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Lysis of Erythrocytes by a Hemolysin Produced by a Group B *Streptococcus* sp.

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An improved procedure for the isolation and purification of the hemolysin produced by a group B streptococcus was developed, and the inactivation of partially purified hemolysin by several enzymes was studied. Hemolysin obtained in buffer containing starch and Tween 80 was inactivated by subtilisin and alpha-amylase, suggesting that the hemolysin may consist of a protein hemolytic moiety complexed to starch which acts as a carrier or stabilizer. Properties of the hemolytic reaction were studied by using sheep erythrocytes as target cells. Experiments to examine the kinetics of hemolysis at different hemolysin concentrations resulted in a family of sigmoidal curves characterized by a short prelytic lag phase followed by a period of rapid release of hemoglobin. The binding of the group B hemolysin at 37°C was rapid; within 3 min, most of the cells had bound sufficient hemolysin to produce lysis. In contrast, the hemolysin did not bind to erythrocytes at 0°C. The length of the prelytic lag period and the rate of hemolysis were also temperature dependent. A decrease in total hemolysis was observed when the target cell/hemolysin ratio was increased, suggesting that a multihit response is required for lysis. Intracellular ^{86}Rb and hemoglobin were released at the same rate from hemolysin-treated cells, indicating that a colloid-osmotic process is not involved in the lytic mechanism.

Group B *Streptococcus* sp. has become an increasingly important etiological agent in human infections (2, 5, 6, 21, 26, 35). In an attempt to define the factors that may be important in the virulence of this organism, extracellular products have been isolated and studied. These include the enzymes hyaluronidase (22, 27), neuraminidase (28, 29), three nucleases (12), and protease (33). The group B streptococci are beta-hemolytic (36), producing narrow hazy to clear zones of hemolysis around their colonies on blood agar plates, and the hemolysin responsible for this reaction has been isolated (26). Little or no hemolytic activity was present in supernatants from broth cultures, but when washed cells were suspended for short periods of time in phosphate-buffered saline (PBS) containing glucose, soluble hemolytic activity was detected in the supernatant. The hemolysin produced under these conditions was rapidly inactivated, however, unless one of several agents, which apparently act as stabilizers or carriers (or both) of the hemolysin, was added. These agents, some of which have been shown to be effective in streptolysin S production (13, 15), included calf serum, albumin, Tween 80, and starch; no hemolysin was produced in the presence of ribonucleic acid. The highest titers of group B hemolysin were obtained in the presence of a starch-Tween 80 mixture. The ability of the bacteria to produce hemolysin was stimulated by the addition of calf serum to the growth medium, but increased glucose concentrations severely repressed hemolysin production.

The soluble hemolysin was inhibited by phosphatidylcholine and phosphatidylethanolamine, but trypan blue and protease (*Streptomyces griseus*, type IV), which inactivate streptolysin S (14), and cholesterol, which inactivates streptolysin O (30, 31), had no effect on the group B hemolysin. The hemolysin appears to be completely unrelated to CAMP factor and streptolysin O, but similar in some respects to streptolysin S.

This report describes an improved method for concentrating and purifying the hemolysin and demonstrates that the hemolysin obtained in the presence of starch can be inactivated by treatment with subtilisin or alpha-amylase. In addition, the lytic properties of the group B hemolysin for sheep erythrocytes were investigated.

MATERIALS AND METHODS

Bacterial strains. Group B *Streptococcus* sp. strain O90/R was used for the isolation of hemolysin

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in the experiments discussed in this paper (26). The culture was maintained on blood agar plates sealed with Parafilm and stored at 4°C. The bacteria were subcultured monthly onto blood agar plates. Fresh cultures were prepared in Todd-Hewitt broth containing 5% sheep blood every 3 months from stocks maintained as a dried bacteria-blood suspension on sterile Ballotini beads. The bacteria were routinely cultured in Todd-Hewitt broth containing 5% calf serum (GIBCO Laboratories).

Hemolysin assay. A 5- to 50-μl amount of sample was added to 2 ml of a 1% sheep erythrocyte suspension in PBS (pH 7.2; whole sheep blood was obtained weekly from a local veterinarian, and unused blood was discarded after 7 to 10 days) and diluted to 2.5 ml with PBS. The tubes were incubated at 37°C for 30 min. After centrifugation (1,500 × *g* for 2 min), the absorbance was read at 540 nm (Coleman 44 linear absorbance spectrophotometer) to measure hemoglobin release. The greatest dilution of sample resulting in 50% hemolysis of 1 ml of a 1% sheep erythrocyte suspension was defined as 1 hemolytic unit (HU).

