays were performed with various dilutions of supernatant containing 0.075 mg of HlyA per ml for 20, 40, 80, and 120 min. Each assay included multiple erythrocyte controls without toxin to ensure that hemolysis over time was not due to autolysis. The sugars used were arabinose (○), inositol (●), cellobiose (△), sucrose (▲), melezitose (□), and raffinose (■). Assays performed in saline are represented by the thicker line. The HlyA concentrations reported are for 1-ml hemolysis assay mixtures. All sugar solutions were 290 mM with 10 mM $CaCl_2$.

mM sugar solutions at different toxin concentrations. At toxin concentrations for which 290 mM disaccharides and trisaccharides protected against osmotic lysis, corresponding 30 mM sugar solutions did not. However, at lower toxin concentrations, the differential patterns of protection can be established with 30 mM sugar solutions, but with less-definite transitions in protection than those seen in Fig. 1 (data not shown).

Osmotic protection is a function of time. We investigated osmotic protection over a range of toxin concentrations as a function of time. Figure 2 shows results from a sample experiment. A set concentration of toxin against which a given sugar protected the erythrocytes in a 20-min assay gave 100% lysis in that sugar solution at a later time point. At 20 min, raffinose and melezitose protect against hemolysis at all toxin concentrations used, while lysis has begun in the disaccharide assays at higher toxin concentrations. Arabinose and inositol assays show lysis with most concentrations of toxin, with 100% hemolysis at higher toxin concentrations. Trisaccharides no longer protect against lysis at most toxin concentrations by 80 min, and higher hemolysis levels also occur in disaccharide assays. All the lysis curves have approached that of saline at 120 min, and the toxin level required for >25% lysis is reduced nearly 20-fold.

Hemolysins from different sources show similar concentration- and time-dependent protection patterns. The previous work on pore formation by plasmid- and chromosomally encoded alleles of *hlyA* which yielded dissimilar lesion size estimations (4) prompted us to investigate the osmotic protection patterns for various sources of plasmid- and chromosomally encoded hemolysins from pig, mouse, and human *E. coli* isolates. All toxin preparations, when normalized to similar HlyA concentrations, showed identical concentration- and time-dependent protection patterns (data not shown).

Osmotic protection and temperature. We conducted experiments to help distinguish among three hypotheses on the formation and structure of the apparent variable-sized hemolysin-induced erythrocyte lesions. The first hypothesis is that the presumed increase in lesion size could be related to progressive aggregation or oligomerization of toxin monomers, in a toxin concentration- and time-dependent manner. The second possible explanation is that insertion of hemolysin could lead to neighboring lipid perturbations in the bilayer that alter membrane permeability and lead to colloid osmotic lysis. These lipid perturbations would increase in size and number with increased hemolysin concentration and incubation time. Finally, our experimental results may be correlated to differential diffusion rates of each protectant through a static-size hemolysin-mediated lesion.

