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Purification and Properties of Staphylococcal Delta Hemolysin

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Large amounts (200 mg per liter of culture supernatant fluid) of highly purified staphylococcal soluble delta hemolysin were obtained by adsorption to and selective elution from hydroxyapatite followed by exhaustive dialysis against water, concentration by polyvinylpyrrolidone or polyethylene glycol 20,000 dialysis, and a final water dialysis. No carbohydrate, phosphorus, or inactive 280-nm absorbing material was detected in the preparation; however, analysis by density gradient centrifugation, gel filtration, analytical ultracentrifugation, carboxymethyl cellulose chromatography, polyacrylamide disc gel electrophoresis, isoelectric focusing, and electron microscopy revealed that the lysin was molecularly heterogeneous. The preparation contained an acidic fibrous lysin ($S_{20,w}$ of 11.9) and a basic lysin component composed of a population of granular aggregates of various sizes, with a maximum $S_{20,w}$ of approximately 4.9. No other staphylococcal products were detected in the preparation. The lysin was active against erythrocytes from many animal species and acted synergistically with staphylococcal beta hemolysin against sheep erythrocytes. It was soluble in chloroform-methanol (2:1), was inactivated by various phospholipids, normal sera, and proteolytic enzymes, but was partially resistant to heat inactivation. Activity was not affected by Ca^{2+}, Mg^{2+}, citrate, ethylenediaminetetraacetic acid, or cysteine. The lysin preparation also disrupted bacterial protoplasts and spheroplasts, erythrocyte membranes, lysosomes, and lipid spherules, was growth-inhibitory for certain bacteria, and clarified egg yolk-agar. Large amounts produced dermonecrosis in rabbits and guinea pigs. The minimum lethal intravenous dose for mice and guinea pigs was approximately 110 and 30 mg/kg, respectively.

Staphylococcal delta hemolysin is differentiated from the other staphylococcal hemolysins by its ability to lyse horse and human erythrocytes (51, 66) and by its ability to be inhibited by phospholipids (F. A. Kapral, Bacteriol. Proc., p. 64, 1967) and normal sera (24, 35, 51). Preparations of delta hemolysin also (i) disrupt bacterial protoplasts and spheroplasts (7, 41), tissue culture cells (24, 29), leukocytes (23, 24, 29, 34), lysosomes (9, 19), mitochondria (H. S. Kantor et al., Bacteriol. Proc., p. 78, 1970), lipid spherules (21), and erythrocyte membranes (28); (ii) are lethal for mice (42); (iii) produce dermonecrosis in guinea pigs (51) and rabbits (22; F. A. Kapral, *personal communication*); (iv) possess limited antibacterial activity (28, 32); (v) act synergistically with staphylococcal beta hemolysin against sheep erythrocytes (51); (vi) are mitogenic for human lymphocytes (19); (vii) clear egg yolk-agar (41); and (viii) possess phospholipase C activity with phosphatidylinositol and phosphatidylserine as substrates (67). Rigorous proof for the belief that all of these effects are caused by delta hemolysin is lacking.

Delta hemolysin has been partially purified and characterized by Marks and Vaughan (51), Jackson and Little (34-36), Yoshida (68), Kayser and Raynaud (42), Kayser (38-41), Hallander (28, 29), Caird and Wiseman (12, 67), Maheswaran and Lindorfer (Bacteriol. Proc., p. 78, 1970), Kantor et al. (Bacteriol. Proc., p. 78, 1970), Kapral (*personal communication*), and Möllby and Wadström (54). In this report, we (i) present a simple and reproducible method for obtaining gram amounts of highly purified delta hemolysin and (ii) describe some of the physical, chemical, and biological properties of the purified preparation.

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MATERIALS AND METHODS

Organism. A mutant of the *Staphylococcus aureus* Wood 46 strain, designated W46M and obtained by ultraviolet irradiation of the Wood 46 strain, was used for delta hemolysin production. It is coagulase-negative and has been estimated to produce only about 5% of the alpha hemolysin produced by the parent strain (7).

Measurement of hemolytic activity. Alpha hemolysin was assayed as described by Bernheimer and Schwartz (8). Beta hemolysin activity was determined as reported by Gow and Robinson (25). Delta hemolysin was estimated as described for alpha hemolysin, with the exceptions that washed horse erythrocytes were used instead of rabbit erythrocytes and bovine serum albumin was not added to the buffer. Unless otherwise mentioned, isotonic phosphate-buffered saline (PBS), *p*H 7, containing 0.067 M phosphate and 0.077 M sodium chloride was utilized as the solvent and diluent for delta hemolysin. In all cases, 1 hemolytic unit (HU) was the amount of lysin required to produce 50% lysis of a 0.7% (v/v) erythrocyte suspension.

Cultivation of organism. Yeast diffusate medium (6.4 liters) was prepared as previously reported (8), with the exception that the yeast extract was obtained from the Fisher Scientific Co. (Fair Lawn, N.J.) rather than from Difco (Detroit, Mich.). The inoculum was prepared by washing the cocci of an overnight broth culture and suspending them in 0.5 volume of sterile 0.9% saline. Each 2-liter flask, containing approximately 533 ml of media, was inoculated with 0.3 ml of washed cocci suspension and incubated for 15 to 18 hr at 37 C on a rotary shaker operating at 220 cycles/min.

Examination for other staphylococcal products. Purified delta hemolysin (1 mg) was examined for the presence of esterase (33, 50), lipase (27, 45, 56), phospholipase C (67), protease (44), gelatinase, deoxyribonuclease (1), ribonuclease (24), coagulase (1), staphylokinase (1, 4), hyaluronidase (1), lysozyme (31), acid phosphatase (49), and alkaline phosphatase (20) activity. The methods used are those described in the appropriate references. Enzymes and substrates other than phospholipids were obtained from the Sigma Chemical Co. (St. Louis, Mo.), Worthington Biochemical Corp. (Freehold, N.J.), and Mann Laboratories (New York, N.Y.). Phospholipids were obtained from the Sylvana Co. (Millburn, N.J.) and Applied Science Laboratories (State College, Pa.).

Sucrose density gradient centrifugation. Zonal centrifugation in a sucrose density gradient was carried out by the method of Martin and Ames (52). A 1-ml amount of a 1% (w/v) solution of soluble delta lysin in PBS was layered on a 28-ml linear gradient of 5 to 20% sucrose in PBS contained in a polyallomer tube (25 by 75 mm). After centrifugation for 24 hr at 25,000 rev/min, a hole was punched in the bottom of the tube and eight-drop fractions were collected. Each fraction was diluted with 1 ml of PBS, and the diluted fractions were assayed for delta hemolysin activity and absorbance at 280 nm.

Gel filtration chromatography. Gel filtration was performed on Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, N.J.) and on Bio-Gel A-5m, 200 to 400 mesh (Bio-Rad Laboratories, New York, N.Y.), packed in water-cooled (5 C) columns (type K25/100, Pharmacia, Piscataway, N.J.). Fractions were collected in a Buchler (Fort Lee, N.J.) refrigerated fraction collector.

Analytical ultracentrifugation. Solutions of delta hemolysin were subjected to sedimentation velocity analysis, by using schlieren and interference optics, in a Spinco An-D analytical rotor at 56,000 rev/min and 20 C with the Spinco model E analytical ultracentrifuge equipped with a temperature control unit. Photographs of the schlieren and interference patterns were taken at 4- or 8-min intervals after the rotor attained two-thirds maximum speed. Photographic plates were read with the aid of a Nikon profile projector (model 6C), and the sedimentation coefficients were estimated by the method described by Schachman (60). With schlieren optics, the sedimentation coefficients were determined from the top (highest point) of the peak. With interference optics, the sedimentation coefficients were determined from the position of the steepest fringe corresponding to the middle portion of the boundary. The observed sedimentation coefficients were corrected to standard conditions by the appropriate viscosity and density corrections.

Carboxymethyl cellulose chromatography. Whatman wet, microgranular, carboxymethyl (CM) cellulose (CM 52, H. Reeve Angel, Inc., Clifton, N.J.) was equilibrated with 0.05 M acetate buffer (*p*H 5) and packed (2.5 by 33.5 cm) in a water-cooled column (type K25/45, Pharmacia, Piscataway, N.J.). After addition of the delta hemolysin (100 mg) dissolved in equilibrating buffer to the column, the column was washed with approximately 250 ml of equilibrating buffer, and the hemolytic activity was eluted from the column with a gradient elution apparatus. The apparatus consisted of an upper reservoir containing 0.05 M acetate buffer (*p*H 5) and 0.5 M sodium chloride which fed into a constant-volume mixing chamber containing approximately 400 ml of 0.05 M acetate buffer (*p*H 5). The constant-volume mixing chamber discharged into the column at a rate of approximately 10 ml/hr, maintained by adjustment at the reservoir. Fractions (approximately 5 ml) were collected and assayed for hemolytic activity and absorbance at 280 nm. The elution gradient was measured with a conductivity bridge (model 31, Yellow Springs Instrument Co., Yellow Springs, Ohio).

