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Purification and Characterization of an Extracellular Cytolysin Produced by *Vibrio vulnificus*

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An extracellular cytolytic toxin produced by the halophilic bacterium *Vibrio vulnificus* was isolated free of detectable contamination with medium constituents and other bacterial products by sequential ammonium sulfate precipitation, gel filtration with Sephadex G-75, hydrophobic interaction chromatography with phenyl-Sepharose CL-4B, and isoelectric focusing in an ethylene glycol density gradient. The cytolysin is a heat-labile, hydrophobic protein that is inhibited by large amounts of cholesterol, is partially inactivated by proteases and trypan blue, has a molecular weight (estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by amino acid analysis) of ca. 56,000, and has an isoelectric point of ca. 7.1. The first 10 amino-terminal amino acid residues of the cytolysin are Gln-Glu-Tyr-Val-Pro-Ile-Val-Glu-Lys-Pro. Lysis of mouse erythrocytes by the purified cytolysin is a multi-hit, at least two-step process consisting of a temperature-independent, toxin-binding step, followed by a temperature-dependent, membrane-perturbation step(s). In addition to possessing cytolytic activity against erythrocytes from 17 animal species and against Chinese hamster ovary cells in tissue culture, the purified cytolysin preparation was lethal for mice (ca. 3 μg/kg, intravenous 50% lethal dose) and had vascular permeability factor activity in guinea pig skin.

Seven new *Vibrio* species have been recognized in the past 9 years as possible causes of human disease (7, 39). One of these, *Vibrio vulnificus* (originally known as lactose-positive *Vibrio* and *Beneckea vulnifica* [4, 11]), has been mistakenly identified as *V. parahaemolyticus* and also called "unnamed marine vibrio" and "halophilic, noncholera vibrio" (12, 15, 38), but it is now known to be a distinct etiological agent of wound infections, septicemia, pneumonia, meningitis, keratitis, and endometritis in humans (7, 39, 44). In addition, mice subcutaneously injected with *V. vulnificus* exhibit extensive edema and tissue necrosis at the portal of entry and a rapidly developing, fatal septicemia (8, 31) similar to that observed during human disease. Putative virulence factors produced by *V. vulnificus* include extracellular cytolysin (22), proteases (35; M. M. Carruthers, and W. J. Kabat, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B59, p. 24; M. D. Poole, J. H. Bowdre, and D. Klapper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B155, p. 43), siderophores (2, 34), and phospholipases (37), and an antiphagocytic surface antigen(s) that possesses protective antigen activity (21, 25). The histological features of lesions and the rapid onset of anemia, refractory shock, and heart block in humans infected with *V. vulnificus* led Craig and Stevens (10) and Zide et al. (46) to suggest that potent bacterial toxins are involved in the pathogenesis of disease caused by *V. vulnificus*. In this paper, we report on the purification and characterization of a previously recognized (22) *V. vulnificus* toxin that possesses cytolytic, lethal, and vascular permeability factor activities.

MATERIALS AND METHODS

Assays. Unless otherwise stated, cytolysin dilutions were prepared with phosphate-buffered saline (PBS; 0.067 M Na_2HPO_4, 0.077 M NaCl [pH 7]) containing 1 mg of crystalline bovine albumin (Miles Laboratories, Inc., Naperville, Ill.) per ml (PBS-BA) (22).

Cytolytic activity against erythrocytes was determined by the method of Bernheimer and Schwartz (6). All cytolytic activity determinations, except those for the hemolytic spectrum studies, were performed with washed mouse erythrocytes.

The final purified cytolysin preparation (stage 5), freed of ethylene glycol by gel filtration with Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.), was assayed for cytotoxicity against Chinese hamster ovary cells, vascular permeability factor activity in guinea pig skin, and lethal activity for mice as previously described for crude preparations of the cytolysin (22).

The final purified cytolysin preparation (100 μl) was examined for protease activity against azocasein as previously described (24), except the assay mixtures were incubated for 20 min instead of 10 min at 37°C.

The final purified cytolysin preparation (100 μl) was examined for the presence of lipopolysaccharide endotoxin (*Escherichia coli* endotoxin standard) by the *Limulus* amebocyte lysate method with commercially available lysate (Pyrostat; Worthington Diagnostics, Inc., Freehold, N.J.) as recommended by the manufacturer.

Protein was estimated by the method of Bradford (9), with bovine gamma globulin as the standard. The standard and the assay reagent were obtained from Bio-Rad Laboratories, Inc., Richmond, Calif.

Bacterium and preparation of seed cultures. *V. vulnificus* E4125 was kindly supplied by R. E. Weaver and D. G. Hollis of the Centers for Disease Control, Atlanta, Ga. Seed cultures were prepared as previously described (22), except the sheep blood was omitted from the Columbia agar plates.

Purification of cytolysin. Unless otherwise noted, all steps were done at approximately 4°C.

Stage 1. Culture supernatant fluids. Twelve 2-liter Erlenmeyer flasks containing 200 ml of heart infusion diffusate broth (23) were inoculated with portions (0.3 to 0.4 ml containing 10 optical density units at 650 nm and ca. 10^{10} CFU) of a seed culture suspension, and the culture super-

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natant fluids were obtained by centrifugation (16,000 × g, 20 min) after incubation of the cultures for 4 to 6 h at 37°C on a Gyrotory shaker (model G-25; New Brunswick Scientific Co., Edison, N.J.) operating at 220 cycles per min.

Stage 2. Ammonium sulfate precipitation. Ammonium sulfate (enzyme grade; Schwarz/Mann, Cambridge, Mass.) was dissolved in the pooled culture supernatant fluids to a final concentration of ca. 50% saturation (350 g/liter). After 16 to 18 h, the precipitate was recovered by centrifugation (16,000 × g, 20 min) and dissolved in 60 ml of deionized water. The solution was centrifuged (20,000 × g, 20 min) to remove a small amount of insoluble residue, and ammonium sulfate was dissolved in the supernatant fluids to a final concentration of ca. 40% saturation (280 g/liter). After 16 to 18 h, the precipitate was recovered by centrifugation (20,000 × g, 20 min) and was dissolved in 10 ml of 10 mM glycine–NaOH buffer (pH 9.8) supplemented with 20 mM NaCl. A small amount of insoluble residue was removed from the solution by centrifugation.

Stage 3. Sephadex G-75 gel filtration. The stage 2 preparation was applied to a column (2.6 by 96 cm) of Sephadex G-75 (Pharmacia) equilibrated with the same glycine buffer used in stage 2 and was eluted at a flow rate of 20 ml/h (ca. 3.8 ml/cm^2 per h). Fractions (5 ml) were assayed for absorbance at 280 nm and for cytolytic activity and were analyzed by fused rocket immunoelectrophoresis. The cytolysin peak fractions (usually eight or nine fractions) were pooled.