Isolation of soluble hemolysin. Four liters of Todd-Hewitt broth plus 5% calf serum was inoculated with 10 ml of strain O90/R and incubated at 37°C for 16 to 18 h. The culture was centrifuged at 6,000 × *g* for 10 min and washed once with PBS. The washed bacterial pellet was suspended in 100 ml of stabilizing solution containing 1% starch (Difco Laboratories) and 3% polyoxysorbitan monooleate (Tween 80; Sigma Chemical Co.) in PBS containing 0.1% glucose (PBSG). The suspension was incubated at 37°C for 10 min, centrifuged at 6,000 × g for 10 min, and the hemolytic supernatant decanted.

Purification procedure. The soluble hemolysin was concentrated by adding 100 ml of cold (0°C) methanol to each 100 ml of crude hemolysin. After standing for 5 min at 0°C, the solution was centrifuged at 6,000 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 20 ml of 0.005 M tris(hydroxymethyl)aminomethane (Tris) buffer (Trizma base; Sigma, pH 7.2). Two resuspended methanol precipitates (40 ml total) were pooled and lyophilized. The lyophilized hemolysin was suspended in 6 ml of Tris buffer and layered onto a Sephacryl S-300 column (2.5 by 50 cm) equilibrated with Tris buffer at 4°C. The column was eluted with Tris buffer at a flow rate of 37 ml/h. Fractions (5 ml) were collected and assayed for hemolytic activity and absorbance at 280 and 230 nm. The fractions possessing peak hemolytic activity were pooled, lyophilized, and suspended in 2 ml of Tris buffer.

The hemolysin was assayed at various stages during the purification by polyacrylamde gel electrophoresis. Polyacrylamide (12%) slab gels were prepared (23) and electrophoresed at 10 mA until the bromphenol blue marker dye entered the separating gel. The amperage was then increased to 30 mA and run for approximately 6 h. Gels were stained for protein with Coomassie brilliant blue. Some gels were stained with carbocyanine (Stains-all reagent; Eastman Kodak Co.) by the method of Green et al. (17). The interaction of various polymers with carbocyanine produces different spectral maxima, so that proteins, glycoproteins, lipoproteins, ribonucleic acid, and deoxyribonucleic acid can be visualized and differentiated.

Inhibition of hemolytic activity. Various enzymes were tested for their ability to inhibit hemolytic activity: alpha-amylase (*Bacillus subtilis* type II A; Sigma); protease (type VI; Sigma), chymotrypsin (type 1-S; Sigma); lipase (type VII; Sigma); esterase (type I; Sigma); and subtilisin (protease type VII; Sigma). Stock solutions of 2 mg/ml were prepared, diluted, and incubated with hemolysin as described below.

Measurement of $^{86}Rb^+$ efflux. Fresh sheep erythrocytes were washed three times in potassium-free PBS buffer (PBS-K: 137 mM NaCl, 10 mM Na_2HPO_4, 3 mM $NaHPO_4$, pH 7.4) and resuspended in PBS-K buffer containing 0.5% glucose (PBSG-K). For each experiment, a 30-ml portion was removed, centrifuged at 3,000 × *g* for 5 min, and suspended in 1.9 ml of PBSG-K. A 0.1-ml sample (100 μCi) of $^{86}Rb^+$ (New England Nuclear Corp.) was added, and the cell suspension was incubated at 37°C for 120 min. The cells were centrifuged, washed once in PBSG-K, and resuspended in 30 ml of the same buffer. Portions (2 ml) were distributed to a series of tubes, and hemolysin was added to a final concentration of 2 HU/ml in a volume of 2.5 ml. In control tubes, PBSG-K was added instead of hemolysin. At the appropriate time intervals, samples were removed and centrifuged for 60 s, and a 0.2-ml sample of each supernatant was quickly removed and placed in a scintillation vial. The samples were then read at 540 nm to determine hemoglobin release. The beta-emitting energy of the $^{86}Rb^+$ samples was counted in a Packard Tri-Carb liquid scintillation spectrometer. The total intracellular amount of hemoglobin and $^{86}Rb^+$ was determined in cell samples lysed by saponin. Control experiments showed that less than 1% of the intracellular $^{86}Rb^+$ leaked out of untreated cells during the 30 min required to carry out each experiment. Virtually no $^{86}Rb^+$ or hemoglobin appeared in the supernatant once the cell had been pelleted by centrifugation.