Polyacrylamide disc gel electrophoresis. Disc gel electrophoresis of soluble delta hemolysin was performed in the 7% gel alkaline system (separation at *p*H 9.5) described by Ornstein (57) and Davis (14) and in the 7.5% gel acidic system (separation at *p*H 4.3) described by Reisfeld et al. (59) with model 12 Canalco equipment (Canal Industrial Corp., Rockville, Md.). After electrophoresis, the gels were extruded, stained and fixed overnight in 1% aniline blue-black in 7% acetic acid, and electrolytically destained.

Chemical analyses. Protein was determined by the method of Lowry et al. (46) with crystalline bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.) as the standard. Carbohydrate was estimated by the orcinol, indole, and anthrone reactions (37), and hexosamine was estimated by a modified Elson-Morgan method, as described by Kabat and Mayer

(37). Total phosphorus was determined as described by De Siervo (15). Free lipid was estimated by extracting soluble delta hemolysin (100 mg) with three portions (10 ml each) of ether, evaporating the ether, and weighing the residue. Bound lipid was determined by extracting acid-hydrolyzed delta hemolysin, prepared by hydrolyzing 100 mg of lysin with 10 ml of 6 N hydrochloric acid for 20 hr at 110 C, with ether (10 ml), washing the ether extract four times with water, evaporating the ether, and weighing the residue. Nitrogen (Dumas method), carbon, hydrogen, and sulfur contents were determined at the Squibb Institute for Medical Research, New Brunswick, N.J. Nitrogen was also determined by the micro-Kjeldahl method, as described by Mayer (53).

Amino acid analysis. Amino acid analyses were performed by the method of Spackman et al. (62). Two samples (1.5 and 3 mg) of soluble delta hemolysin were hydrolyzed for 22, 48, 72, and 97 hr at 110 C with 6 N HCl (1 ml) in sealed Pyrex tubes after evacuation and degassing. The hydrolysates were dried under vacuum, and amino acid analyses were performed with a Beckman model 120C amino acid analyzer. Color constants for each amino acid were determined by analyzing standard amino acid mixtures shortly before each determination. The amounts of threonine and serine were calculated by extrapolation to zero hydrolysis time. The amounts of valine, leucine, and isoleucine were calculated from the 97-hr hydrolysis samples. The amounts of all other amino acids were calculated from the averaged values of all of the samples. The tryptophan content was estimated by the spectrophotometric method of Edelhoch (18). Half-cystine was determined as cysteic acid by the performic acid oxidation method described by Moore (55).

Spectral analysis. The ultraviolet absorption spectrum was determined with a model 14 Cary recording spectrophotometer and quartz cuvettes having a light path of 1 cm. The infrared absorption spectrum of the soluble lysin (KBr pellet) was determined with a Perkin-Elmer 421 grating infrared spectrophotometer.

Electron microscopy. Lysin, dissolved in distilled water, was negatively stained with 2% (w/v) ammonium molybdate (*p*H 6.8; certified reagent, Fisher Scientific Co., Pittsburgh, Pa.) on Formvar-supported, carbon-coated grids (11). The preparations were examined at an accelerating voltage of 80 kv in a Siemens Elmiskop 1A equipped with a decontamination device and employing a 35-μm objective aperture.

Inactivation studies. The capacity of various lipids and normal sera to inhibit the hemolytic activity of soluble delta hemolysin was assayed by mixing various amounts of test material (in 0.5 ml of PBS) with a fixed amount (3 HU, in 0.5 ml PBS) of delta lysin, allowing the mixtures to stand for 10 min at room temperature, and adding 1 ml of a 0.7% washed horse erythrocyte suspension. After 30 min at 37 C, the mixtures were centrifuged briefly, and the hemoglobin in the supernatant fluids was estimated colorimetrically at 545 nm. Fifty per cent hemolysis was used as the end point of the titrations. Phosphatidylcholine and diphosphatidylglycerol were obtained from Sylvana Co., Millburn, N.J.; phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and sphingomyelin were from Applied Science Laboratories, Inc., State College, Pa.; and cholesterol was from Matheson Coleman and Bell, East Rutherford, N.J.

The capacity of various proteolytic enzymes to destroy the hemolytic activity of soluble delta lysin was determined by incubating 100 and 10 μg of enzyme dissolved in PBS (0.1 ml) with 1 mg of lysin dissolved in PBS (1 ml) for 30 min at 37 C and determining the residual hemolytic activity. Trypsin (bovine pancreas, twice crystallized) was obtained from Sigma Chemical Co., St Louis, Mo.; crystalline chymotrypsin was from Armour and Co., Chicago, Ill.; Pronase (grade B) was from Calbiochem, Los Angeles, Cal.; and crystalline papain was from Worthington Biochemical Corp., Freehold, N.J.

The ability of soluble delta hemolysin to resist heat inactivation was examined by heating 0.1% solutions of lysin in PBS for 1 hr at various temperatures, followed by assaying for residual hemolytic activity.

Lysis of bacterial protoplasts and spheroplasts. Protoplasts of *Streptococcus faecalis* (ATCC 9790) were prepared by the method of Shockman and Slade (61) by use of 1 mg of lysozyme per ml for 16 hr at 37 C. The protoplasts were sedimented by centrifugation and were suspended in 0.5 M sucrose in 0.05 M phosphate buffer (*p*H 6.6) containing 0.01 M Mg^{2+}. *Micrococcus lysodeikticus* protoplasts were prepared with lysozyme as reported by Bernheimer (5). Protoplasts of *Bacillus megaterium* KM and *Sarcina lutea* and spheroplasts of *Escherichia coli* W [prepared with lysozyme and ethylenediaminetetraacetic acid (EDTA)] and *Vibrio metschnikovii* were obtained as previously described (10).

Test preparations (1 ml) having an optical density at 500 nm of approximately 1.0 were examined for susceptibility to lysis by soluble delta hemolysin by incubation for 30 min at 37 C with various amounts of lysin dissolved in the protoplast- or spheroplast-suspending buffer (1 ml). The amount of lysin required to produce half-maximal lysis (half-maximal decrease in optical density) was determined by interpolation.

Disruption of erythrocyte membranes. Rabbit erythrocyte membranes were prepared as described by Dodge et al. (16). The membrane suspension (0.5 ml) having an optical density at 650 nm of approximately 0.4 was examined for susceptibility to lysis by soluble delta hemolysin by incubation for 30 min at 37 C with various amounts of lysin dissolved in the membrane-suspending hypotonic (7.6×10^{-3} M, *p*H 7.4) phosphate buffer (0.5 ml). The amount of lysin required to produce half-maximal lysis (half-maximal decrease in optical density) was determined by interpolation.

Disruption of lysosomes. Rabbit polymorphonuclear leukocyte lysosomes were isolated as described by Weissmann et al. (65) and were suspended in 0.34 M sucrose. The lysosome suspension (0.9 ml), having an optical density at 520 nm of approximately 0.5, was tested for susceptibility to disruption by delta lysin by incubation for 30 min at 37 C with various amounts of lysin dissolved in PBS containing 0.34 M sucrose (0.1 ml). The amount of lysin required to produce half-maximal lysis was determined by interpolation.

Lipid spherule disruption. Spherules were prepared,

assayed for chromate leakage, and examined in the electron microscope as previously reported (21). The lysin was dissolved in distilled water.

Antibacterial activity. The ability of soluble delta hemolysin to inhibit the growth of various bacteria was examined by the tube dilution method. Doubling dilutions (1 ml) of lysin in 0.9% sterile saline were added to double concentrated (1 ml) Trypticase Soy Broth (TSB, BBL). Todd-Hewitt Broth (BBL) was used instead of TSB for the growth of the group A *Streptococcus*. Each assay mixture was inoculated with one loop (5 mm diameter) of a 24-hr culture of the test bacterium, and the mixtures were incubated at 37 C for 4 days. The tubes were examined daily for growth.

Clearing of egg yolk-agar. Egg yolk-agar was composed of 1.25% (v/v) egg yolk, 1.5% (w/v) agar (Difco), and 0.01% (w/v) Merthiolate in 0.05 M tris (hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 7.6). Ten millimeter holes were cut in the agar, and various amounts of soluble delta hemolysin, dissolved in saline (0.2 ml), were added to the wells. The plates were incubated for 5 days at 37 C and were examined daily.

Toxicity. Various amounts of soluble delta hemolysin, dissolved in 0.9% sterile saline, were injected intravenously into female Swiss mice (20 to 25 g) and intracardially into female guinea pigs (330 to 350 g). Animals surviving for less than 24 hr were scored as deaths.

Various amounts of soluble delta hemolysin and 12S-free alpha hemolysin (8), dissolved in 0.1 ml of 0.9% saline (containing 0.1% gelatin for the alpha lysin) and sterilized by membrane (Millipore Corp.) filtration, were injected intradermally into shaved female New Zealand white rabbits and guinea pigs. The animals were examined daily. The ability of lecithin to inhibit the dermonecrosis produced by delta lysin and alpha lysin was examined by allowing the lysins to stand at room temperature for 10 min with 0.5 mg of lecithin before injection.