Stage 4. Hydrophobic interaction chromatography. The stage 3 pool was applied to a column (1.6 by 30 cm) of phenyl-Sepharose CL-4B (Pharmacia) equilibrated with the same glycine buffer used in stage 2 and the column was washed (40 ml/h; 4-ml fractions collected) with 3 to 4 bed volumes (180 to 240 ml) of 25% ethylene glycol (EG) in 10 mM glycine–NaOH buffer (pH 9.8). The cytolysin was eluted by washing the column (8 to 12 ml/h; 2- to 3-ml fractions collected) with 50% EG in 2 mM glycine-NaOH buffer (pH 9.8). The fractions were assayed for absorbance at 280 nm and for cytolytic activity, and were analyzed by fused rocket immunoelectrophoresis. The cytolysin peak fractions (usually 10 fractions) were pooled.

Stage 5. Isoelectric focusing. The stage 4 pool was fractionated by high-speed electrofocusing (45) in a pH 6 to 8 EG density gradient formed at 15 W for 18 h with an LKB 8100-1 column (LKB Instruments, Inc., Gaithersburg, Md.). The pH of each fraction (4 ml) was determined at 4°C, and the fractions were assayed for cytolytic activity and analyzed by fused rocket immunoelectrophoresis. The cytolysin peak fractions (usually five fractions) were pooled and stored at −70°C.

Preparation of antiserum. A crude cytolysin preparation was obtained from the culture supernatant fluids by precipitation with ammonium sulfate (350 g/liter) as described in the stage 2 protocol, except the preparation was lyophilized after being dissolved in deionized water and centrifuged to remove a small amount of insoluble residue. New Zealand white rabbits, weighing ca. 2.5 kg each at the start of the vaccination schedule, were injected subcutaneously with 0.75 ml of an anhydrous oil vaccine containing ca. 36 mg (ca. 30,000 hemolytic units [HU]) of the crude cytolysin preparation suspended in complete Freund adjuvant. The rabbits were injected subcutaneously 4 and 8 weeks later with 18 and 9 mg, respectively, of the crude cytolysin preparation suspended in 0.75 ml of incomplete Freund adjuvant. The rabbits were exsanguinated ca. 3 weeks after the last injection, and the pooled sera were lyophilized and stored at 4°C.

Immunodiffusion and immunoelectrophoretic procedures. Fused rocket immunoelectrophoresis and crossed immunoelectrophoresis were performed with the LKB 2117-401 immunoelectrophoresis kit and a Tris-barbital buffer (0.02 M, pH 8.6). The general methodology described in the LKB instruction manual and application note 249 (42) and in the handbook of immunoprecipitation-in-gel techniques edited by Axelsen (3) was followed. Samples (18 μl) of fractions analyzed by fused rocket immunoelectrophoresis were placed, in sequence, into wells (4-mm diameter) cut in 1.2% (wt/vol) agarose (Bio-Rad Laboratories) in Tris-barbital buffer and were allowed to diffuse for 30 min before electrophoresis. The upper part of each gel (ca. 61 cm^2) consisted of a 1.2% agarose gel (11 ml) containing 0.75 ml of anti-crude cytolysin antiserum. After diffusion, electrophoresis was performed at 2 V/cm for 18 h at 15°C, and the gels were washed, dried, and stained with Coomassie brilliant blue R-250.

Slab SDS-PAGE. Slab sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel electrophoresis (PAGE) was performed with a Protean electrophoresis cell (16-by-14-by-0.15-cm gel configuration; Bio-Rad Laboratories) by a modification of the method of Laemmli (26). The stacking gel contained 4% acrylamide, 0.1% bis-acrylamide, and 0.1% SDS in 0.12 M Tris-hydrochloride buffer (pH 6.8), and the gradient separating gel contained 4 to 30% acrylamide, 0.1 to 0.5% bis-acrylamide, and 0.1% SDS in 0.38 M Tris-hydrochloride buffer (pH 8.8). The running buffer was 0.02 M Tris-hydrochloride–0.19 M glycine (pH 8.3) containing 0.1% SDS. Samples (15 to 113 μl) of cytolysin preparations and molecular weight standards (Pharmacia) were mixed with 25 μl of disruption solution (4% SDS, 2% β-mercaptoethanol, 20% glycerin, and a few crystals of bromophenol blue) and boiled for 2 min in a water bath before being loaded onto the gel. In some experiments, the β-mercaptoethanol was omitted from the disruption solution. Electrophoresis was performed at 250 V until the blue tracking dye reached the separating gel and at 400 V until the dye reached the bottom of the gel (ca. 4 h, total running time). The temperature of the gel was maintained at ca. 10°C during electrophoresis. The gels were fixed and stained in methanol-water-glacial acetic acid (5:4:1 [vol/vol/vol]) containing 0.2% Coomassie brilliant blue R-250 and 0.5% cupric sulfate, destained with water-methanol-glacial acetic acid (73:20:7 [vol/vol/vol]) containing 0.5% cupric sulfate, and scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, Calif.). In some experiments, the concentration of SDS and β-mercaptoethanol in the disruption solution was increased to 8 and 10%, respectively. In some experiments, the stage 5 cytolysin preparation was carboxymethylated by the following procedure before examination by SDS-PAGE. A sample (110 μl) of the preparation was boiled for 2 min with 11 μl of 10% SDS and 11 μl of 0.5 M dithiothreitol, and the mixture was stored at 25°C for 1 h and incubated in the dark with 11 μl of 1 M iodoacetamide for 30 min at 25°C. The mixture was applied to the gel after adding 11 μl of glycerin containing a few crystals of bromophenol blue.

Molecular weight estimation. The molecular weight of the denatured and reduced stage 5 cytolysin was estimated by the relative mobility method of Weber et al. (43), using the slab SDS-PAGE protocol described in this paper. The apparent molecular weight of the native stage 5 cytolysin was estimated by gel filtration as previously described (22) for a crude *V. vulnificus* cytolysin preparation, except Sephadex G-75 was used instead of Sephadex G-100.

Analytical isoelectric focusing. Analytical thin-layer isoelectric focusing in polyacrylamide gel was performed with