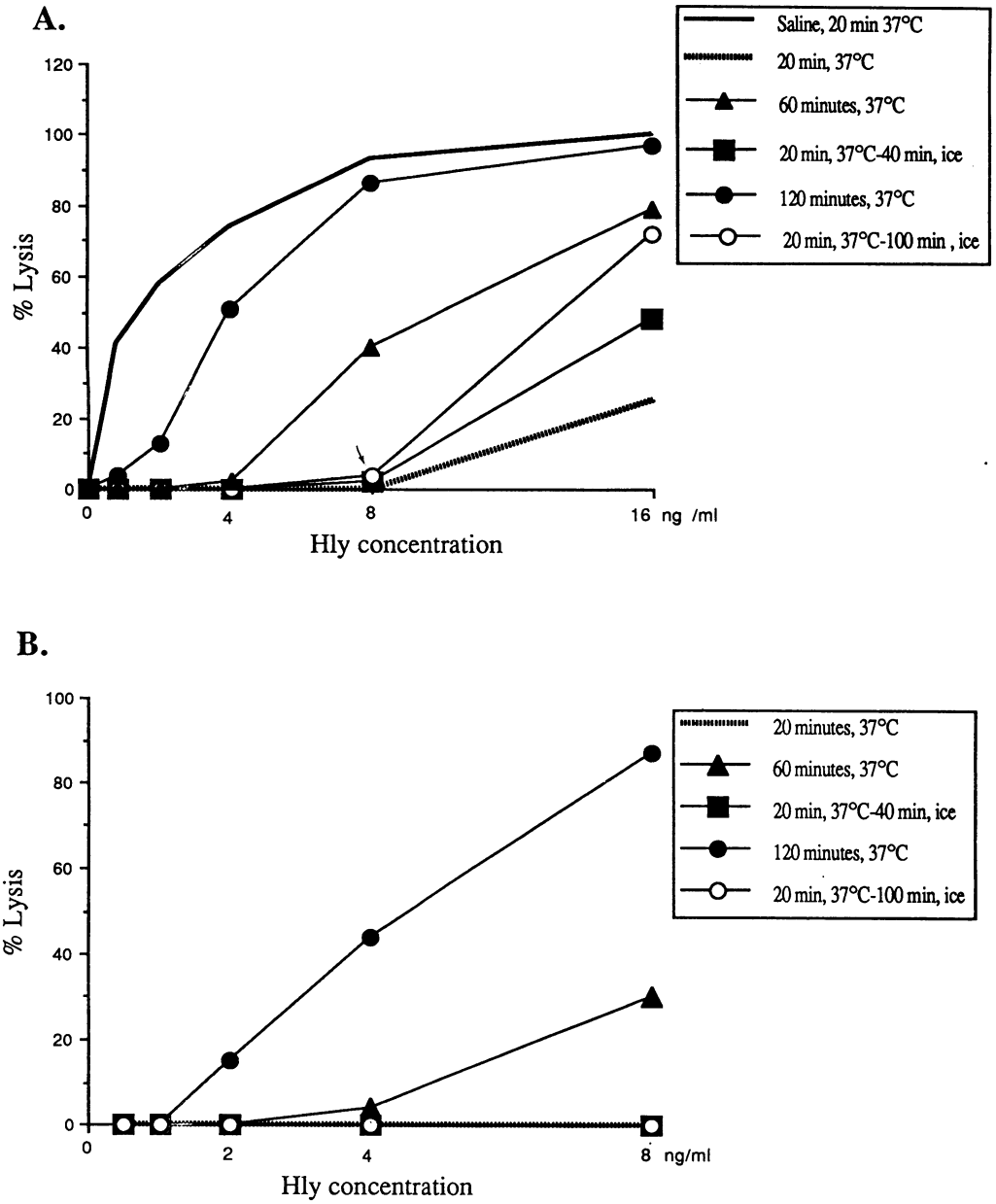

FIG. 3. Temperature shift experiments. In these representative experiments a fixed number of hemolysin molecules were bound to erythrocytes, and toxin-treated cells were incubated at 37°C for 20 min prior to being shifted to 4°C for 40 or 100 min. (A) Representative graph from experiments in which melezitose was the protective agent. The arrow indicates the concentration at which all dilutions were still protected prior to the temperature shift to 4°C. (B) A similar experiment, performed with raffinose, with lower toxin concentrations, such that all dilutions can be protected upon the shift to ice. In all experiments toxin was prebound to erythrocytes on ice for 30 min, and excess toxin was removed by centrifugation prior to the indicated incubations. HlyA concentrations reported are for input toxin in 1-ml assay mixtures.

Experiments were performed in which a fixed number of hemolysin molecules were bound to erythrocytes at 0 to 4°C and the toxin-treated cells were shifted to 37°C for set periods of time to initiate the lesion-forming process and then shifted to 0 to 4°C before any lysis had occurred. Presumably, the shift in temperature to 0 to 4°C would significantly affect potential toxin multimerization in or on membranes, as well as the putative neighboring lipid perturbations, but not the diffusion rate of sugars through a discrete, static, protein-lined hollow pore. To eliminate the variable of the reduced incubation temperature (on ice) slowing down the rate of toxin interaction with erythrocytes simply by reducing Brownian motion, we bound toxin molecules to erythrocytes on ice for 30 min prior to beginning each assay. Hemolysin can associate with erythrocytes on ice without causing lysis, and excess unbound toxin can be removed by centrifugation of toxin-bound erythrocytes (5 s, 8,000 × *g*, 4°C) followed by resuspension in the appropriate sugar solution. The dissociation rate of bound toxin is negligible (data not shown). Figure 3 shows results from a sample temperature shift experiment. After their formation at 37°C, hemolysin-induced lesions could be stabilized in size by shifting assay mixtures to ice. Over time, erythrocytes with