RESULTS

Purification procedure. All procedures were done at approximately 5 to 10 C. The cultures were pooled and centrifuged to remove the cocci. The supernatant fluid (approximately 6 liters, containing 300 to 400 HU/ml) was mixed for 3 hr with 120 g of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories, New York, N.Y.), allowed to stand overnight, and tested for residual delta hemolysin activity. Approximately 98% of the initial activity was adsorbed. The supernatant fluid was discarded, and the hydroxyapatite was washed free from adhering supernatant fluid with 0.01 M potassium phosphate buffer (*p*H 6.8) followed by six washings (500 ml each) with 0.4 M phosphate buffer (*p*H 6.8) for 30 to 60 min each time. These washings, which were discarded, contained approximately 5 to 10% of the adsorbed hemolytic activity and the majority of the adsorbed pigment. The adsorbed delta hemolysin activity was released by washing the hydroxyapatite five to six times with 1 M phosphate (*p*H 7.4) buffer (400 ml each time) for 30 to 60 min. The hemolytic supernatant fluid, containing about 50% of the adsorbed activity, was centrifuged to remove traces of hydroxyapatite and exhaustively dialyzed against water to remove the phosphate ions. The precipitate appearing during dialysis was removed by centrifugation, washed three times with water, and lyophilized. This precipitate, a buff-colored powder, was called "insoluble delta hemolysin." The fluid in the dialysis sac (approximately 3.7 liters) was concentrated to approximately one-fifth to one-sixth its original volume by dialysis against either polyvinylpyrrolidone (A. H. Thomas, Co., Philadelphia, Pa.) or polyethylene glycol 20,000 (Fisher Scientific Co., Fair Lawn, N.J.), exhaustively dialyzed against water, centrifuged to remove a trace of precipitate, and lyophilized. This final product, a fluffy-white powder, was designated "soluble delta hemolysin." Each liter of culture supernatant fluid yielded approximately 50 to 70 mg of insoluble delta hemolysin having about 400 HU/mg and approximately 200 to 250 mg of soluble delta hemolysin having about 200 HU/mg. The activity of the soluble delta hemolysin was observed to vary by as much as 25 to 50% with different lots of horse erythrocytes. The specific activity of the soluble delta hemolysin (HU/milligram of protein) was about 15 times greater than that of the culture supernatant fluid. The total activity of both forms of delta lysin represents approximately 20 to 30% of the culture supernatant fluid activity.

Solubility and stability in various buffers and organic solvents. Soluble delta hemolysin was

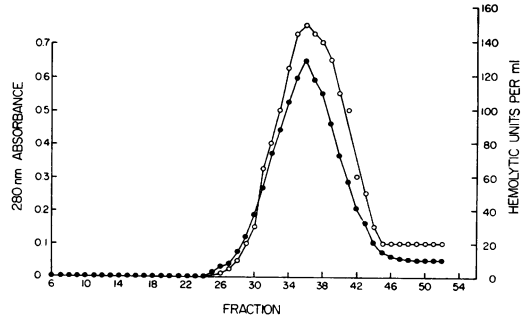


FIG. 1. *Sucrose density gradient centrifugation of soluble delta hemolysin. Fractions (eight drops) were diluted with phosphate-buffered saline (1 ml) and assayed for hemolytic activity* (○) *and for absorbance at 280 nm* (●).

soluble and stable for at least 7 days at 5 C in water, a variety of 0.1 M buffers ranging from *p*H 5 to 9, 0.1 M sodium hydroxide and acetic acid, 6 M guanidine hydrochloride, 8 M urea, and 0.1% sodium lauryl sulfate. The lysin was insoluble in chloroform, acetone, and ether but was soluble in chloroform–methanol (2:1), methanol, and 75% ethanol. The insoluble delta hemolysin was poorly soluble (<0.05 mg/ml) in water and in a variety of 0.1 M buffers unless they contained 8 M urea. It was also soluble and stable in 0.1 M sodium hydroxide and chloroform–methanol (2:1).

Examination for other staphylococcal products. Esterase, lipase, caseinase, gelatinase, deoxyribonuclease, ribonuclease, coagulase, staphylokinase, hyaluronidase, lysozyme, acid phosphatase, and alkaline phosphatase activities were not detected in the soluble delta hemolysin preparation. No phospholipase C activity was detected when phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol, and sphingomyelin were used as substrates.

Sucrose density gradient centrifugation. No inactive 280-nm absorbing peaks were observed. One broad active peak trailing toward the top of the gradient was observed (Fig. 1).

Gel filtration chromatography. No inactive 280-nm absorbing peaks were observed. Activity eluted from Sephadex G-150 (Fig. 2) as a void volume peak trailing almost the entire length of the column. Activity eluted from Bio-Gel A-5m (Fig. 3) as two peaks. One peak eluted slightly after the void volume. Both this material and the more slowly eluted lysin had similar activities per milligram (dry weight); however, the slowly eluted lysin had approximately twice the activity of the rapidly eluted lysin per 280-nm absorbance unit; i.e., the rapidly eluted lysin had about twice the absorbance at 280 nm per hemolytic unit as the slowly eluted lysin.

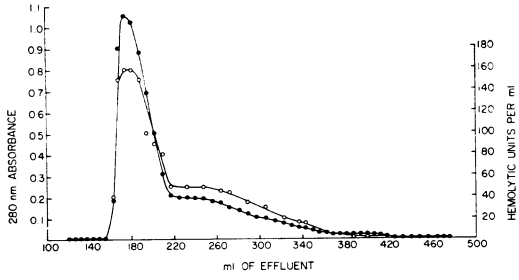


FIG. 2. *Sephadex G-150 gel filtration of soluble delta hemolysin. Lysin (70 mg) was applied to a column (2.5 by 90 cm) and eluted at a flow rate of approximately 15 ml/hr with 0.05 M phosphate buffer (pH 7.2) containing 0.5 M sodium chloride. Fractions (5 to 7 ml) were assayed for hemolytic activity (○) and for absorbance at 280 nm (●). Column void volume was approximately 166 ml.*

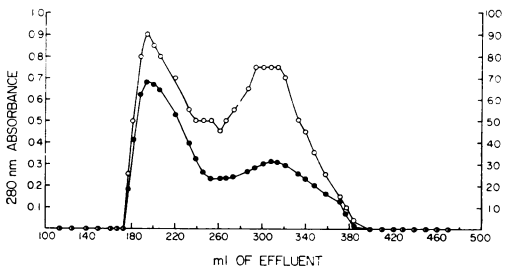


FIG. 3. *Bio-Gel A-5m gel filtration of soluble delta hemolysin. Lysin (50 mg) was applied to a column (2.5 by 92 cm) and eluted at a flow rate of approximately 10 ml/hr with 0.05 M tris (hydroxymethyl) aminomethane-hydrochloride buffer (pH 7.2) containing 0.5 M sodium chloride. Fractions (3 to 4 ml) were assayed for delta hemolysin activity (○) and for absorbance at 280 nm (●). Column void volume was approximately 170 ml. Alpha and beta hemolysin activity was not detected in the fractions.*

Analytical ultracentrifugation. Sedimentation of delta hemolysin in various solvents (Fig. 4) showed that the soluble lysin sedimented at *p*H 7 as one peak having a polydisperse trailing edge and that it could be converted into more slowly sedimenting units by high and low *p*H, guanidine hydrochloride, urea, and sodium lauryl sulfate. Sedimentation analysis of the lysin fractions which were rapidly and slowly eluted from Sephadex G-150 (Fig. 4B) showed that the soluble delta lysin preparation was composed of a mixture of 11.9*S* lysin (Fig. 5) and a population of more slowly sedimenting molecules, with the most rapid being approximately 4.9*S*. The sedimentation coefficient of the 11.9*S* lysin showed a marked concentration dependence (Fig. 5). When dissolved in 0.05 M sodium hydroxide, both the insoluble and soluble lysins had molecules with an $S_{20,w}$ of approximately 1.9; however, the insoluble lysin preparation also showed a peak with a sedimentation coefficient of approximately 16*S*. By means of a separation cell, a portion of the 1.9*S* component was isolated free from the 16*S* component. The hemolytic activity resided in the 1.9*S* component. We have observed that the conversion of soluble delta hemolysin to the 1.9*S* form by alkali and urea is reversible upon removal of these reagents. It cannot, therefore, be conclusively stated that the hemolytic moiety of delta lysin has a sedimentation coefficient of 1.9*S*. The hemolytic assay involves dilutions in PBS to remove the