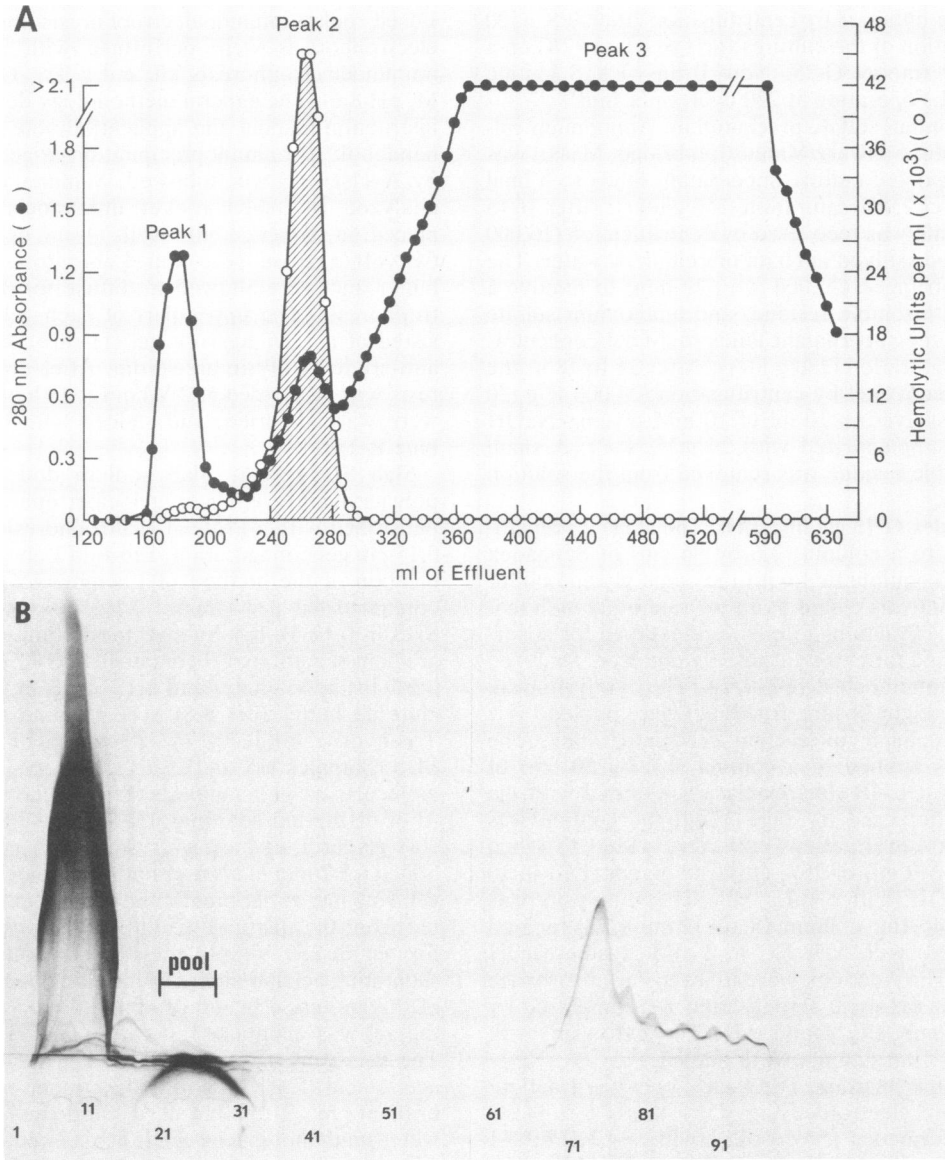

FIG. 1. Sephadex G-75 gel filtration and fused rocket immunoelectrophoretic analysis of stage 2 cytolysin preparation. (A) Gel filtration pattern. The cross-hatched area beneath peak 2 indicates the fractions that were pooled to form the stage 3 cytolysin preparation. (B) Antigen distribution profile obtained by fused rocket immunoelectrophoresis of every other fraction. Fractions 1 to 13 (peak 1), 146 to 204 ml of effluent; fractions 19 to 29 (peak 2), 235 to 287 ml of effluent; fractions 37 to 89 (peak 3), 330 to 640 ml of effluent.

the LKB 2217 Ultrophor electrophoresis apparatus and commercial PAG plates (pH 3.5 to 9.5) as recommended by the manufacturer, except the cytolysin preparations and the isoelectric point standards (Pharmacia) were applied directly to the gel surface (4 to 6 cm from the cathode).

Amino acid analyses. Amino acid analyses were performed by the method of Spackman et al. (36). Samples of a stage 5 cytolysin preparation were dialyzed against cold PBS, precipitated with ammonium sulfate (75% saturation; 525 g/liter), and dialyzed against cold deionized water and lyophilized. Samples of the lyophilized preparation were hydrolyzed for 24, 48, and 72 h at 110°C with 6 N HCl in sealed glass tubes after evacuation and degassing. The hydrolysates were dried under nitrogen, and amino acid analyses were performed with a Dionex model D-500 Mark II amino acid analyzer (Dionex Corp., Sunnyvale, Calif.). Half-cystine was determined as cysteic acid by the performic acid oxidation method of Moore (28). Tryptophan content was estimated after alkaline hydrolysis for 48 h at 135°C, as described by Hugli and Moore (16). The amounts of serine and threonine were estimated by extrapolation to zero hydrolysis time, and the amounts of leucine, isoleucine, and valine were calculated from the 72-h hydrolysis sample. The amounts of all other amino acids were calculated from the averaged values of all of the samples. N-terminal amino acid sequence analyses were performed with a model 470A protein sequencer (Applied Biosystems, Foster City, Calif.) (14) and identification of the phenylthiohydantoinyl amino acids with a high-pressure liquid chromatography system (Waters Associates, Milford, Mass.) (17).

Inactivation studies. The effect of various proteases on the cytolysin was examined by incubating samples (100 HU) of