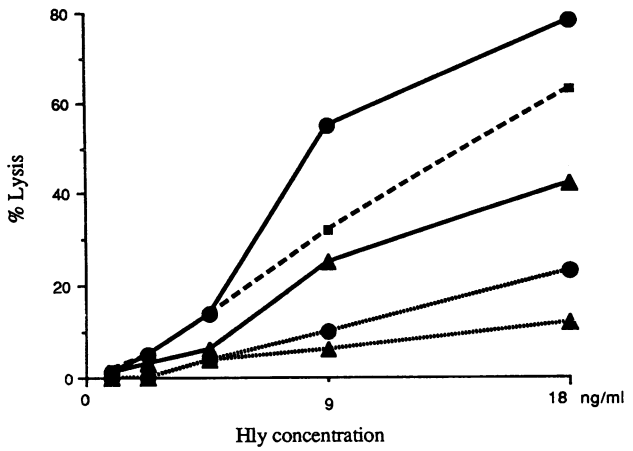

FIG. 4. Representative experiment with a temperature shift to 22°C in which cellobiose was the protective agent. Toxin was prebound to erythrocytes on ice for 30 min, and excess toxin was removed by centrifugation prior to the indicated incubations. Assays were set up for 15- and 30-min incubation periods at 22 and 37°C. The temperature shift assay included an initial 15 min at 37°C followed by 15 min at 22°C. ●, 30-min incubations; ▲, 15-min incubations; . . . . ., incubation at 22°C; ———, incubation at 37°C; – – –, temperature shift assay results. HlyA concentrations reported are for input toxin in 1-ml assay mixtures.

lesions stabilized in this manner were protected by sugars larger than their "frozen" lesions, while parallel assay mixtures at 37°C approached 100% lysis in similar-size protectants. The crucial factor in these experiments is that the lesion at the time of the temperature shift be smaller than the protectant used, and no lysis should occur. If lysis has initiated at the time of the temperature shift, then, over time on ice, the few lesions larger than the protectant would result in further lysis. This was observed at the highest concentration of hemolysin used for Fig. 3A, in which lysis increases with time even at 4°C but at a much slower rate than at 37°C. For a similar assay with cellobiose, for example, stabilization of the lesion size is possible only at toxin concentrations lower than those required for lysis in the presence of trisaccharides. At the higher concentrations appropriate for lysis with melezitose and raffinose, some lesions will be large enough to allow cellobiose influx.

Assays were also set up with an initial 15-min incubation at 37°C, 30 to 45 s of rapid temperature reduction on ice, and a second 15-min incubation at 22°C. Parallel protection assays were performed at 22°C for 15 and 30 min as well as at 37°C for 15 and 30 min. These times are representative of those used in other experiments performed with multiple time points that were chosen so that maximal lysis did not occur and differential protection could still be observed. In Fig. 4, results of an assay with cellobiose show the dependence of presumed lesion growth on temperature. Lysis occured less at 22°C than at 37°C, over ranges of concentration and time. Assays in which an incubation period was divided between two temperatures showed lysis patterns between the patterns for each individual temperature.

Influx experiments. To determine whether our observations could be explained by differential diffusion of osmotic protectants, the comparative rates of diffusion of mono-, di-, and trisaccharides were measured by performing radioactive marker influx experiments. We performed experiments as described previously (6), including concentrations below 100 HU as well as a negative control (no hemolysin). A concentration of 128 HU is roughly equivalent to 100 ng of HlyA per ml or 60 HlyA molecules per erythrocyte. The concentration of 128 HU was adequate for 100% lysis of a 33% erythrocyte suspension, in saline, over 20 min, while concentrations of 10 HU can give up to 35% lysis. Results from repeated influx experiments indicate that no statistically significant difference in diffusion patterns of inositol, sucrose, and raffinose can be distinguished. Standard deviations for duplicate samplings pooled from six independent experiments are within a range which includes no change in marker concentration in all samplings at each toxin concentration, and at each time point, for all three sugars. Figure 5 summarizes the results of six influx experiments and clearly demonstrates the range of variation in pooling independent influx experiments.

Sugar switch experiments. In order to further test the prevailing molecular model of hemolysin membrane pores, we postulated that a discrete uniformly sized hemolysin pore would impose differential diffusion rates on sugars based on their hydrated sizes in relation to the pore size. A dynamic lesion, however, would dictate a dynamic diffusion rate. We tested our hypotheses with the following experiment. A protection assay was set up in which erythrocytes harboring a set number of pores of presumed identical size were established in the presence of a trisaccharide over a time period when lysis had yet to occur. The trisaccharide was then substituted by a monosaccharide, and the time needed to observe a certain lysis pattern was compared with the time needed to achieve a similar lysis pattern with monosaccharides alone. The experiments were performed to investigate whether the incubation time during which toxin-treated erythrocytes are protected from lysis can affect the rate of lysis of these erythrocytes in a subsequent incubation with a monosaccharide.