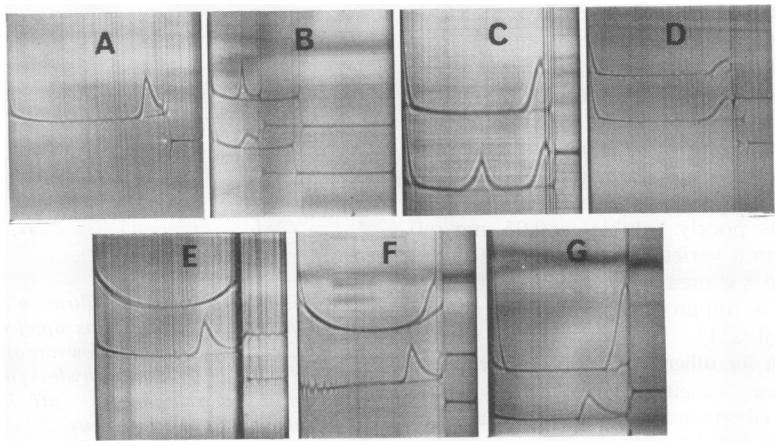


FIG. 4. *Schlieren patterns obtained during ultracentrifugation of delta hemolysin in different solvents. Photographs were taken at 4- or 8-min intervals after the rotor had attained two-thirds maximum speed. (A) Soluble hemolysin [0.6% (w/v)] in 0.05 M phosphate buffer (pH 7), 16 min. (B) Top pattern is lysin eluted at void volume of Sephadex G-150, and bottom pattern is lysin eluted more slowly from Sephadex G-150. Both are 0.45% (w/v) solutions in 0.05 M phosphate buffer (pH 7). 12 min. (C) Top pattern is soluble lysin and bottom pattern is insoluble lysin. Both are 0.6% (w/v) solutions in 0.05 M sodium hydroxide, 24 min. (D) Soluble lysin dissolved in 0.05 M acetic acid. Bottom pattern is a 0.5% (w/v) solution and top pattern is a 0.25% (w/v) solution, 32 min. (E) Top pattern is 0.6% (w/v) soluble lysin in 0.05 M phosphate buffer (pH 7) containing 6 M guanidine hydrochloride and 0.5% (v/v) mercaptoethanol, 32 min. (F) Top pattern is 0.6% (w/v) soluble lysin in 0.05 M phosphate buffer (pH 7) containing 8 M urea, 32 min. (G) Top pattern is 0.6% (w/v) soluble lysin in 0.05 M tris (hydroxymethyl) aminomethane-hydrochloride buffer (pH 7) containing 0.1% (w/v) sodium lauryl sulfate, 32 min. Bottom patterns in (E), (F), and (G) are of 0.6% (w/v) soluble lysin in buffers without dissociating agents.*

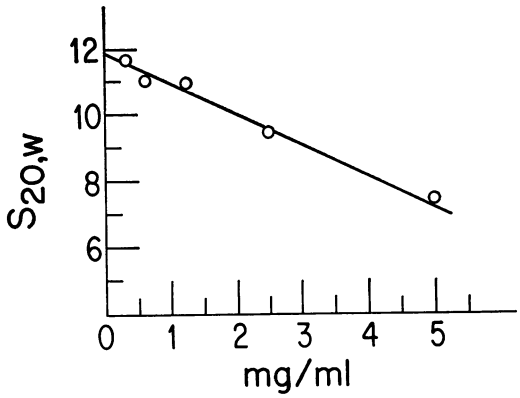


FIG. 5. *Sedimentation coefficient of soluble delta hemolysin eluting at the void volume of Sephadex G-150 as a function of concentration.*

dissociating agents and may allow reaggregation to the active moiety.

Isoelectric focusing. No inactive 280-nm absorbing peaks were observed. Two distinct delta hemolysin peaks, both inhibitable by lecithin and rabbit serum, were observed (Fig. 6). The main peak had an isoelectric *p*H (*p*I) of approximately 9.5 ± 0.3 and represented approximately 70% of the total activity. Most of the remaining activity focused at *p*I 5.0 ± 0.2. Both lysins had similar activities per milligram (dry weight), but the basic lysin had approximately twice the activity of the acidic lysin per 280-nm absorbance unit; i.e., the acidic lysin had about twice the 280-nm absorbance per hemolytic unit as the basic lysin. Refocusing of the basic lysin yielded additional acidic lysin; however, refocusing of the acidic lysin did not yield additional basic lysin. Isoelectric focusing of soluble delta hemolysin in a column containing 6 M urea gave results similar to those obtained without urea.

CM cellulose chromatography. Approximately 20% of the input hemolytic activity did not adsorb to the column. The remainder was eluted in a broad peak at a sodium chloride concentration of approximately 0.3 M (Fig. 7). About 70% of the adsorbed activity was recovered.

Polyacrylamide disc gel electrophoresis. Two bands were observed in the acidic system and in the basic system (Fig. 8). In the acidic system, both bands migrated into the separating gel. The bands were close to one another, but one of the bands stained more deeply and was less diffuse than the other band. In the basic system, one of the bands stayed near the top of the

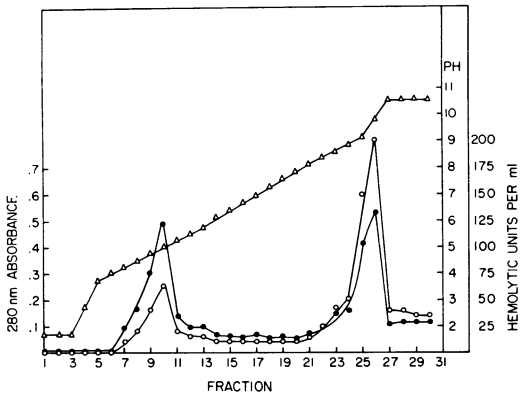


FIG. 6. *Isoelectric focusing of soluble delta hemolysin. The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 8% (w/v) ampholine (pH 3 to 10; LKB Instruments, Rockville, Md.), and 20 mg of soluble delta hemolysin and a more dense solution consisting of 32 ml of water, 8.5 ml of 8% (w/v) ampholine (pH 3 to 10), and 25 g of sucrose. Focusing was done at about 4 C for 2 days in a 110-ml electrolysis column (LKB Instruments) with a final potential of 600 v. Fractions (4 ml) were examined for absorbance at 280 nm (●), delta hemolysin activity (○), and pH at approximately 25 C (△). The 280-nm absorbance values were corrected for background absorbance contributed by the ampholine and sucrose. About 90% of the input activity was recovered. Alpha and beta hemolysin activity was not detected in the fractions.*

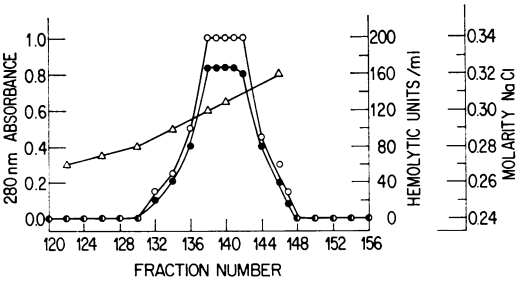


FIG. 7. *Carboxymethyl cellulose chromatography of soluble delta hemolysin. Fractions (5 ml) were assayed for delta hemolysin activity (○), absorbance at 280 nm (●), and sodium chloride concentration (△). Alpha and beta hemolysin activity was not detected in the fractions.*

separating gel but the other migrated further down the gel.

Chemical analysis. The insoluble delta hemolysin preparation contained approximately 1 to 2% phosphorus. The soluble delta hemolysin preparation contained less than 0.025% phosphorus, less than 1% carbohydrate (as glucose) and amino sugar (as glucosamine), and less than

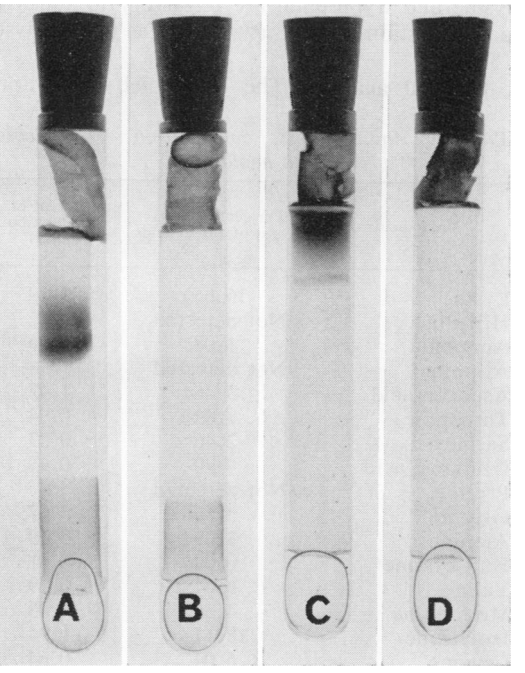


FIG. 8. *Polyacrylamide disc gel electrophoresis of soluble delta hemolysin. (A) A 75-μg amount of lysin in the acidic (pH 4.3) 7.5% gel system of Reisfeld et al. (59). Migration is toward the cathode. (B) Control acidic gel (no lysin). (C) A 130-μg amount of lysin in the basic (pH 9.5) 7% gel system of Ornstein (57) and Davis (14). Migration is toward the anode. (D) Control basic gel (no lysin).*

1% ash. The soluble lysin contained approximately 1% bound lipid but less than 0.5% free lipid. Elemental analysis yielded 53.77% C, 7.27% H, 13.38% Dumas N, 11.90% Kjeldahl N, and 0.82% S.

Amino acid composition. Isoleucine (18.17%), lysine (16.66%), and aspartic acid (13.46%) were the major amino acids in the preparation (Table 1). The hydrophobic amino acids, isoleucine, leucine, and valine, accounted for approximately 31% of the amino acids in the preparation. No histidine, arginine, proline, or tyrosine was detected even when 3.0 mg of hydrolyzed lysin preparation was examined. Qualitative tests for these four missing amino acids in the unhydrolyzed protein were also negative. The value for nitrogen content, as calculated from the amino acid composition data, agrees well with that obtained by the Dumas method. The low value for nitrogen content, as determined by the Kjeldahl method, is most likely caused by incomplete digestion of the protein with Cu^{2+} as the catalyst. It is known that

lysine and tryptophan are refractory to sulfuric acid digestion when Cu^{2+} is used as the catalyst (53).