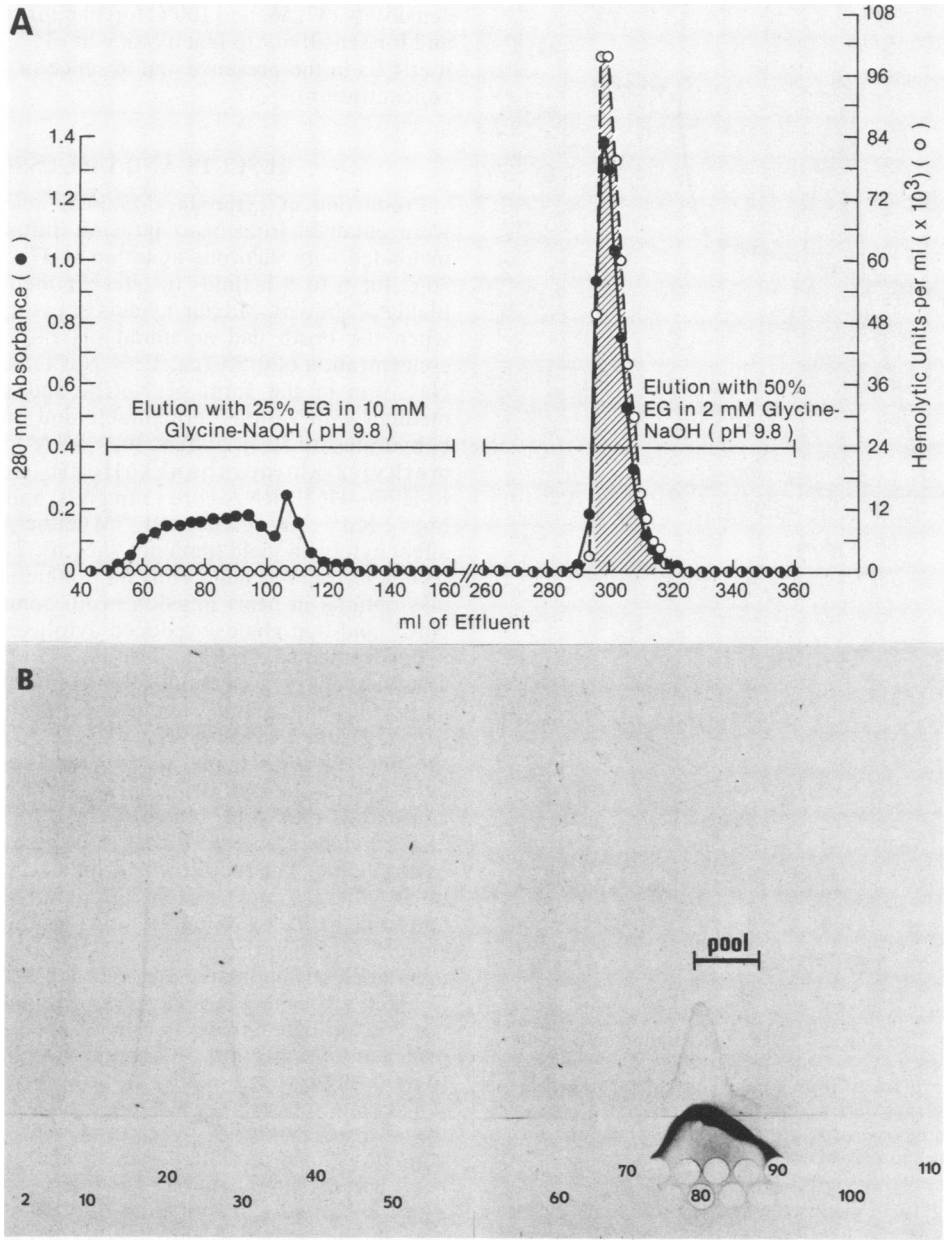

FIG. 2. Hydrophobic interaction chromatography and fused rocket immunoelectrophoretic analysis of stage 3 cytolysin preparation. (A) Hydrophobic interaction chromatography elution pattern with phenyl-Sepharose CL-4B. The cross-hatched area indicates the fractions that were pooled to form the stage 4 cytolysin preparation. (B) Antigen distribution profile obtained by fused rocket immunoelectrophoresis of every other fraction. Fractions 2 to 54, 15 to 235 ml of effluent; fractions 56 to 112, 239 to 361 ml of effluent.

the stage 5 cytolysin preparation in PBS (1 ml, final volume) for 30 min at 37°C with soluble Pronase-CB (Calbiochem-Behring, La Jolla, Calif.), bovine pancreatic trypsin and chymotrypsin (3× crystallized; Worthington Diagnostics), and subtilisin BPN, subtilopeptidase A, papain (activated with dithiothreitol, 10^{-3} M), thermolysin, and proteinase K (Sigma Chemical Co., St. Louis, Mo.), and assaying the mixtures for residual cytolytic activity.

Samples of cytolysin (100 HU in 0.1 ml PBS) also were tested for sensitivity to inactivation by trypan blue (Eastman Kodak Co., Rochester, N.Y.), glycophorin (Sigma), and suspensions of various lipids (cholesterol, mixed bovine brain gangliosides, and cardiolipin [Sigma]; sphingomyelin and phosphatidylcholine [Supelco, Inc., Bellefonte, Pa.]; and phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine [Serdary Research Laboratories, London, Ontario, Canada]). The organic solvents in which the lipids were dissolved were evaporated with a stream of nitrogen, and the lipids were suspended in 0.9 ml of PBS by blending with a Vortex mixer for 1 min. The cytolysin-reagent mixtures (1 ml, final volume) were incubated at 25°C for 15 min before assay for residual activity.

The effect of dithiothreitol (electrophoresis grade; Bio-Rad Laboratories), various divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}), and chelating agents [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA) and disodium EDTA] on cytolysin activity was examined by assaying the cytolysin in the presence of the reagents (i.e.,

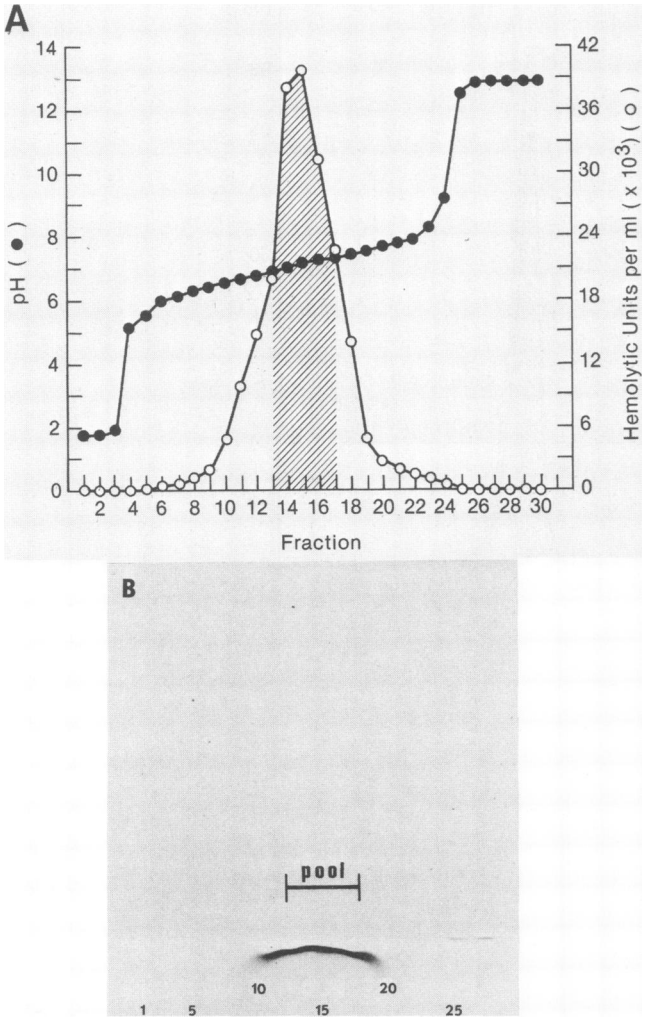

FIG. 3. Isoelectric focusing and fused rocket immunoelectrophoretic analysis of stage 4 cytolysin preparation. (A) Isoelectric focusing pattern in a pH 6 to 8 gradient. The cross-hatched area indicates the fractions that were pooled to form the stage 5 cytolysin preparation. (B) Antigen distribution profile obtained by fused rocket immunoelectrophoresis of every fraction.

without incubating the cytolysin with the reagents before assay). The assays in the presence of Ca^{2+} and Mg^{2+} were performed with Tris-buffered saline (0.02 M Tris-hydrochloride, 0.15 M NaCl [pH 7]) supplemented with 1 mg BA per ml, instead of PBS-BA.

Samples of cytolysin (100 HU) also were tested for heat sensitivity (37, 56, and 100°C for 30 min) in PBS and PBS-BA and for sensitivity to inactivation at pH 4, 6, 7, 8, and 10 (24 h at 4°C) in the presence and absence of BA (1 mg/ml, final concentration).

RESULTS AND DISCUSSION

Production of cytolysin. Maximal amounts of cytolysin were obtained from heart infusion diffusate broth cultures incubated with vigorous agitation at 37°C for 4 to 6 h or at 30°C for 6 to 8 h (mid- to late exponential growth phase) (data not shown). Maximal yields of cytolysin were obtained when the broth had an initial pH of 7.4 and a Na^+ ion concentration of 0.5% (ca. 1.3% NaCl, assuming that all the Na^+ was in the form of NaCl), and supplementing the medium with various fermentable and nonfermentable carbohydrates (0.5%), buffers [0.1 M *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid–NaOH and K_2HPO_4–HCl], RNA-core (1 mg/ml), and metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}; 10^{-4} M) either reduced or did not affect cytolysin yield (data not shown). Tison and Kelly (40) recently observed that cytolysin production by *V. vulnificus* was optimal in heart infusion broth containing 0.5 to 1.5% NaCl, and that glucose reduced cytolysin yield.