Protein determination. The protein content of the samples was measured by the method of Lowry et al. (25), with bovine serum albumin as a standard.

Starch determination. Starch content was quantitated by a modification of an anthrone reagent assay (18) as previously described (26).

RESULTS

Purification of hemolysin. The group B streptococcal hemolysin prepared by incubating washed bacteria in a 1% starch–3% Tween 80 solution (see above) was concentrated by precipitation with cold methanol. This step precipitated the hemolytic activity along with the starch and effectively removed the Tween 80, which remained in the supernatant. Methanol precipitation resulted in considerably better recovery (Table 1) than did the ammonium sulfate precipitation procedure previously used (26). Two methanol-precipitated pellets were resuspended in Tris buffer, pooled, lyophilized to remove residual methanol, then resuspended in 6 ml of Tris buffer. The limited solubility of starch prevented more than two precipitates from being combined.

TABLE 1. *Purification of group B hemolysin*

Procedure	Vol (ml)	Starch[a] (g)	Protein[b] (mg)	Activity (HU)	Sp act (HU/mg) of starch	Recovery (%)
Crude preparation	600	11.8	ND[c]	375,000	31.8	
Methanol precipitation	21	6.7	26.4	362,712	54.1	97
Sephacryl S-300[d]	5.5	0.55	4.7	148,711	270.4	40

[a] Determined with anthrone reagent (8).
[b] Determined by the method of Lowry et al. (25).
[c] Not determined. Tween 80 interfered with the protein determination.
[d] Fractions containing peak hemolytic activity were pooled and lyophilized.

The soluble hemolysin was partially purified by column chromatography on a Sephacryl S-300 column. The elution profile closely resembled that previously reported (26), and fractions containing the highest hemolytic activity were pooled, lyophilized, and suspended in 2 ml of Tris buffer. The resuspended hemolysin from three column runs was pooled to give a final volume of 5.5 ml. The purification scheme resulted in a ninefold increase in the specific activity (Table 1), and the recovery of hemolytic activity was more than twice that previously reported (26).

The Sephacryl S-300 material was analyzed by polyacrylamide gel electrophoresis in nondissociating gels. Three major Coomassie blue-staining bands were seen, one of which appeared to be a glycoprotein based on its appearance in carbocyanin-stained gels (data not shown). The glycoprotein band migrated near the tracking dye and corresponded to the fastest-migrating band seen with Coomassie blue. No hemolytic activity has been detected in any region of the gel.

Inactivation of hemolytic activity. Several degradative enzymes were tested for their ability to inactivate the hemolysin. In each experiment a constant amount of hemolysin (185 HU) was added to 50 U of the indicated enzyme and incubated for 30 min at 37°C. Controls consisted of hemolysin incubated under the same conditions in PBS without enzyme. In addition, sheep erythrocytes treated with each enzyme before the addition of the hemolysin were as sensitive to lysis as were untreated erythrocytes, and none of the enzymes were hemolytic under the experimental conditions. The data in Table 2 show that alpha-amylase and subtilisin were the most effective inactivators of the hemolysin. All of the enzymes were shown to be active on appropriate substrates under the experimental conditions.

The hemolysin was also sensitive to heat inactivation (Fig. 1). A 10-min incubation at 60°C resulted in greater than 90% loss of hemolytic activity.

Kinetics of erythrocyte lysis. The characteristics of cell lysis were investigated by using sheep erythrocytes. Initially, the kinetics of hemolysis as a function of hemolysin concentration were studied. Hemolysin was added to 2.5-ml samples of a 1% sheep erythrocyte suspension to a final concentrations of 0.7, 1.0, 1.2, and 2 HU/ml, and after incubation at 37°C for the indicated times (Fig. 2), the tubes containing each hemolysin concentration were assayed for he-

TABLE 2. *Inactivation of group B hemolysin*

Enzyme[a]	Sp act (U/mg)	Concn (mg/ml)	% Inactivation[b]
Esterase	138	0.36	0
Lipase	19,200	0.003	0
Protease VI	4.4	11.2	25
Chymotrypsin	47	1.06	34
Subtilisin	12.2	4.1	90
Alpha-amylase	695	0.07	82

[a] In each experiment 184 HU of hemolysin was incubated with 50 U of the indicated enzyme for 30 min at 37°C. The hemolysin was cooled in an ice bath, and the titer was determined. As a control, 184 HU of hemolysin was incubated with PBS under the same conditions.
[b] The percent inactivation represents the loss of hemolytic activity after enzyme treatment compared with the PBS control.