Spectral analysis. The ultraviolet absorption spectrum (Fig. 9) was similar to that reported by Kayser and Raynaud (42). It had a maximum at approximately 282 nm, a minimum at approximately 250 nm, and a distinct shoulder at about 291 nm. The 280:260 absorbance ratio was approximately 1.5. The infrared absorption spectrum (Fig. 10) did not indicate the presence of significant amounts of lipid. A distinct carbonyl absorption peak was not observed.

TABLE 1. *Amino acid composition of soluble delta hemolysin*

Amino acid	Per cent of total weight of amino acids	Per cent of total amino acid N
Lysine	16.66	3.19
Histidine	Not detected	
Ammonia	1.08	0.95
Arginine	Not detected	
Aspartic acid	13.46	1.42
Threonine	8.02	0.94
Serine	2.46	0.33
Glutamic acid	4.90	0.47
Proline	Not detected	
Glycine	3.60	0.67
Alanine	3.09	0.49
Half-cystine[a]	0.09	0.01
Valine	6.64	0.79
Methionine	4.56	0.43
Isoleucine	18.17	1.94
Leucine	6.04	0.64
Tyrosine	Not detected	
Phenylalanine	7.93	0.67
Tryptophan	3.30	0.45
Total	100.00	13.39

[a] Determined as cysteic acid.

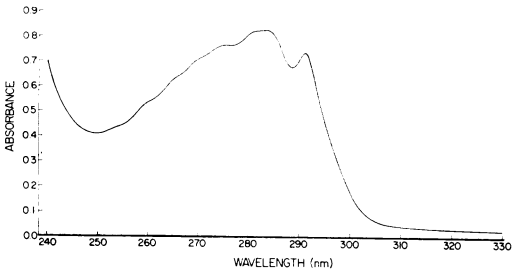


FIG. 9. *Ultraviolet absorption spectrum of soluble delta hemolysin. The lysin (1 mg/ml) was dissolved in phosphate-buffered saline (pH 7).*

Electron microscopy. The soluble delta hemolysin preparation was composed of a mixture of fibers and granular aggregates of various sizes. Examination of the Sephadex G-150, Bio-Gel A-5m, and isoelectric focusing fractions revealed that the fibrous material (Fig. 11A) was the lysin which (i) eluted in the void volume of Sephadex G-150, (ii) was the main component in the first peak eluting from the Bio-Gel A-5m column, and (iii) had a *p*I of approximately 5.0. The granular material (Fig. 11B) was the lysin which (i) was found in the trailing portion of the Sephadex G-150 curve, (ii) was the main component in the second peak eluting from the Bio-Gel A-5m column, and (iii) had a *p*I of approximately 9.5.

Inactivation studies. All of the phospholipids and normal sera tested inhibited delta hemolysin activity (Table 2). Cholesterol had no effect. All of the proteolytic enzymes tested inactivated the lysin; however, trypsin and Pronase were most potent (Table 3). The lysin was resistant to heat inactivation. Heating for 1 hr at temperatures as high as 80 C had no effect. Heating for 1 hr at 100 C reduced activity by approximately 50%. Even after autoclaving (121 C) for 1 hr, there was still about 10% residual activity. Citrate, EDTA, Mg^{2+}, and Ca^{2+} at concentrations as high as 10^{-3} M had no effect on activity. The lysin was not cysteine-activable.

Lysis of erythrocytes from different animal species. Soluble delta hemolysin lysed erythrocytes from all of the animal species tested; however, horse, rabbit, and human erythrocytes were most sensitive (Table 4).

Synergism of delta and beta hemolysins. Staph-

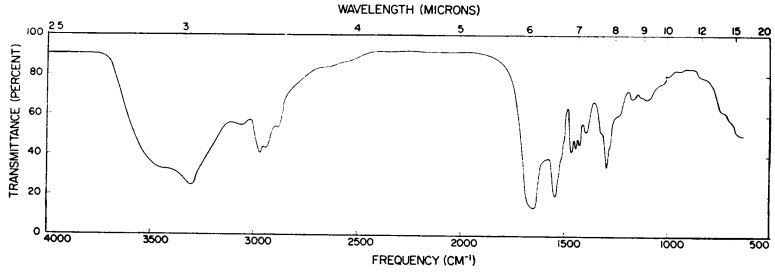


FIG. 10. *Infrared absorption spectrum of soluble delta hemolysin. The lysin was examined as a potassium bromide pellet.*

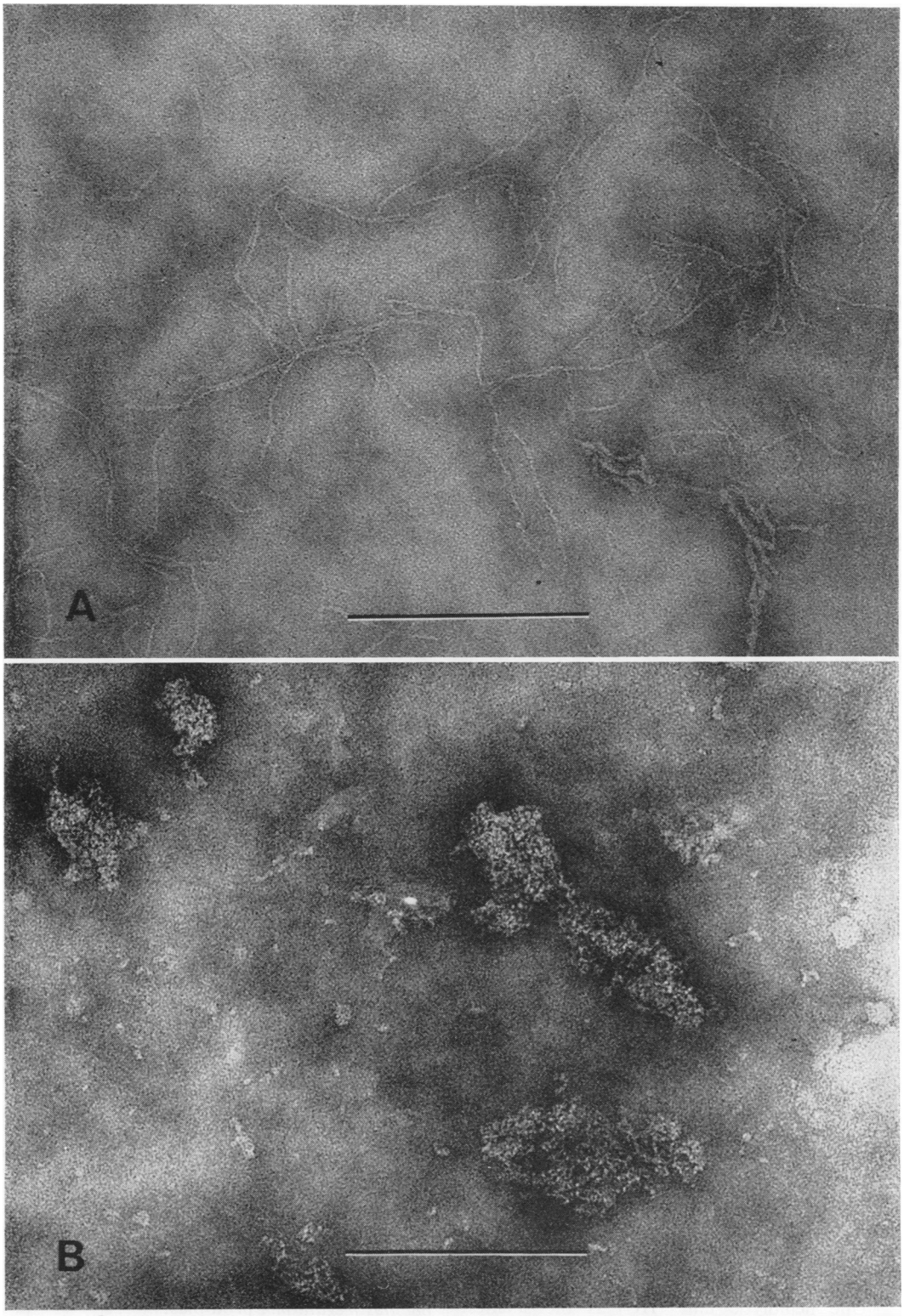


FIG. 11. *Electron micrographs of soluble delta hemolysin. (A) Lysin eluting at the void volume of Sephadex G-150. Note the fibrous appearance. (B) Lysin found in the fractions of the trailing edge during Sephadex G-150 filtration. Note the granular aggregates of various sizes. Line markers denote distance of 0.25 μm. × 150,000.*

TABLE 2. *Inhibition of soluble delta hemolysin by phospholipids and normal sera*

Substance	Amt required to inhibit ¾ of the test amt[a] of delta lysin (μg)
Phosphatidylcholine (lecithin)	2
Phosphatidylserine	2
Phosphatidylinositol	4
Phosphatidylethanolamine	8
Diphosphatidylglycerol (cardiolipin)	4
Sphingomyelin	8
Cholesterol	>1,000
Horse serum	2[b]
Human serum	1[b]
Rabbit serum	2[b]

[a] Three hemolytic units.
[b] Measured as microliters.