Purification of cytolysin. The behavior of the *V. vulnificus* cytolysin during sequential gel filtration, hydrophobic interaction chromatography, and isoelectric focusing is shown in Fig. 1, 2, and 3, respectively, and the quantitative results of the purification scheme are summarized in Table 1. The behavior of the cytolysin during hydrophobic interaction chromatography (i.e., binding at pH 9.8 in the presence of 25% ethylene glycol) indicates that the cytolysin is strongly hydrophobic. The recovery and specific activity of cytolysin in six different stage 5 preparations ranged from 20 to 25% and from 70,000 to 90,000 HU/mg protein, respectively. The stage 5 cytolysin preparation was homogeneous by crossed immunoelectrophoresis (Fig. 4) and analytical thin-layer isoelectric focusing in polyacrylamide gel (Fig. 5A) and did not contain detectable amounts of protease activity (<0.25 protease units per ml) or lipopolysaccharide endotoxin activity (<0.1 ng/ml).

The electrophoretic patterns obtained by SDS-PAGE of the stage 5 cytolysin preparation were influenced by the conditions used to denature the preparation (Fig. 6). Cytolysin preparations denatured in the absence of a reducing agent exhibited a major band (ca. 99% of the Coomassie blue-staining material) with a molecular weight of ca. 56,000 and a minor band consisting of an aggregate with a molecular weight of >200,000. SDS-PAGE of preparations denatured under reducing conditions (β-mercaptoethanol or dithiothreitol in the disruption solution) did not show the minor,

TABLE 1. Purification of extracellular cytolysin produced by *V. vulnificus* E4125

Purification stage	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Activity (HU/ml)	Total activity (HU)	Recovery (%)	Sp act (HU/mg of protein)
1. Culture supernatant fluids	2,275	0.185	421	1,100	2,502,500	100	5,946
2. Ammonium sulfate precipitation	12.2	10	122	140,000	1,708,000	68	14,000
3. Sephadex G-75 gel filtration	47	0.345	16.2	22,000	1,034,000	41	63,768
4. Hydrophobic interaction chromatography	19.6	0.550	10.8	44,000	862,400	34	80,000
5. Isoelectric focusing	20	0.330	6.6	27,000	540,000	22	81,818

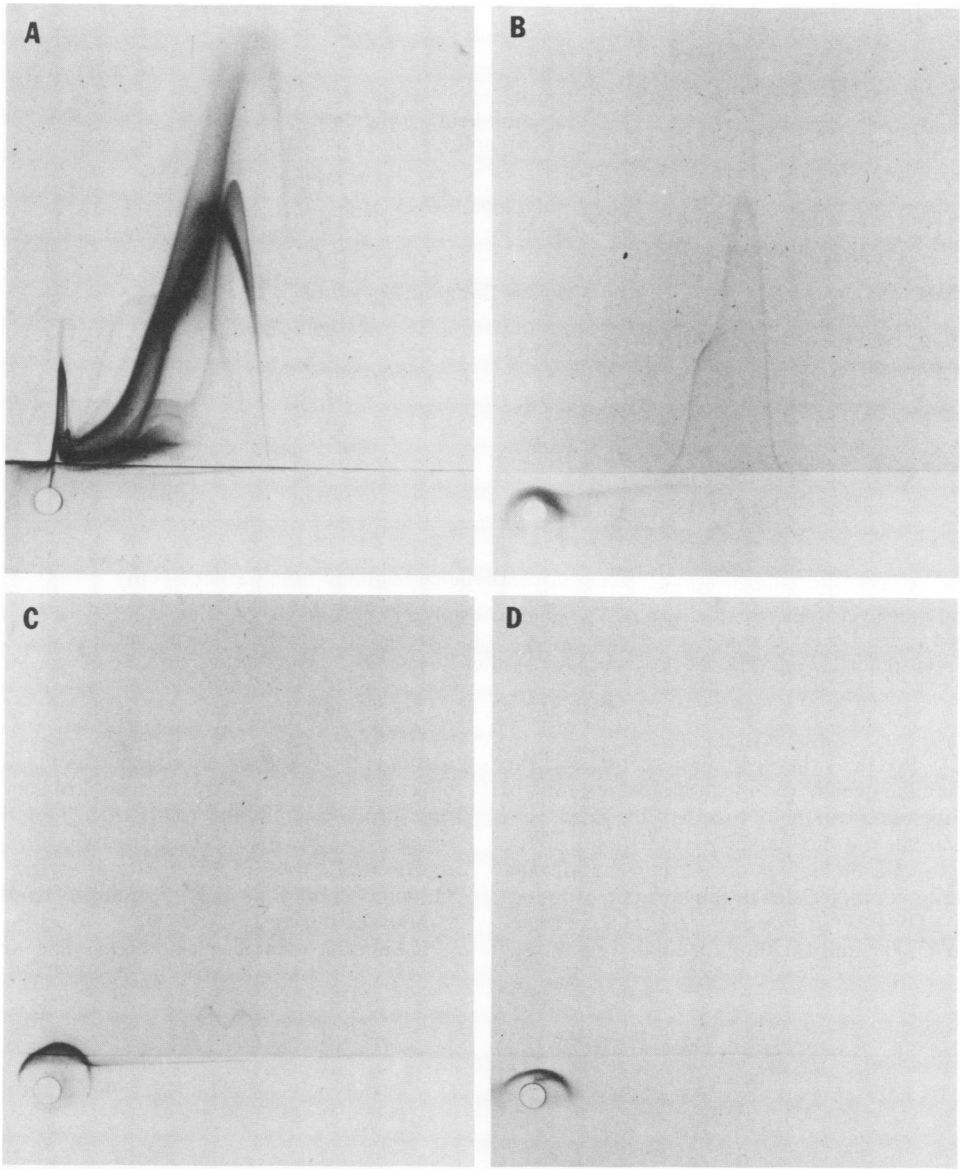

FIG. 4. Crossed immunoelectrophoresis of *V. vulnificus* cytolysin preparations. In the first dimension, samples (18 μl) were placed into wells (4-mm diameter) cut in gels composed of 1.2% (wt/vol) agarose (Bio-Rad Laboratories) in Tris-barbital buffer and were electrophoresed (anode to right) at 10 V/cm for 75 min at 12°C. In the second dimension, the upper part of each gel (ca. 61 cm^2) was composed of a 1.2% agarose gel (11 ml) containing anti-crude cytolysin antiserum. Electrophoresis (anode at top) was performed at 2 V/cm for 18 h at 15°C. (A) Stage 2, 16 μl (178 μg); 0.5 ml antiserum. (B) Stage 3, 20 μl (5 μg); 0.75 ml antiserum. (C) Stage 4, 17 μl (8 μg); 0.25 ml antiserum. (D) Stage 5, 20 μl (16 μg); 0.25 ml antiserum. Gels were stained with Coomassie brilliant blue R-250.

high-molecular-weight component seen in nonreduced preparations, but revealed a major band (88% of the Coomassie blue-staining material) with a molecular weight of ca. 56,000 and two minor bands with molecular weights of ca. 48,000 and 16,500 and containing ca. 8 and 4% of the Coomassie blue-staining material, respectively. The similarity of the combined molecular weight of the two minor components (64,500) and the molecular weight of the major component (56,000), and the unlikely possibility that contaminants would be associated with the cytolysin by disulfide bonds during purification of the cytolysin, suggest that the two minor components are fragments of the cytolysin.