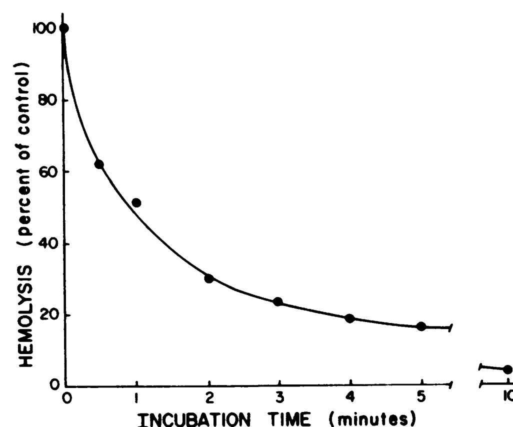

FIG. 1. *Effect of temperature on hemolytic activity. A solution of hemolysin (200 HU/ml) was incubated at 60°C, and at the indicated times a sample was removed and the titer was determined. The titer of the control sample was determined at 0 time.*

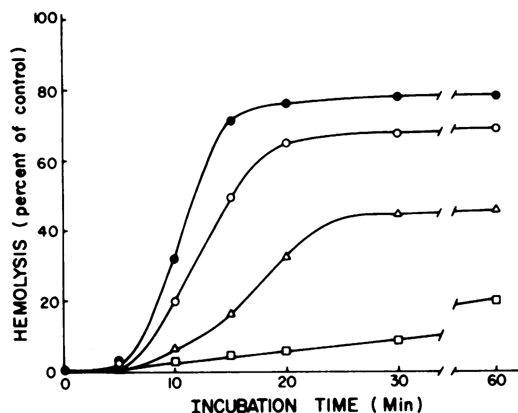

FIG. 2. *Effect of increasing hemolysin concentration on erythrocyte lysis. To 20 ml of a 1% sheep erythrocyte suspension, hemolysin was added to a final concentration of 0.7 HU/ml (○), 1.0 HU/ml (△), 1.2 HU/ml (○), or 2 HU/ml (●). The suspensions were incubated at 37°C, and 2.5-ml samples were removed at the times indicated. The amount of hemoglobin released was determined spectrophotometrically. A control sample (100% hemolysis) was lysed with saponin.*

moglobin release. The resultant curves were sigmoidal, except at the lowest hemolysin concentration. A 5-min lag period, a stage of rapid lysis, and a plateau of hemoglobin release were characteristic for hemolysin concentrations of 1 HU/ml or greater.

Binding of hemolysin to erythrocytes. It has been demonstrated that other hemolysins bind to target cells before the beginning of lysis (11, 30). Therefore, the interaction between the group B hemolysin and erythrocytes during the prelytic lag period was studied. Sheep erythrocytes were incubated with 2 HU of hemolysin per ml at 37°C for up to 5 min, and samples were removed at 1-min intervals and centrifuged. No hemolysis occurred during this initial incubation period. The supernatant was carefully removed, and the erythrocyte pellet was resuspended in PBS and incubated at 37°C for 30 min. The amount of hemolysis that occurred during the second 37°C incubation period was determined spectrophotometrically. By 3 min, enough hemolysin had become associated with the erythrocytes to cause 80% lysis (Fig. 3). Washing the cells three times in PBS did not remove the bound hemolysin (data not shown). The amount of hemolytic activity remaining in the supernatant after the initial incubation period was determined by incubating the supernatant with fresh erythrocytes at 37°C for 30 min. The hemolytic activity in the supernatant progressively decreased with increased incubation time.

Similar experiments were performed with erythrocytes and hemolysin incubated at 0°C. In these studies no hemolysis was detected when the erythrocytes were centrifuged, suspended in fresh PBS, and incubated at 37°C for 30 min. Even after a 1-h incubation at 0°C, less than 10% of the hemolytic activity had adsorbed to the erythrocytes, and no lysis occurred at 37°C.