TABLE 3. *Inactivation of soluble delta hemolysin by proteolytic enzymes*

Enzyme	Per cent of control hemolytic activity[a] remaining
Trypsin	
100 μg	1
10 μg	40
Chymotrypsin	
100 μg	10
10 μg	80
Pronase	
100 μg	2
10 μg	40
Papain	
100 μg	40
10 μg	100

[a] Control hemolytic activity was 200 hemolytic units.

TABLE 4. *Lysis of erythrocytes from different animal species by soluble delta hemolysin*

Animal	HU[a]/mg
Horse	200
Rabbit	200
Human	100
Guinea pig	50
Pig	40
Calf	40
Sheep	40
Goat	20
Cat	20
Chicken	20

[a] One hemolytic unit (HU) is the smallest amount of lysin, contained in 1 ml of phosphate-buffered saline, which will produce 50% lysis of 1 ml of a 0.7% (v/v) washed erythrocyte suspension after incubation for 30 min at 37 C.

TABLE 5. *Synergistic effect of delta and beta hemolysins on sheep erythrocytes as a function of delta hemolysin concentration*

Amt of delta lysin (μg)	Per cent hemolysis[a]
5.00	100
2.50	90
1.25	50
0.63	30
0.31	20
0.16	10
0	10
Control[b]	0

[a] Determined after incubation of various amounts of delta lysin and a constant amount (10 hemolytic units in the hot-cold assay system) of staphylococcal beta hemolysin (kindly supplied by W. Chesbro, Department of Microbiology, University of New Hampshire) in 1 ml of buffer used in the beta hemolysin assay with 1 ml of a 0.7% washed sheep erythrocyte suspension for 30 min at 37 C.
[b] Five micrograms of delta lysin but no beta lysin.

TABLE 6. *Synergistic effect of delta and beta hemolysins on sheep erythrocytes as a function of beta hemolysin concentration*

Amt of beta lysin (HU)[a]	Per cent hemolysis[b]
10.0	100
5.0	100
2.5	0
1.3	0
0.6	0
0	0
Control[c]	10

[a] Hemolytic units.
[b] Determined after incubation of various amounts of beta lysin and a constant amount (5 μg, approximately 0.2 HU with sheep erythrocytes) of delta lysin in 1 ml of buffer used in the beta hemolysin assay with 1 ml of a 0.7% washed sheep erythrocyte suspension for 30 min at 37 C.
[c] Fifteen HU of beta lysin but no delta lysin.

TABLE 7. *Lysis of bacterial protoplasts and spheroplasts by soluble delta hemolysin*

Prepn	Concn of lysin required to produce half-maximal lysis (μg/ml)
Sarcina lutea protoplasts	12
Streptococcus faecalis protoplasts	15
Bacillus megaterium protoplasts	12
Escherichia coli spheroplasts	10
Micrococcus lysodeikticus protoplasts	24
Vibrio metschnikovii spheroplasts	>1,000

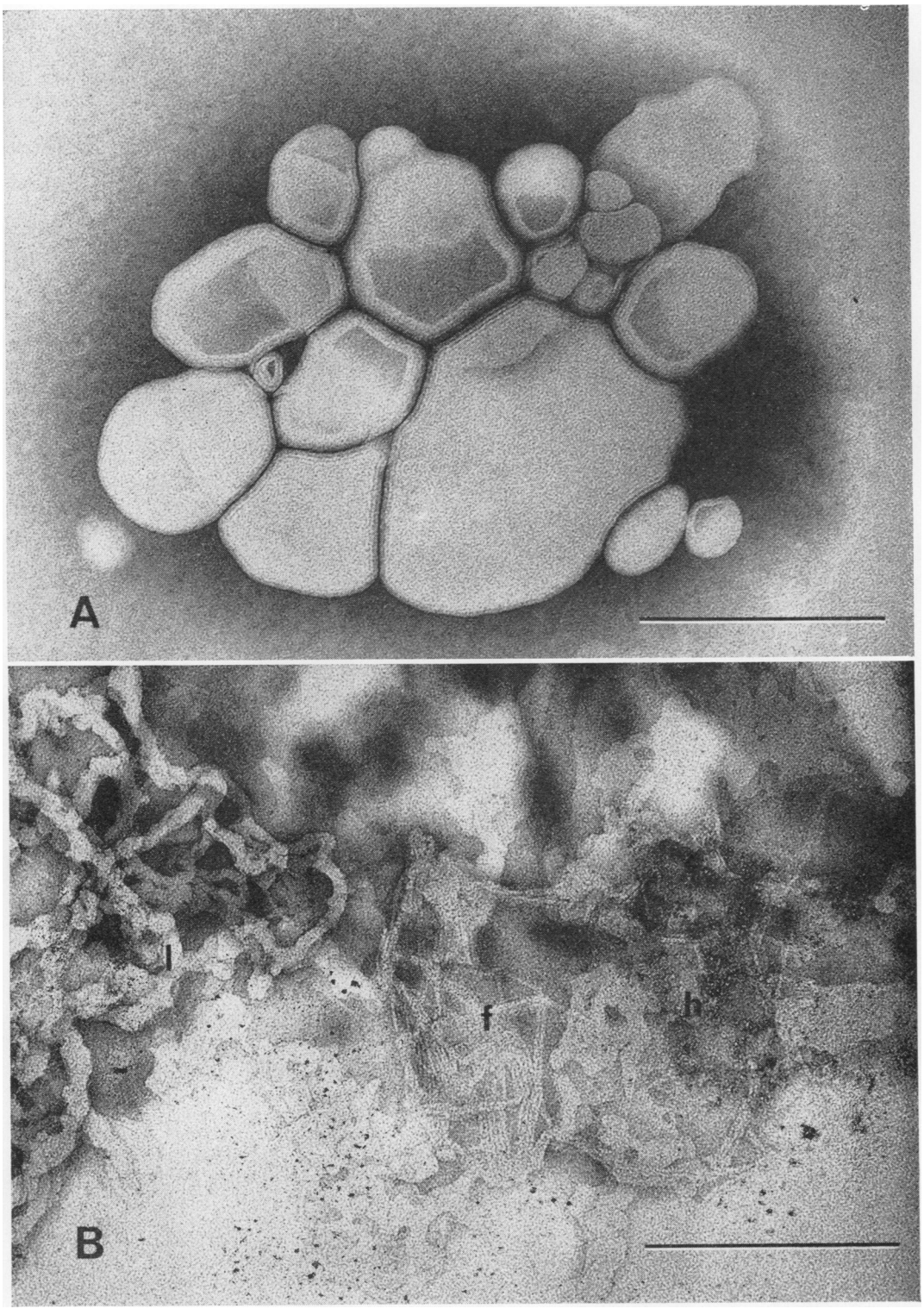


FIG. 12. *Disruption of lipid spherules by soluble delta hemolysin. (A) Normal spherule preparation; (B) spherule preparation incubated for 1 hr at 37 C with 200 μg of soluble delta lysin. Note the presence of holes (h), fibrous material (f), and disorganized lipid (l). Line markers denote distance of 0.25 μm. × 150,000.*

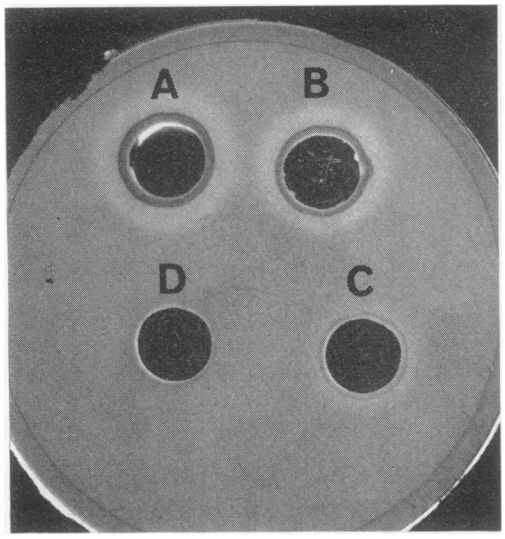


FIG. 13. *Clearing of egg yolk-agar by soluble delta hemolysin. Egg yolk-agar was incubated for 3 days at 37 C with various amounts of lysin in saline (0.2 ml). (A) A 1-mg amount, (B) 0.5 mg, (C) 0.25 mg, (D) saline control. The dark area around the wells is the opaque area of precipitation.*

ylococcal beta and soluble delta lysins exhibited a synergistic effect on sheep erythrocytes (Tables 5 and 6). The ratio of delta to beta hemolysin having the optimum synergistic effect was approximately 0.2 HU (with sheep erythrocytes) of delta lysin (approximately 5 μg) to 5 to 10 HU of beta lysin.