Increasing the exposure of the cytolysin to the disruption solution from 2 min to 1 h, increasing the concentrations of SDS and β-mercaptoethanol in the disruption solution from 4 to 8% and from 2 to 10%, respectively, and carboxymethylating the reduced cytolysin preparation with iodoacetamide before electrophoresis did not cause a detectable increase in the amounts of the 48,000- and 16,500-molecular-weight bands (data not shown). Therefore, it is unlikely (i) that only a small percentage of the cytolysin molecules were converted to the 48,000- and 16,500-molecular-weight fragments because of incomplete denaturation and reduction or (ii) that all of the cytolysin molecules were converted under reducing conditions to the two fragments, but most of the fragments were reoxidized to the native 56,000-molecular-weight form because of the absence of reducing agent in the polyacrylamide gel.

At the present time, the idea we favor to explain the observation of the 48,000- and 16,500-molecular-weight com-

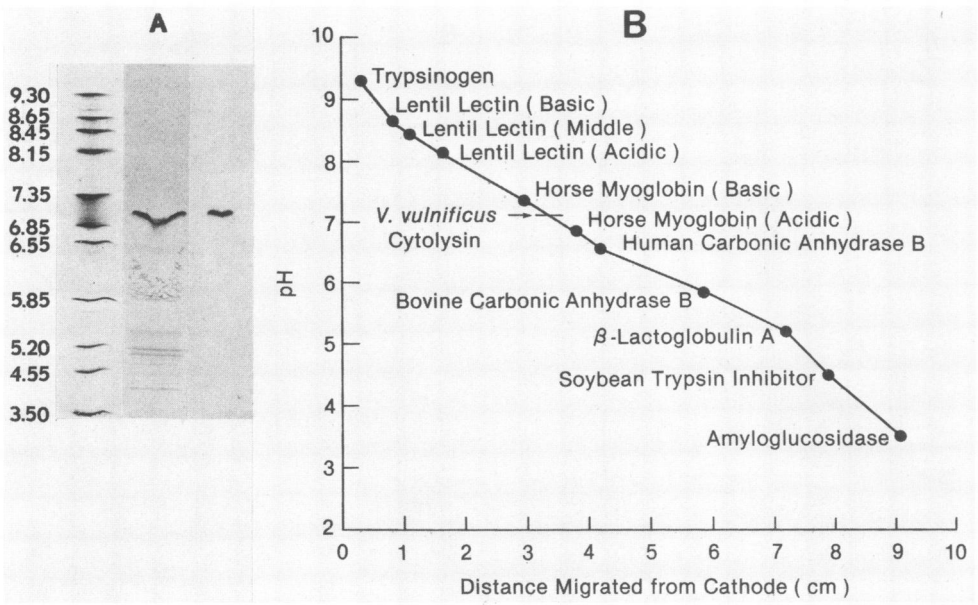

FIG. 5. Analytical thin-layer isoelectric focusing of *V. vulnificus* cytolysin preparations. (A) Gel stained with Coomassie brilliant blue R-250. Cathode at top of gel. Lanes: 1, pI markers named in panel B (pIs are indicated to the left of the lane); 2, stage 2 preparation (90 μg in 40 μl); 3, stage 5 preparation (12 μg in 15 μl). (B) Estimation of pI of *V. vulnificus* cytolysin. Results are the mean values of two separate experiments.

ponents during SDS-PAGE is that a small percentage of the native, 56,000-molecular-weight cytolysin molecules exist in a proteolytically nicked form held together by intrachain disulfide bonds. The nicked molecules are indistinguishable from the intact cytolysin molecules during purification and comigrate with the intact molecules in the absence of reducing agents during SDS-PAGE; however, the nicked molecules separate into 48,000- and 16,500-molecular-weight fragments in the presence of reducing agents during SDS-PAGE. Our idea is supported by the recent observations (30, 32, 33) that proteolytically nicked *Penicillium notatum* phospholipase B, staphylococcal enterotoxin C_3, and *Pseudomonas aeruginosa* exotoxin A migrate as single bands under nonreducing conditions but as multiple bands under reducing conditions during PAGE.

Estimation of molecular weight. The apparent molecular

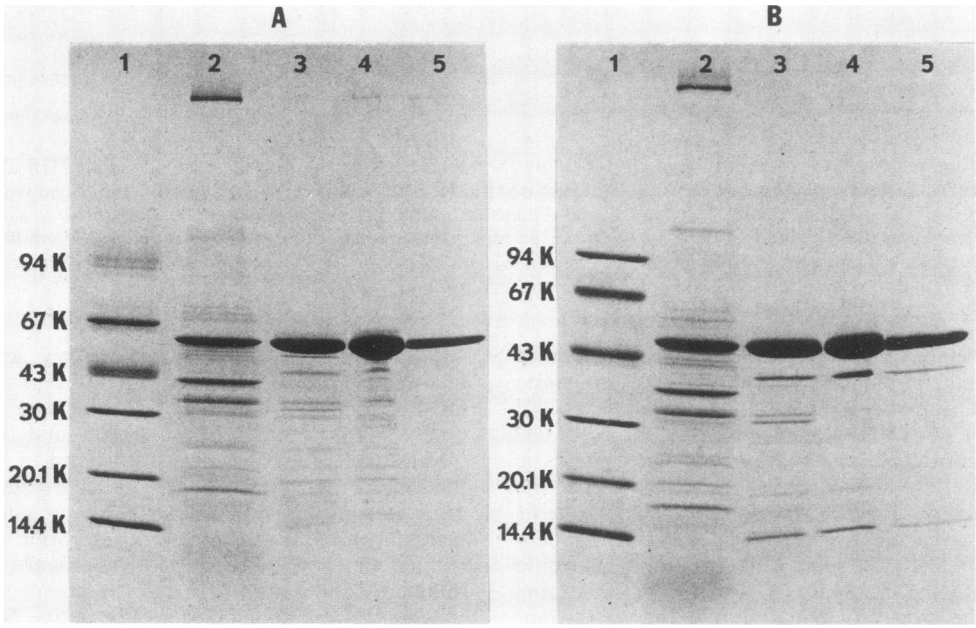

FIG. 6. SDS-PAGE of *V. vulnificus* cytolysin preparations. Cathode at top of gel. (A) Preparations were denatured under nonreducing conditions. (B) Preparations were denatured under reducing conditions (β-mercaptoethanol in the disruption solution). Lanes: 1, molecular weight markers (Pharmacia); 2, stage 2 (70 μg); 3, stage 3 (35 μg); 4, stage 4 (50 μg); 5, stage 5 (25 μg). Gels were stained with Coomassie brilliant blue R-250.