Figure 6 demonstrates results from two sugar switch experiments representative of eight similar experiments. At the switch time (40 or 60 min), HlyA-associated erythrocytes were pelleted by centrifugation (5 s), and the trisaccharide solution was removed and replaced by an equal volume of monosaccharide solution. These assay mixtures were incubated for additional amounts of time with the monosaccharide (20 and 70 min in the experiments shown in Fig. 6). Standard monosaccharide assays were performed for periods equivalent to the total and postswitch assay times in the switch experiments (80 and 20 min, respectively, for Fig. 6A and 110 and 70 min, respectively, for Fig. 6B). The results of these experiments indicate that the protection patterns take into account the trisaccharide incubation period and do not resemble the protection patterns of the shorter inositol-only assays. This indicates that events during the raffinose protection period subsequently alter the rate of lysis in inositol.

Osmotic protection with hybrid RTX toxins. The putative pore-forming domain of RTX toxins has been assigned to a hydrophobic stretch of amino acids in the N-terminal region of the molecules (29, 33). The results of osmotic protection experiments for the *P. haemolytica* leukotoxin indicated a 0.9-nm pore diameter for this toxin (10). We decided to indirectly investigate the lesion-forming domain of leukotoxin as well as the lesion size by utilizing a hybrid protein consisting of the N-terminal 383 amino acids of *P. haemolytica* LktA and the 632 C-terminal amino acids of HlyA (19). This hybrid toxin has hemolytic activity against sheep erythrocytes, while wild-type leukotoxin does not. The hybrid showed protection patterns, as functions of toxin concentration and time, identical to those of the full-length HlyA clone (data not shown).