Lysis of bacterial protoplasts and spheroplasts. Soluble delta hemolysin disrupted five of the six protoplast and spheroplast preparations (Table 7). *M. lysodeikticus* protoplasts required about twice the amount of lysin to produce half-maximal lysis than did the other four preparations. The lysin had no effect on *V. metschnikovii* $_S$pheroplasts.

TABLE 8. *Lethality of soluble delta hemolysin for mice and guinea pigs*

Amt of lysin injected (mg)	No. of animals dead/no. of animals injected	Approx survival time (min)
Mice[a]		
2.5	12/12	15
2.0	6/12	35
1.5	0/12	
1.0	0/12	
0	0/12	
Guinea pigs[b]		
20	8/8	10
10	8/8	12
5	0/8	
0	0/8	

[a] A 0.25-ml amount injected intravenously.
[b] A 2-ml amount injected intracardially.

Disruption of rabbit erythrocyte membranes and leukocyte lysosomes. Amounts of lysin of 500 and 25 μg/ml produced half-maximal lysis of the rabbit erythrocyte membrane and leukocyte lysosome suspensions, respectively. The activities were inhibited by pretreatment of the lysin with rabbit serum.

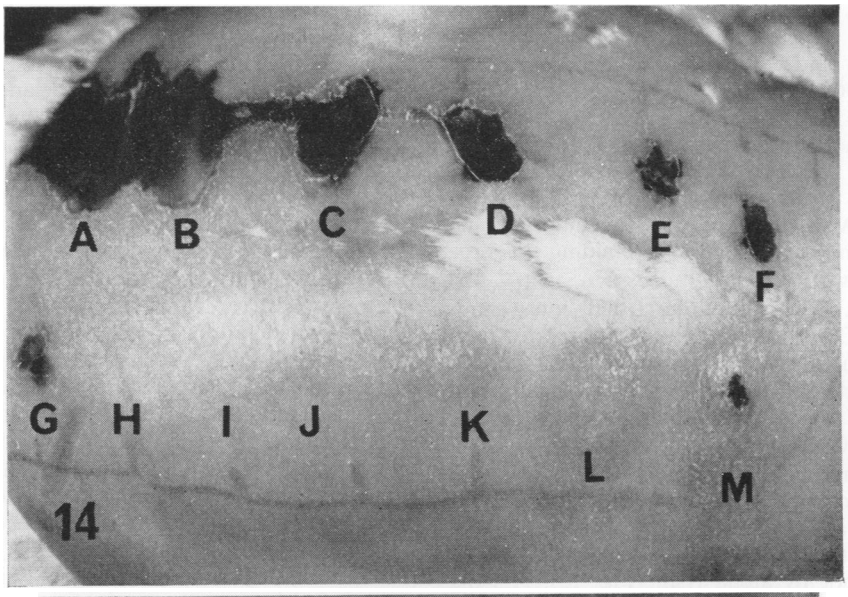
Lipid spherule disruption. Five HU of soluble delta hemolysin incubated for 1 hr at 37 C with the spherule preparation released approximately 90% of the chromate ions trapped in the spherule preparation. Disruption of a normal spherule preparation (Fig. 12A) by lysin could also be observed in the electron microscope. Treated preparations contained holes, fibrous material, and disorganized lipid (Fig. 12B).

Antibacterial activity. Growth of *B. megaterium* KM was prevented for 4 days by 63 μg of soluble delta hemolysin per ml. Growth of *M. lysodeikticus* and a group A *Streptococcus* (strain C203s) was inhibited for 1 day by 500 μg/ml, after which time growth commenced. Growth of *Gaffkya tetragena* (ATCC 10875), *Lactobacillus casei*

FIG. 14. *Dermonecrosis produced in rabbits by staphylococcal alpha and delta hemolysins. Results shown are 7 days postinjection. A through F show dermonecrosis produced by 12S-free alpha hemolysin. (A) A 10.8-μg amount, (B) 5.4 μg, (C) 2.7 μg, (D) 1.35 μg, (E) 0.68 μg, (F) 0.34 μg. G through M show results obtained with delta hemolysin. (G) A 1-mg amount (lot 39, kindly supplied by F. A. Kapral, Department of Medical Microbiology, Ohio State University), (H) saline control, (I) 0.06 mg of our purified soluble delta hemolysin, (J) 0.13 mg, (K) 0.25 mg, (L) 0.5 mg, and (M) 1.0 mg.*

FIG. 15. *Inhibition by lecithin of dermonecrosis produced in rabbits by delta hemolysin. Results shown are 6 days postinjection. (A) saline-lecithin (0.5 mg) control, (B) 2.7 μg of alpha hemolysin and 0.5 mg of lecithin, (C) 2.7 μg of alpha lysin, (D) 1 mg of delta lysin and 0.5 mg of lecithin, (E) 1 mg of delta lysin, (F) 0.5 mg of delta lysin, (G) 0.5 mg of delta lysin (lot 17, from F. A. Kapral), (H) 1 mg of delta lysin (from F. A. Kapral) and 0.5 mg of lecithin, and (I) 1 mg of delta lysin (from F. A. Kapral).*

FIG. 16. *Dermonecrosis produced in guinea pigs by staphylococcal alpha and delta hemolysins. Results shown are 7 days postinjection. (A) A 1-mg amount of delta lysin (lot 39, from F. A. Kapral), (B) saline control, (C) 0.063 mg of our purified soluble delta lysin, (D) 0.125 mg, (E) 0.25 mg, (F) 0.5 mg, (G) 1 mg, and (H) 5.4 μg of alpha lysin.*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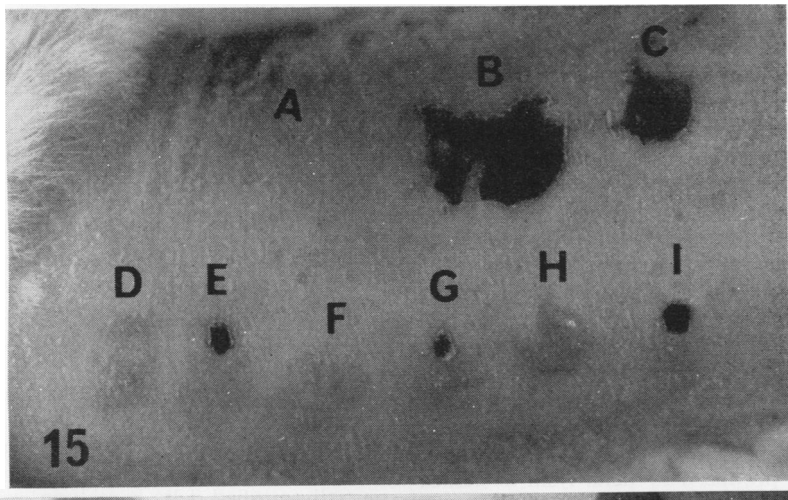
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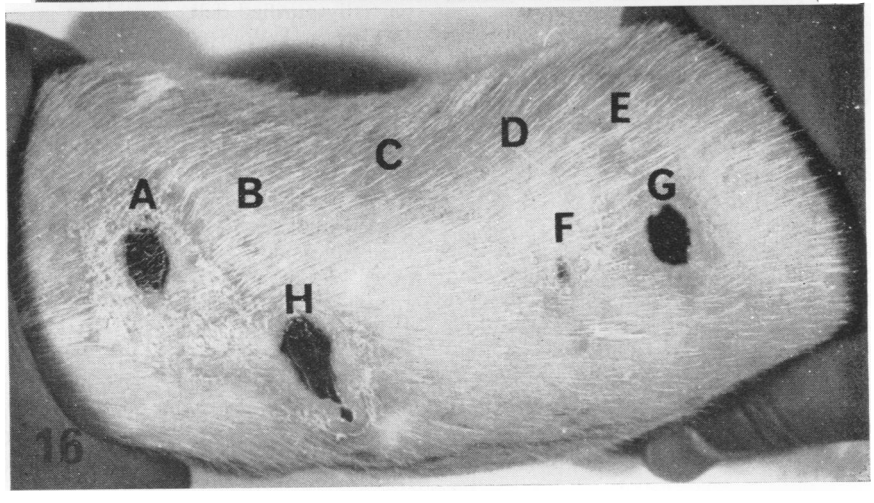
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(subspecies *rhamnosus*, ATCC 7469), *Corynebacterium diphtheriae* (mitis C7), *S. aureus* (Wood 46 and W46M strains), *B. subtilis*, *B. cereus* (ATCC 10876a), *S. faecalis* (ATCC 9790), *E. coli* K-12, *Pseudomonas aeruginosa*, and *S. lutea* was not affected by as much as 1,000 μg/ml.

Clearing of egg yolk-agar. Soluble delta hemolysin produced clearing of egg yolk-agar (Fig. 13). An opaque area of precipitation around the well and a clear halo surrounding this area were observed.

Toxicity. The minimum lethal intravenous dose (MLD) of soluble delta hemolysin for mice and guinea pigs was approximately 2.5 and 10 mg, respectively (Table 8). On a weight basis, the MLD was approximately 110 mg/kg for mice and 30 mg/kg for guinea pigs.