TABLE 2. Amino acid composition of *V. vulnificus* cytolysin

Amino acid	Concn found (mol%)	No. of residues per molecule of cytolysin[a]
Aspartic acid	12.93	63
Threonine	5.92	28
Serine	9.46	44
Glutamic acid	8.92	44
Proline	3.64	17
Glycine	7.76	33
Alanine	5.90	26
Valine	7.36	35
Methionine	0.42	2
Isoleucine	3.43	17
Leucine	5.90	28
Tyrosine	4.54	23
Phenylalanine	3.72	19
Lysine	5.34	26
Histidine	1.78	9
Arginine	5.68	29
Half-cystine	1.67	8
Tryptophan	5.63	29

[a] Based on a molecular weight of 56,000.

weight of the native stage 5 cytolysin was estimated by gel filtration to be ca. 15,000 (data not shown), and this value was substantially lower than the molecular weight estimated by SDS-PAGE (ca. 56,000). The most likely explanation for our findings is that the highly purified, hydrophobic cytolysin interacted with the dextran-based gel and was retarded in its passage through the gel column. Therefore, the 56,000-molecular-weight value obtained by SDS-PAGE is more correct than the lower value obtained by gel filtration. Similar observations have been made during molecular weight estimations of *Clostridium perfringens* delta toxin (1) and *P. aeruginosa* elastase (24). The difference in the molecular weight estimated by gel filtration of the stage 5 cytolysin preparation and the previously reported (22) molecular weight estimated by gel filtration of crude cytolysin preparations (ca. 38,500) may be explained by the difference in the amount of contaminants in the two preparations. If the contaminants have an affinity for the dextran gel, they could have interfered with cytolysin-gel interaction. Alternatively, the contaminants could have interacted with the cytolysin and enhanced its elution from the gel.

Estimation of isoelectric point. Toxinogram analysis (data not shown), with an overlay of washed mouse erythrocytes in agarose (41), of the bands obtained by analytical thin-layer isoelectric focusing of partially purified (stage 2) and highly purified (stage 5) cytolysin preparations indicated that the cytolysin has an isoelectric point (pI) of ca. 7.1 (Fig. 5B). This value is similar to the pI obtained during preparative isoelectric focusing of the stage 4 cytolysin preparation (Fig. 3A). However, the pI is substantially higher than the pI of 3.8 previously estimated (37) by preparative isoelectric focusing of crude cytolysin preparations. This difference suggests that the value obtained by preparative isoelectric focusing of the crude cytolysin may have been artificially low because of cytolysin interaction or complex formation with acidic contaminants in the preparation.

Amino acid analyses. The amino acid composition of the purified cytolysin is shown in Table 2. The minimal molecular weight calculated from the total 480 residues was 55,700. The similarity of the molecular weights estimated by amino acid analysis and SDS-PAGE indicates that the cytolysin does not contain appreciable amounts of non-amino acid material. Basic, acidic, and nonpolar hydrophobic amino acid residues accounted for ca. 13, 22, and 36% of the total residues, respectively. However, many of the aspartic acid and glutamic acid residues must occur as asparagine and glutamine because the pI of the cytolysin is ca. 7.1. The presence of eight half-cystine residues suggests that the cytolysin could have four intra- or interchain disulfide bonds. The first 10 amino-terminal amino acid residues of the purified cytolysin preparation were Gln-Glu-Tyr-Val-Pro-Ile-Val-Glu-Lys-Pro, and only a single amino acid sequence was detected in the preparation.

Inactivation studies. The ability of the purified cytolysin to lyse erythrocytes was not affected by phospholipids (cardiolipin, sphingomyelin, phosphatidylserine, phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine; 100 μg), mixed gangliosides (100 μg), glycophorin (100 μg), dithiothreitol (5×10^{-3} M), chelating agents (EDTA and EGTA, 1×10^{-3} M), and divalent cations (Ca^{2+}, Mg^{2+}, and Zn^{2+}; 1×10^{-3} M) (data not shown). However, the cytolysin was heat labile (56°C, 30 min), was partially inactivated by proteases and trypan blue, and was almost totally inactivated by an amount of cholesterol that is ca. 100-fold more than the dose commonly used to inactivate thiol-activated (oxygen-labile) cytolysins (Table 3). In addition, the cytolysin was unstable at pH 4, 6, 7, 8, and 10 (4°C for 24 h) unless crystalline BA was added (1 mg/ml) to the buffers (data not shown). The amino acid composition data does not support the idea that the cytolysin contains nonprotein structures; therefore, the only partial sensitivity of the cytolysin to various proteases suggests that the cytolysin has a tertiary structure in which the active site(s) is only partially exposed.

TABLE 3. Inactivation of *V. vulnificus* cytolysin by heat, proteases, trypan blue, and cholesterol

Treatment[a]	Residual activity (%)
37°C	100[b]
56°C	2
100°C	2
37°C, PBS, 30 min (control)	65
Pronase	50
Trypsin	70
Chymotrypsin	40
Subtilisin BPN	40
Subtilopeptidase A	20
Papain	50
Thermolysin	50
Proteinase K	30
25°C, PBS, 15 min (control)	70
Trypan blue	
50 μg	30
25 μg[c]	50
Cholesterol	
100 μg	3
50 μg	40
25 μg	50
1 μg[d]	70

[a] The three heat treatments were for 30 min in PBS-BA. The eight protease treatments (100 μg/ml, final concentration) were at 37°C in PBS for 30 min. The trypan blue and cholesterol treatments were at room temperature (ca. 25°C) in PBS for 15 min.

[b] 100 HU/ml.

[c] Dose commonly used to inactivate streptolysin S (13).

[d] Dose commonly used to inactivate thiol-activated cytolysins (5).

Cytolytic activity and variables influencing erythrocyte lysis. The cytolysin was active against erythrocytes from 17 animal species, but it was most active against pig, monkey, burro, cat, sheep, pigeon, and mouse erythrocytes (Table 4). The purified cytolysin preparation also was cytolytic for Chinese hamster ovary cells in tissue culture (data not shown). The minimal cytotoxic dose of purified cytolysin for the tissue culture cells was similar to the previously reported (22) minimal cytotoxic dose of crude cytolysin preparations and was ca. 0.1 HU (ca. 1 ng).

Variables affecting the hemolytic process were studied by using washed mouse erythrocytes as target cells. A decrease in percent hemolysis was observed when the target cell/cytolysin ratio was increased (Fig. 7). In addition, the absolute amount of hemolysis (measured by absorbance at 545 nm) increased to a peak and then decreased. These observations suggest that more than one molecule of the cytolysin (a multi-hit process) is required to lyse a single erythrocyte (18–20, 27, 29). When the dose-response data in Fig. 8 were used to construct a multi-hit survival curve as described by Inoue et al. (18) (the logarithm of the fraction of unlysed erythrocytes was plotted versus the cytolysin concentration), the ordinate intercept of the linear part of the curve indicated that hemolysis by the *V. vulnificus* cytolysin requires two or three hits (data not shown).

The manner in which rate of lysis depends upon concentration of cytolysin also was revealed by analysis of the curves shown in Fig. 8. The result of plotting the slope of the linear part of each curve as a function of cytolysin concentration indicated that the rate of erythrocyte lysis is directly proportional to cytolysin concentration (data not shown).