Effect of incubation temperature on hemolysis. The experiments just described suggested that the hemolysin bound to and lysed erythrocytes rapidly at 37°C, but not at 0°C. Therefore, the rate of hemolysis at a variety of temperatures was studied. Group B hemolysin was added to 25 ml of an erythrocyte suspension to a final concentration of 2 HU/ml. The flasks were incubated at various temperatures, and at different times, a 2.5-ml portion was removed from each flask and assayed for hemolysis. At temperatures lower than 37°C the length of the prelytic lag period increased significantly (Fig. 4), and at 15°C, more than 60 min elapsed before any hemolysis was detected. As the incubation temperature increased, the length of the prelytic period shortened and the rate of hemoglobin release increased. At 42°C there was essentially no lag period, and 40% of the cells had lysed within 5 min.

Effect of erythrocyte concentration on hemolysis. The effect of erythrocyte concentration on the lytic reaction was studied by incu-

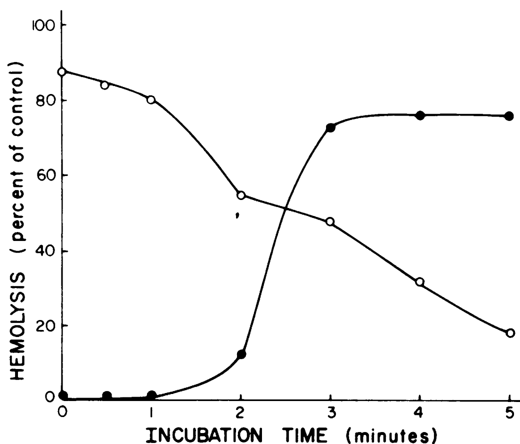

FIG. 3. *Adsorption of hemolysin to sheep erythrocytes at 37°C. A 1% suspension of sheep erythrocytes was incubated with hemolysin (2 HU/ml) at 37°C for the indicated times. The suspension was then centrifuged, and the supernatant was decanted. The erythrocyte pellet was resuspended in PBS and incubated at 37°C for 30 min. The degree of hemoglobin released was measured spectrophotometrically (●). The decanted supernatant was added to a fresh pellet of untreated erythrocytes and incubated at 37°C for 30 min (○) to determine the amount of hemolytic activity remaining unadsorbed.*

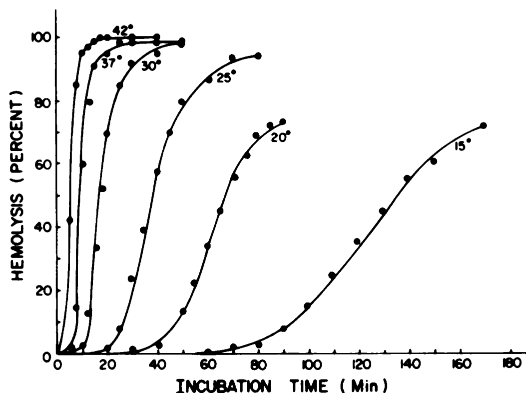

FIG. 4. *Kinetics of hemolysis at various temperatures. To 25 ml of a 1% sheep erythrocyte suspension, hemolysin was added to a final concentration of 2 HU/ml. After incubation at various temperatures for the indicated times, a 2.5-ml sample was removed and assayed for hemolysis.*

bating the cells with 2 HU of hemolysin per ml for 30 min. With increased cell concentrations, the total hemoglobin released by the hemolysin was drastically reduced (Fig. 5). Almost no lysis was seen in the 4% erythrocyte suspension. Longer incubation of the more concentrated cell suspensions did not result in an increase in the amount of hemoglobin released. These observations suggest that more that one molecule of hemolysin is required to lyse a single erythrocyte.

$^{86}Rb^+$ release from hemolysin-treated cells. A study of the comparative release of $^{86}Rb^+$ and hemoglobin was undertaken to determine the nature of the membrane lesion produced by the group B hemolysin. In these experiments, $^{86}Rb^+$ was used to measure electrolyte efflux from toxin-treated cells; rubidium and potassium are known to be transported and maintained in a similar manner by erythrocytes (32, 34). Fresh sheep erythrocytes were washed and labeled as described above. The extracellular hemoglobin and $^{86}Rb^+$ counts in control tubes (spontaneous release) at each sample time were subtracted from the appropriate experimental tubes. The amount of $^{86}Rb^+$ and hemoglobin lost from toxin-treated cells at each time point was determined and plotted as a percentage of the total intracellular hemoglobin and rubidium. A typical experiment is shown in Fig. 6. There was no difference in the rate of loss of $^{86}Rb^+$ and hemoglobin under these experimental conditions.