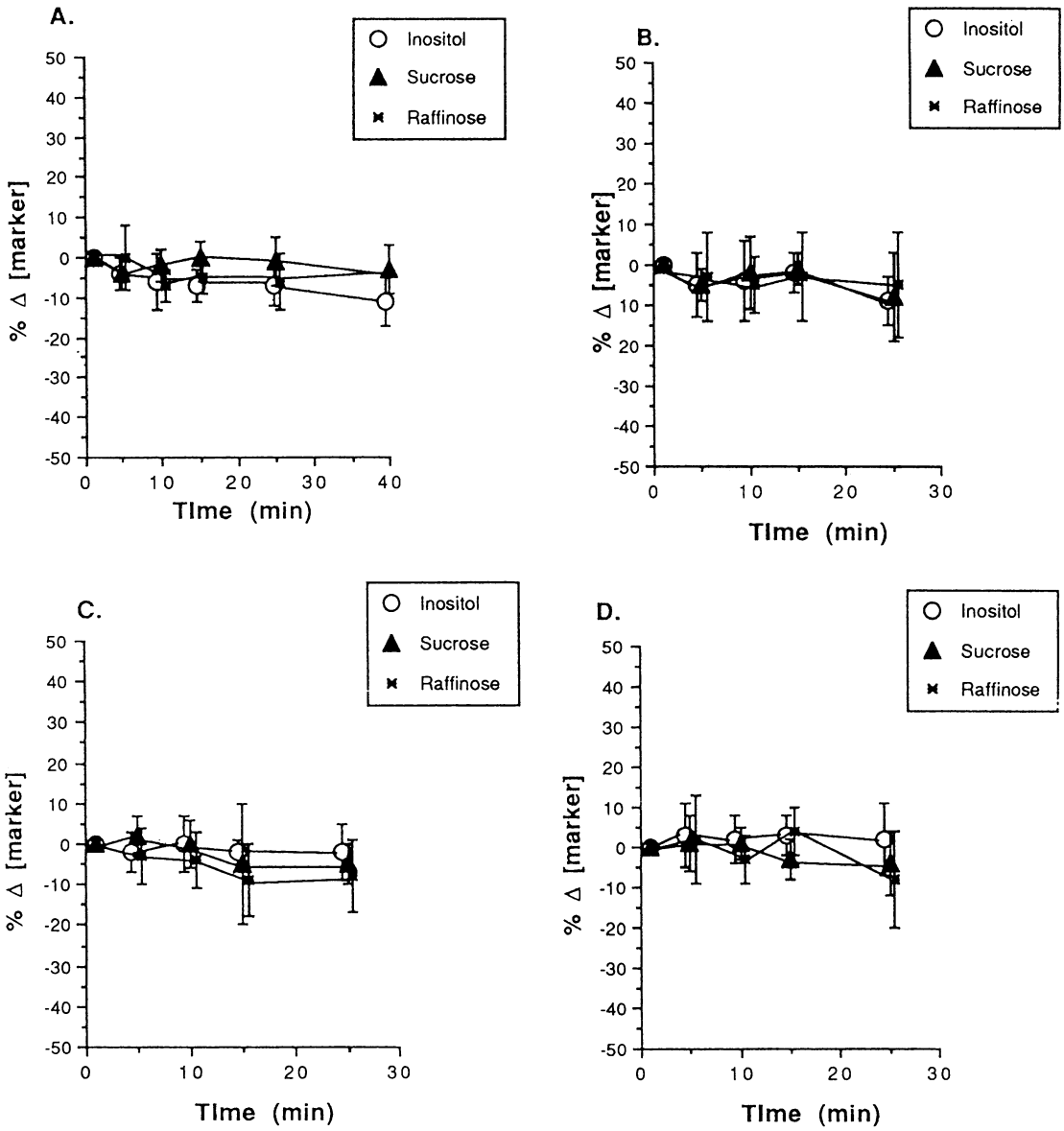

FIG. 5. Influx experiments. Erythrocyte suspensions (33%) were prepared in dextran 4 buffer with three different radioactive markers. For each experiment and each marker, three different concentrations of toxin (128 [A], 10 [B], or 5 [C] HU) or no hemolysin (D) was used. Following addition of toxin, duplicate samples were removed at 1, 5, 10, 15, and 25 min and centrifuged, and radioactivity in supernatants was determined. Each point is the average of 12 values from six pooled experiments. Experiments for panels B and C were performed with the same PEG-precipitated hemolysin sample. Experiments for panel A were performed with the same culture supernatant sample. The *y* axis in each panel represents the percent change in concentration of radioactive marker in an 80-μl volume of supernatant as measured for each time point. The same time points of 1, 5, 10, 15, and 25 min were utilized for all three sugars but have been plotted in scattered fashion here (±0.5 min) for better presentation of the standard deviations associated with each point.

DISCUSSION

We present data that provide insight into the nature of *E. coli* hemolysin-induced lysis of erythrocytes. Previous osmotic protection experiments and planar lipid bilayer studies (5, 6, 31) suggested a pore-forming function for the hemolysin. These investigations did not arrive at a consensus size for the hemolysin-induced pore (4–6, 30, 31). The data addressing monomer-versus oligomer-dependent pore formation by hemolysin are also contradictory (4, 5, 31).

Our work indicates that care should be taken in arriving at conclusions about pore formation from osmotic protection experiments. In most osmotic protection experiments, a single concentration of RTX toxin was used to arrive at a specific pore size. In these experiments, there is no consistency among osmotic protectant concentrations, assay times, or the protection end points chosen by individual investigators (6, 10, 17, 24, 27). We examined the effects of these variables for the *E. coli* hemolysin and found the apparent lesion size deduced from osmotic protection experiments to be dependent on the toxin concentration, osmotic protectant concentration, assay time, and incubation temperature. We showed with protection experiments in isotonic 290 mM sugar solutions that lesions