Large amounts of soluble delta lysin were required to produce dermonecrosis in rabbits (Fig. 14) and guinea pigs (Fig. 16). Lecithin prevented the dermonecrosis produced by delta lysin but not by alpha lysin (Fig. 15). A 1-mg amount of delta lysin injected intradermally into rabbits produced, after 1 day, a large (approximately 28 mm in diameter) erythematous, hard, indurated lesion with a small (4 mm in diameter) whitish-yellow center. This small area became necrotic by 3 days postinjection. The other amounts of lysin injected produced erythematous, indurated lesions which never became necrotic. Some desquamation was observed by 2 days postinjection with 1 and 0.5 mg. Samples of soluble delta hemolysin kindly supplied by F. A. Kapral (Department of Medical Microbiology, Ohio State University) were more potent, both in the presence and absence of lecithin, than our purified lysin. A 0.5-mg amount of his lysin also produced dermonecrosis in rabbits (Fig. 15).

The lesions produced by delta lysin in guinea pigs resembled those observed in rabbits; however, the necrotic areas developed more rapidly and were larger in the guinea pig than in the rabbit. A 0.5-mg amount of delta lysin produced a small (2 mm in diameter) necrotic lesion in the guinea pig but not in the rabbit.

DISCUSSION

Perhaps the most important aspect of the current study is that it provides a simple and reproducible method for obtaining large amounts of highly purified delta lysin which can be used for further experimentation. Our success may depend upon use of a mutant *Staphylococcus* which produces very large amounts of delta lysin but relatively small amounts of other staphylococcal exoproducts. Further comparison of the culture supernatant fluids of the parent and mutant strains should show whether this is the correct explanation.

As previously observed with crude and partially purified delta hemolysin, our highly purified lysin preparation (i) disrupts erythrocytes obtained from many animal species, bacterial protoplasts and spheroplasts, lysosomes, lipid spherules, and erythrocyte membranes; (ii) acts synergistically with staphylococcal beta hemolysin; (iii) is lethal and produces dermonecrosis when administered in large amounts; (iv) possesses limited antibacterial activity; and (v) clears egg yolk-agar. These confirmatory findings support the belief that all of the effects are caused by delta hemolysin and not by a contaminating staphylococcal product. In addition, the observation that the relative sensitivities of the different protoplasts and spheroplasts to disruption by delta lysin were similar to those reported (5) for alpha lysin supports the idea (7) that the protoplast-lysing activity of alpha lysin preparations is due to contamination with delta hemolysin.

Hallander (28) reported that delta lysin obtained from the S6 strain of *S. aureus* was more negatively charged at *p*H 8.2 than lysin from the 196E strain. In addition to the difference between the two delta lysins studied, each lysin was molecularly heterogeneous when examined by agarose gel filtration and by Sephadex gel electrophoresis. Hoffmann and Streitfeld (32) suggested that two lysins, separable by paper chromatography, might be present, delta A and delta B. Jackson and Little (36) described one heat-stable ethanol-soluble and one heat-labile ethanol-insoluble lysin fraction. Guyonnet and Plommet (26) think that the heat-labile ethanol-insoluble lysin was, in reality, staphylococcal gamma hemolysin. Isoelectric focusing of a partially purified delta hemolysin preparation by Möllby and Wadström (54) yielded only one peak of activity; however, Maheswaran and Lindorfer (Bacteriol. Proc., p. 78, 1970) isolated delta lysins with three different isoelectric points. Our findings support the idea that staphylococcal delta hemolysin is molecularly heterogeneous, i.e., it may, as staphylococcal alpha lysin (8, 64) and beta lysin (13, 30, 48), exist in more than one active physicochemical form. Many other bacterial toxins are known to be physicochemically heterogeneous (2).

There are at least five major unexplained differences between our purified delta lysin preparation and the purified preparation studied by Caird and Wiseman (12, 67). First, their preparation possesses phospholipase C activity with phosphatidylinositol and phosphatidylserine

as substrates. We have not detected this activity in our preparation. Second, their preparation exhibits two peaks in the analytical ultracentrifuge. Ours shows one peak having a polydisperse trailing edge. Third, the hemolytic activity per milligram of protein of their preparation is approximately 45-fold greater than the specific activity of our preparation. The specific activity of their starting culture supernatant is approximately 20-fold greater than ours. Fourth, they report proline to be the N-terminal amino acid of the lysin. We have not detected proline in our preparation. Fifth, their lysin is inhibited by cholesterol, as reported by Gladstone and van Heyningen (23). Our lysin is not inhibited by cholesterol, as reported by Gladstone and Yoshida (24).

Hallander found (28) that partially purified delta lysin inhibited the growth of a strain belonging to the genus *Corynebacterium*. Hoffmann and Streitfeld (32) did not observe inhibition of *C. diphtheriae* or *B. megaterium* by partially purified delta lysin but reported inhibition of *G. tetragena*, *L. casei*, and *M. lysodeikticus*. We observed inhibition of *B. megaterium* and *M. lysodeikticus* but not of *G. tetragena*, *L. casei*, or *C. diphtheriae* (mitis C7).

The question of whether delta lysin is heat-stable is also controversial. Partially purified delta lysin has been reported to be stable to heating at 100 C for 30 min (28) and 120 min (50) and at 115 C for 20 min (23). One of the two fractions described by Jackson and Little (36) was stable at 60 C for 120 min. However, the lysin obtained by Yoshida (68) lost 85% of its activity after 30 min at 100 C. Our purified lysin preparation was partially heat-stable. It was not affected by heating at 80 C for 60 min but lost approximately 50% of its activity after 60 min at 100 C and about 90% of its activity after 60 min at 121 C.

Yoshida (68) mentioned the poor solubility of partially purified delta lysin in buffers at a neutral *p*H and a low ionic strength. Möllby and Wadström (54) observed the formation of a white flocculent delta lysin precipitate during isoelectric focusing; precipitation was prevented by the addition of a nonionic detergent, Triton X-100, to the *p*H gradient. Our purified delta hemolysin preparation, however, is soluble at neutral *p*H in low ionic strength buffer and does not precipitate during isoelectric focusing.

Partially purified delta hemolysin has been reported to be soluble in 75% ethanol (36) and in chloroform–methanol (2:1) (68). We have confirmed these observations. Our results do not support the idea that delta lysin is a lipid or lipoprotein; however, the high content of hydrophobic amino acids in the protein may explain its unusual solubility in certain organic solvents.

There are at least five important unanswered questions concerning delta hemolysin. First, what is the interrelationship between the insoluble delta lysin, the soluble, acidic, fibrous lysin, and the soluble, basic, granular lysin(s)? Is one an aggregate or a carrier-bound version of another? Kayser (38–40) and Bernheimer (6) have proposed that delta lysin may exist in a carrier-bound form. Most of the chemical and biological property studies reported in this paper were performed with the lysin preparation which was a mixture of the acidic and basic components. Additional comparative studies of the acidic and basic lysins should be done.

Second, what is the mechanism of action of delta lysin? Wiseman and Caird (67) have proposed that it is a phospholipase C; however, we have not found this activity in our preparation. In addition, Marks and Vaughan (51), Jackson and Little (35), and Bernheimer (6) have observed that, when a fixed amount of delta lysin is incubated with various numbers of erythrocytes, the absolute number or the per cent of total cells lysed decreases with increasing cell concentration. This observation suggests that delta hemolysin is a nonenzymatic lysin, since if it were an enzyme it should act on many cells sequentially rather than only once, and per cent hemolysis would remain constant with increasing cell concentration, as has been observed with staphylococcal beta hemolysin (a known enzyme). A nonenzymatic mechanism of action is also suggested by the observation by Galston (*personal communication*) that our delta lysin preparation is surface-active.

Third, is delta lysin immunogenic? Delta hemolysin preparations show precipitin bands when diffused against staphylococcal antiserum; however, with the exception of Kayser and Raynaud (42) and McLeod (47), investigators (24, 28, 43, 58) have not been able to produce significant amounts of neutralizing antibody in rabbits with delta lysin. Sera from a variety of animal species will inactivate the lysin; however, this activity is not primarily present in the gamma globulin fraction (24, 35). In addition, the similar amounts of inhibitor present in many human sera (17) and its occurrence in fetal calf serum (J. A. Donahue, Bacteriol. Proc., p. 93, 1969) suggest that it is not antibody. The normal serum inhibitor of delta hemolysin has not been purified and characterized. Phospholipids are known inhibitors of this lysin, and it has been suggested (17) that the neutralizing activity of serum is due to serum lipid(s)

and that the lines of precipitation seen when delta lysin preparations are diffused against staphyloccal antiserum are insoluble complexes resulting from combination of delta lysin with normal serum lipoproteins (6). Streptolysin S has been reported (63) to be inhibited by serum lipoprotein(s).

Fourth, what is the role of delta hemolysin in staphylococcal metabolism? A very large amount of this material is produced and excreted in vitro, but there does not seem to be any clue as to its function.

Fifth, what is the role of delta lysin in staphylococcal infections? Partially purified lysin has been reported (23, 24, 29, 34) to kill leukocytes in vitro. If the lysin is produced and can function in vivo, it might aid in establishing or perpetuating staphylococcal lesions. Arbuthnott et al. (3) have suggested that delta lysin may play a part in the pathogenesis of Ritter's type of toxic epidermal necrolysis and in extensive impetigo.

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