In a separate experiment, bovine serum albumin was added to the extracellular medium. If hemolysis involved a colloid-osmotic process (19) the addition of bovine serum albumin should retard hemoglobin release, but $^{86}Rb^+$ efflux should not be hindered. The release of hemoglobin was not retarded by this procedure

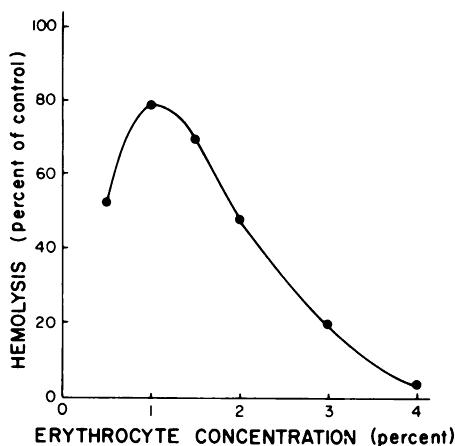

FIG. 5. *Hemolysis of sheep erythrocyte suspensions with increasing target cell/hemolysin ratios. Suspensions containing increasing concentrations of erythrocytes were incubated for 30 min at 37°C with a constant concentration (2 HU/ml) of hemolysin. The control hemolysis was determined by lysing a 1% sheep erythrocyte suspension with saponin.*

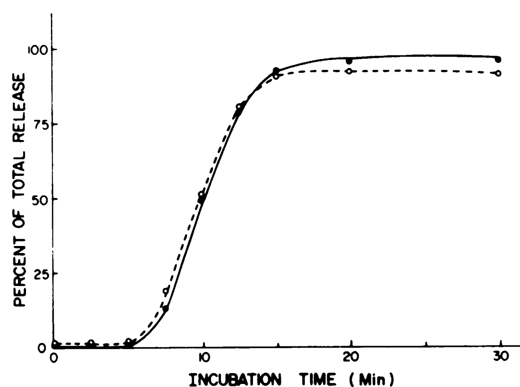

FIG. 6. *Release of $^{86}Rb^+$ (○) and hemoglobin (●) from sheep erythrocytes exposed to group B hemolysin. Washed erythrocytes were suspended in potassium-free PBSG. $^{86}Rb^+$ (200 μCi) was added, and the suspension was incubated at 37°C for 120 min. The cells were centrifuged, washed in PBSG-K, and suspended to the original volume in PBSG-K. Portions (2.5 ml) were distributed to a series of tubes and incubated at 37°C with either 2 HU of group B hemolysin per ml or control solution (PBSG-K). At the indicated times, one hemolysin and one control sample were removed and centrifuged. The optical density was read at 540 nm to determine hemoglobin release, and the amount of $^{86}Rb^+$ in a 0.2-ml sample of each supernatant was determined by liquid scintillation spectrometry. The total intracellular amount of hemoglobin and $^{86}Rb^+$ was determined in saponin-lysed samples.*

(data not shown); the rate of release was very similar to that seen in Fig. 6, although the lag period was slightly longer, and the linear portion of the curve was less steep for both $^{86}Rb^{+}$ and hemoglobin. These results suggest that the group B hemolysin produces large lesions in the membrane which allow large and small molecules to escape from the cell at the same rate. Electron microscopy studies will be required to confirm this hypothesis.

DISCUSSION

This report describes an improved procedure for isolating group B streptococcal hemolysin. The use of cold methanol to precipitate the hemolysin allowed increased recovery of hemolytic activity and had the additional advantage of separating Tween 80 from the starch-hemolysin complex. Resuspension of the methanol precipitate or lyophilized hemolysin in Tris buffer rather than PBS eliminated the need for solubilization of the sample in hot 2 M urea and allowed more concentrated hemolysin samples to be separated by gel filtration. The procedures detailed here resulted in a significant increase in hemolysin recovery. During the course of these experiments, we attempted to increase the production of hemolysin by altering the concentration of starch and Tween 80 and in the stabilizing solution. Reducing the concentration of starch resulted in the production of comparable titers of hemolytic activity which were unstable (data not shown). Reducing the concentration of Tween 80 resulted in a decrease in the amount of hemolysin recovered. Although detergents other than Tween 80 are useful in obtaining streptolysin S (9), Tween 20 and Tween 40 were less effective for the isolation of the group B hemolysin.