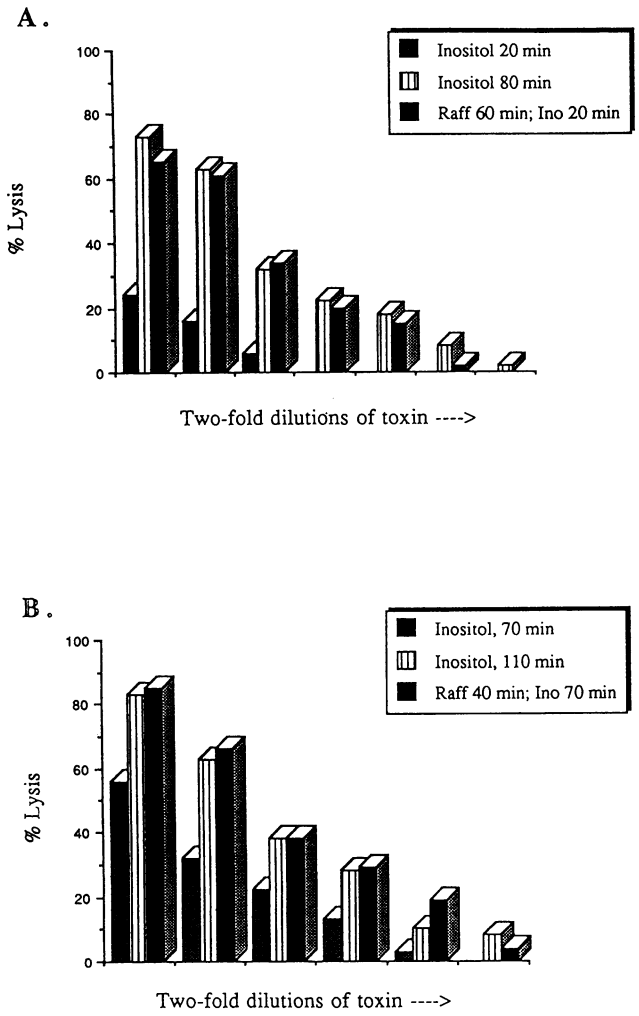

FIG. 6. Sugar switch experiments. Representative experiments comparing lysis patterns for standard monosaccharide assays and sugar switch assays. Hemolysis assays utilizing twofold dilutions of a low-toxin-concentration supernatant were set up with raffinose for 60 min (A) or 40 min (B). At these switch points erythrocytes were pelleted by centrifugation for 5 s, and raffinose was removed and replaced with an equal volume of inositol. Parallel assays were run with inositol for periods equivalent to the total and postswitch assay times of the switch experiments (80 and 20 min, respectively, in panel A; 110 and 70 min, respectively, in panel B). The final approximate concentrations of toxin under the least diluted conditions were 2.5 (A) and 2.0 (B) ng of HlyA per ml of assay mixture. In all experiments toxin was prebound to erythrocytes on ice for 30 min, and excess toxin was removed by centrifugation prior to the indicated incubations.

ranging in size from smaller than 0.6 nm to greater than 1.4 nm can be inferred. The lesions appear to increase in size over time. Analysis of time dependency was performed with a fixed number of toxin molecules associated with erythrocytes by initiating the assays on ice and removing unbound toxin. Thus, the decrease in osmotic protection observed with trisaccharides over time was not the result of new toxin insertion events. It was also demonstrated that the perceived differences in protection patterns among various *E. coli* hemolytic strains (i.e., plasmid versus chromosomal locus and human versus animal isolate) are likely related to the levels of toxin they produce and not to structural differences in hemolysin.

Experiments with a hybrid toxin made of the N-terminal portion of LktA and the C-terminal sequences of HlyA indicate that the hybrid toxin forms lesions with the same initial size and the same protection patterns, as functions of concentration and time, as for those formed by hemolysin. Earlier reports suggested that the RTX toxin pore-forming domains lie in the N-terminal half of the molecules (4, 29, 33) and that the leukotoxin forms a discrete pore 0.9 nm in diameter (10). If the leukotoxin does indeed have a lesion size different from that of the hemolysin, the pore-forming domain of HlyA or LktA is not solely the product of the N-terminal half of the molecule.

We propose three hypotheses for the observed concentration- and time-dependent changes in the hemolysin-induced lesion: a concentration- and time-dependent aggregation or oligomerization of toxin molecules, the induction of dynamic lipid perturbations in target cell membranes upon insertion of toxin molecules; or differential rates of diffusion for osmotic protectants, leading to differential lysis patterns.

Among the several possible aggregation or oligomerization events, an aggregation or oligomerization process that takes place in the solution over time seems unlikely. Dilutions of highly concentrated toxin preparations that would possess the putative large preformed oligomers do not initially yield larger pores. Obviously, this argument would not hold true for an unstable aggregate. An aggregation or oligomerization event that occurs at the binding step is unlikely because the association of toxin with erythrocytes at 4°C for an extended time also does not yield initial lesions larger than the diameter of raffinose. A toxin aggregation or multimerization event involving the lateral movement of the toxin in the erythrocyte membrane could be involved. Such a process would probably be susceptible to reduced temperature (20).

Oligomerization of toxin by lateral aggregation after membrane association has been observed with other toxins. This type of oligomerization event can involve a specific number of monomers coming together in a defined fashion, such as seen with the *Staphylococcus aureus* alpha-toxin (7), or it may be a more random event (3). Oligomerization has been previously suggested for HlyA. The oligomers were postulated to be unstable compared with those of the *Aeromonas hydrophila* aerolysin and *S. aureus* alpha-toxin. Models for the formation of dimers at lower concentrations of HlyA and aggregates of higher numbers of HlyA molecules at higher concentrations have been presented (5). Planar lipid bilayer experiments by Benz et al. indicate that increases in membrane conductance are HlyA concentration and time dependent. Small changes in HlyA concentration led to large variations in membrane conductance in a nonlinear fashion. The investigators concluded that several HlyA molecules are involved in the formation of single ion channels. The data indicated the existence of an ongoing association-dissociation equilibrium for the oligomers (5). Attempts to isolate HlyA oligomers from deoxycholate-solubilized membranes were inconclusive (6). To date, we have been unable to isolate and identify HlyA oligomers by various membrane solubilization and electrophoretic methods.

The oligomerization event suggested by our results is not required for the onset of lysis in saline. We demonstrate that under high-erythrocyte-concentration conditions (50%), when less than one toxin molecule per erythrocyte is present, lysis is proportional to the number of erythrocytes which receive one hit of hemolysin (data not shown). The HlyA molecule may not necessarily act by forming a protein-lined pore. It could easily perturb the membrane by other means (discussed below). Also, the lysis in saline by a single-hit phenomenon is not exclusive of normal oligomeric lytic function for HlyA. Even if

the events described here may not be due to an oligomerization event, the existence of an oligomeric hemolysin species as a functional entity responsible for induction of membrane lesions remains a possibility. Ludwig and coworkers (28) recently showed the complementation of mutations in one HlyA molecule by wild-type sequences in another mutant HlyA and vice versa. Their data indicate the cooperation of more than one molecule of HlyA as a functional unit for hemolysis.

The second hypothesis, that HlyA causes lysis by induction of lipid bilayer perturbations, is consistent with the new data here. Previous studies on complement-mediated lysis suggest that lipid bilayer perturbations after complement insertion into membranes can lead to ion fluxes, osmotic gradient formation, and colloid osmotic lysis. This prominent model of complement lysis does not require the existence of protein-lined pores formed by complement components in target membranes (18). This "leaky patch" model of complement lysis is supported by data showing a range of pore sizes for the complement lesion, ranging from 0.7 to 15 nm in diameter. These size estimates were made from osmotic protection experiments, marker influx-efflux studies, and planar lipid bilayer methods (8, 9, 12, 21, 23, 32, 36–38, 42–44, 48). The range of different pore sizes depends on factors identical to those described here for the hemolysin lesions, including preparation method, dose, incubation temperature, and time.

The leaky patch model of complement can be applied to hemolysin membrane lesions in the following manner. The hemolysin inserts into membranes and causes local bilayer perturbations that alter membrane permeability. These perturbations are still subject to molecular sieving and osmotic protection effects. These lesions differ in size because of the fluid nature of the bilayer, but they will be smaller at lower concentrations of hemolysin and will physically increase in size and stability at higher toxin concentrations. At high concentrations, hemolysin may function as a detergent, as has been suggested for complement (35). As a detergent-like species it would cause the micellization of membranes because of extreme lipid perturbations. Recent experiments detected such a detergent-like function for hemolysin at high concentrations (34). It should be noted that membrane conductance changes that resemble channels in bilayers and positive data from osmotic protection experiments are not evidence for a protein lined pore, per se. Detergents such as SDS or Triton X-100 at lower concentrations have similar activities (1, 2, 41). HlyA-mediated ion channels in planar lipid bilayers had limited lifetimes and displayed rapid fluctuations between open and closed states (5). Benz et al. concluded that the lesion formed by HlyA under these conditions was not a rigid structure. HlyA concentration, time, and applied membrane potential led to molecular changes in channels that were reflected by changes in conductance (5).

The third applicable hypothesis is that the differential protection patterns and apparent cooperativity for an increasing lesion size are the results of different rates of protectant diffusion across a uniformly sized, stable pore. We have performed various experiments to address the possibility of differential diffusion. The effects of temperature on the dynamics of protection as presented in Fig. 3 argue against a role for differential diffusion because sugar diffusion should continue through a static pore at 4°C. The data in Fig. 3A show that only when an HlyA-mediated membrane lesion that allows diffusion of a sugar is created will a shift to 4°C not prevent lysis. If no lysis has occurred at 37°C, a shift to ice will protect against lysis. The issue of differential diffusion of osmotic protectants has been raised in experiments with complement. Regardless of their molecular size, all solutes are restricted to the same degree during diffusion through a complement lesion (44). Investigators concluded that as the size of the diffusing protectant increases, the fraction of target cell population permeable to the molecule decreases. This occurs because there are fewer lesions with a size sufficient to accommodate the larger molecules. The number of such lesions changes with time and concentration of functional complement complexes (44). Such a model is consistent with the data as shown in Fig. 3.

It can be argued that the results of the temperature shift experiments reflect changes in the diffusion rates of sugars due to temperature. It is unlikely that slower diffusion rates would lead to complete protection against lysis at 4°C. A related argument is that while a functional lesion is formed at 37°C, a shift to 4°C alters the conformation of a monomeric protein-lined pore in a manner which closes the pore. Such an event has been described for the hemolysin channel in lipid bilayer studies. If this were true, a dynamic lesion formed by a single monomer could exist.