Group B streptococci do not produce streptolysin S (14), but there are several similarities between group B hemolysin and that group A toxin. Most notable is the requirement for the addition of carrier or stabilizer molecules to obtain soluble hemolytic activity (14, 16, 26). However, ribonucleic acid-core, an agent useful in obtaining streptolysin S (8) is unable to act as a carrier-stabilizer for the group B hemolysin (26). Streptolysin S has been shown to be inactivated by the enzymes subtilisin, chymotrypsin, pronase, and papain, but not by trypsin, lipase, leucine amino peptidase, or carboxypeptidase Y (14, 24). Of the enzymes listed above, only subtilisin significantly inhibited the activity of the group B hemolysin, although protease and chymotrypsin partially affected hemolytic activity. Digestion of the starch by alpha-amylase also inactivated the hemolysin. One explanation of these results is that the group B hemolysin may consist of a streptococcal protein that becomes associated with, or is stabilized by, starch: the enzymatic degradation of either portion results in inactivation of hemolytic activity. Destruction of the streptolysin S carrier molecule also results in the inactivation of that toxin (14).

The most striking difference between the group B hemolysin and streptolysin S is in the mechanism of hemolysis. Time course experiments (7) showed that 1 to 2 HU of streptolysin S had an extended lag period (up to 20 min), whereas the group B hemolysin has a much shorter lag period (Fig. 2), more closely resembling lysis by streptolysin O or theta-toxin of *Clostridium perfringens* (7). The fact that streptolysin S hemolysis is the result of a colloid-osmotic process (10) may account for the delay in observable hemoglobin release. In such a process the ability of the membrane to maintain an ion gradient is disrupted, resulting in cell swelling and subsequent lysis. In contrast, Rb^{+} (K^{+}) and hemoglobin are released at the same rate in group B hemolysin-treated cells, indicating that a colloid-osmotic process is not involved. The group B hemolysin probably causes large lesions in the membrane allowing for the simultaneous escape of both large and small molecules.

Binding of the hemolysin was rapid (Fig. 3) at 37°C, and by approximately 3 min enough hemolysin associated with erythrocytes to result in 80% lysis. Although the amount of hemolytic activity in the supernatant decreased throughout the incubation period, the lysis of treated cells did not increase significantly after 3 min of exposure to the hemolysin. The loss of activity could be caused by continued binding of hemolysin to damaged erythrocytes or erythrocyte ghosts. This result indicates that the group B hemolysin bound to erythrocytes irreversibly, unlike streptolysin S, whereas most of the hemolytic activity remained in the supernatant (10).

The binding of the hemolysin and the length of the prelytic lag period and the rate of hemolysis were temperature dependent. Streptolysin O is known to bind to erythrocytes at 0°C, but no lysis occurs at that temperature (1, 30). In contrast, the group B hemolysin and streptolysin S (10, 11) do not bind to erythrocytes at 0°C (or bind very slowly). It was proposed that the temperature-dependent fluidity of the membrane may be necessary for streptolysin S adsorption to erythrocytes (10), and a similar process may be important for the group B hemolysin. As the incubation temperature increased, the length of the prelytic period decreased and the rate of

lysis increased. Hemolysin action may require that the toxin become inserted into the lipid bilayer, and this process, as well as the subsequent disruption of the membrane which results in cell lysis, may be facilitated by higher temperatures.

Our results suggested that more than one hemolysin molecule is required to lyse a single cell (Fig. 5). When the logarithm of the fraction of unlysed erythrocytes was plotted against the hemolysin concentration, a typical multihit survival curve was obtained (4). Extrapolation of the linear part of the curve to the ordinate provided the number of molecules required to produce hemolysis of a single cell. This value was 5 for the lysis of sheep erythrocytes by the group B hemolysin. Similar studies with streptolysin O and *C. perfringens* theta-toxin resulted in values of 5 and 2, respectively (20).

A large number of bacteria produce membrane-damaging toxins which are known to cause lysis of eucaryotic cells. Several different molecular mechanisms have been defined for the action of these toxins (3), including enzymatic degradation of membrane phospholipids, detergent-like solubilization of membrane components, and formation of functional pores as a result of specific interactions with membrane lipids. The category into which the group B hemolysin falls is not clear at this point. Although the hemolysin is inhibited by phospholipids, no degradation is apparent and the specific target of hemolysin action is still unknown. The exact nature of the membrane lesion will require further study.

ACKNOWLEDGMENTS

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