The possibility that different diffusion rates for the osmotic protectants lead to differential lysis is not supported by the results of the sugar switch experiments. These results suggest a lesion that increases in size with time. In these experiments, the assays were performed with various dilutions of toxin with a trisaccharide. The toxin concentrations and assay time were selected such that the trisaccharide completely protected the erythrocytes from lysis. After the initial incubation period, the trisaccharide solution was removed and replaced by a monosaccharide solution, and the assay mixtures were incubated further for various time periods. The switched assays would now be starting at a "pseudo zero time point," and the diffusion of the monosaccharide replacement would begin in the same manner as in a regular monosaccharide-based assay. If differential rates of diffusion through a discrete-sized pore alone explain the protection differences between monosaccharides and trisaccharides, lysis patterns in this second monosaccharide incubation should be similar to those for other monosaccharide assays of equal length. After an incubation with the monosaccharide at 20 or 70 min, the protection patterns should resemble those for standard 20- or 70-min monosaccharide assays performed with the same toxin dilutions. However, if the dimensions or the nature of the lesion is altered with time, then during the trisaccharide incubation period, the lesion will have changed in size, and the switch assays will incorporate the time effects of the initial incubation with trisaccharide. It was observed that the incubation period with trisaccharide (raffinose) affects the protection patterns seen in switch assays. The patterns of sugar protection in switch assays resembled that for the longer standard monosaccharide (inositol) assays. The time of these longer monosaccharide assays equalled the total time of the switch assays. Parallel raffinose assays that were extended well beyond the switch time did not show lysis. These data support a model for a dynamic, growing membrane lesion.

One possible explanation of the results of the sugar switch experiments which would support the existence of a static protein-lined channel is that after binding of toxin to erythrocytes at 4°C and then shifting to 37°C, all toxin molecules do not insert into membranes as functional units for lysis at the same rate. In that case, the results could indicate an increased number of active insertions, over time, in both raffinose and inositol assays. The inositol assays with shorter incubations simply do not have the time required for a maximum of toxin insertion events. This would be similar to having an unequal number of hemolysin molecules present in each assay. This scenario is unlikely because after incubation of the toxin with

erythrocytes at 4°C, removal of unbound hemolysin, and subsequent incubation for 20 min at 37°C, 25% lysis is observed with inositol, whereas a parallel assay run in saline shows >75% lysis. This clearly indicates that most erythrocytes receive an adequate number of functional hemolysin lytic hits.

The influx of radiolabelled inositol, sucrose, and raffinose into hemolysin-treated erythrocytes was measured by the methods of Bhakdi et al. (6) but with three toxin concentrations and a negative control. There are no significant differences in diffusion rates among the three sugars and the negative control in the data presented in Fig. 5. The experiments by Bhakdi et al. employed one toxin concentration in what appears to be a representative experiment without a report of the associated error. They found 10 to 20% influx of sucrose and 17 to 23% influx of mannitol over 5 to 20 min (6). Because the negative control in the experiments cited above is inapparent, analysis of the data is difficult. Jorgensen et al. performed similar influx experiments with sucrose and concluded that significant influx occurred at a single toxin concentration over 16 min of incubation, but the data were also published as a representative experiment (25). Perhaps no significant difference in influx is seen over the time and toxin concentration ranges utilized in these influx experiments because cooperative toxin multimerization events that are required for a lesion that would allow the influx of all three sugars cannot occur because of the high erythrocyte-to-toxin ratio. It remains surprising that no differential influx is observed with the high toxin concentration of 128 HU/ml, which is adequate for lysis of 100% of the erythrocytes in the assay.

A puzzling aspect of our alternative models to the discrete-sized pore model is the apparent stable osmotic protection provided by dextran 4 (diameter = 3 nm). Dextran 4, which cannot be prepared at 290 mM concentrations, protects against lysis at concentrations as low as 5 mM (data not shown). The use of polymers like dextrans in osmotic protection experiments was previously questioned because of their highly hydrated state and the observation that lytic protection is proportional to the weight concentration of the polymer and independent of its molecular weight (13, 40). Ostolaza et al. recently showed that at high concentrations of HlyA, the toxin has a detergent-like function that leads to the influx of molecules as large as a 17-kDa dextran (34). This indicates that it is possible for the hemolysin-induced lesion size to exceed the protective diameter of dextran 4.

It is possible that the events described here are limited to hemolysin-erythrocyte interactions. Erythrocytes are incapable of withstanding osmotic gradients in a manner that is possible for nucleated cells. In pathogenesis of disease by RTX toxin-producing pathogens, sublytic-dose effects of these toxins on nucleated cells may be more important in affecting target cells than frank lytic events (46). In that regard, it is fascinating that osmotic protection against complement-induced colloid osmotic lysis of nucleated targets does not prevent their death (26). Investigation of the interaction of hemolysin with nucleated cells at the lesion formation level as well as further inquiry into lesion formation by other RTX family members are important areas to be pursued.

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