The rate of erythrocyte lysis by the cytolysin was temperature-dependent and was optimal between 30 to 37°C (Fig. 9). Hemolysis was not observed at 4°C. The observation that the *V. vulnificus* cytolysin did not lyse mouse erythrocytes at 4°C prompted a series of experiments to determine whether the cytolysin binds to mouse erythrocytes at 4°C. Cytolysin preparations (2 HU/ml of PBS-BA) were incubated with equal volumes of an erythrocyte suspension (0.7% [vol/vol]) at 4°C for up to 5 min, and samples were centrifuged at 1-min intervals. The supernatant fluids were carefully removed, and the cells were washed and suspended in PBS-BA and incubated at 37°C for 30 min. The amount of hemolysis that occurred during the 37°C incubation period was determined spectrophotometrically. Enough cytolysin had become associated with the erythrocytes after 1 min at 4°C to cause 60% hemolysis (data not shown). The amount of cytolytic activity remaining in the supernatant fluids after incubation at 4°C was determined by incubating

TABLE 4. Sensitivity of erythrocytes from various animal species to *V. vulnificus* cytolysin

Species[a]	Sp act (HU/mg protein)[b]	Relative sensitivity (%)[c]
Mouse	75,000, 77,000, 80,000, 82,000	100
Sheep	66,000, 120,000, 184,000, 240,000	83–300
Pig	160,000	200
Monkey	122,000, 138,000, 150,000, 154,000	153–193
Burro	137,000	167
Cat	137,000	167
Pigeon	66,000, 80,000	83–100
Dog	74,000	92
Rat	61,000, 66,000, 70,000	76–88
Horse	55,000	69
Hamster	54,000, 54,000	67
Chicken	50,000	62
Cow	43,000	54
Human	26,000, 31,000, 31,000, 40,000	33–50
Guinea pig	33,000, 33,000, 33,000, 35,000	41–44
Rabbit	30,000, 30,000, 33,000, 33,000	37–41
Goat	26,000	33

[a] The sources of the blood samples used to prepare the erythrocyte suspensions were as previously described (23).

[b] Multiple values were obtained with erythrocyte suspensions prepared from individual mice, sheep, monkeys, pigeons, rats, hamsters, humans, guinea pigs, and rabbits.

[c] Compared with sensitivity of mouse erythrocytes (ca. 80,000 HU/mg protein).

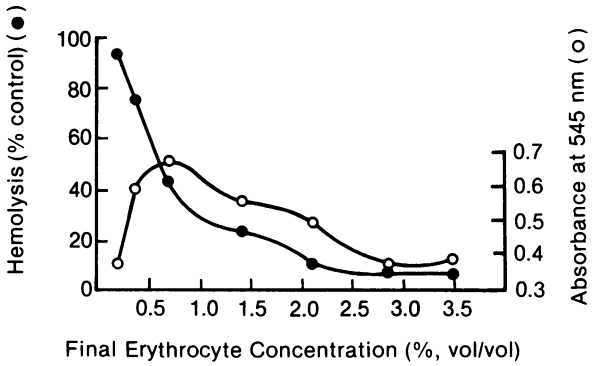

FIG. 7. Effect of erythrocyte concentration on erythrocyte lysis by *V. vulnificus* cytolysin. Samples (1 ml) of washed mouse erythrocyte suspensions were incubated with stage 5 cytolysin (2 HU in 1 ml of PBS-BA) for 30 min at 37°C, the erythrocyte-cytolysin mixtures were centrifuged (750 × *g*, 5 min) to sediment the unlysed erythrocytes, and the absorbance of the supernatant fluids was determined at 545 nm and compared with that of control, saponin-lysed erythrocyte suspensions (0.18 to 3.5% [vol/vol]).

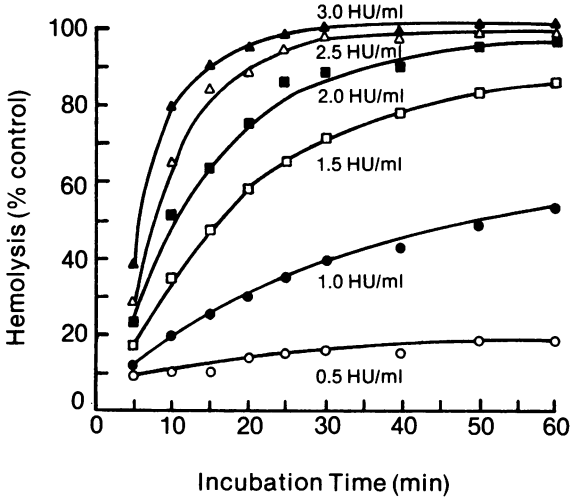

FIG. 8. Kinetics of erythrocyte lysis by *V. vulnificus* cytolysin as a function of cytolysin concentration. Samples (1 ml) of a suspension (0.7%, [vol/vol]) of washed mouse erythrocytes were incubated with 0.5 to 3 HU of stage 5 cytolysin (in 1 ml of PBS-BA) for 5 to 60 min at 37°C, and the supernatant fluids of the mixtures were obtained and examined as described in the legend to Fig. 7.

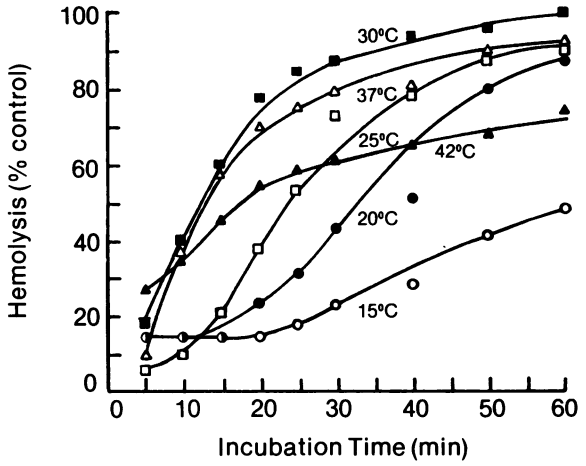

FIG. 9. Kinetics of erythrocyte lysis by *V. vulnificus* cytolysin as a function of temperature. Samples (1 ml) of a suspension (0.7% [vol/vol]) of washed mouse erythrocytes were incubated with stage 5 cytolysin (2 HU in 1 ml of PBS-BA) for 5 to 60 min at 15, 20, 25, 30, 37, and 42°C, and the supernatant fluids of the mixtures were obtained and examined as described in the legend to Fig. 7. Hemolysis was not observed at 4°C.

the supernatant fluids with fresh erythrocytes at 37°C for 30 min. The cytolytic activity in the supernatant fluids progressively decreased with increased incubation time. Our results indicate that hemolysis by the *V. vulnificus* cytolysin is at least a two-step process, consisting of a temperature-independent, cytolysin-binding step, followed by a temperature-dependent, membrane-perturbation step(s) that leads to cell lysis.

Lethal and vascular permeability factor activities. The 50% lethal dose of the purified cytolysin preparation by the intravenous route (ca. 6 HU or 0.08 μg per mouse [ca. 3 μg/kg]) was similar to the value previously estimated (22) for a crude cytolysin preparation administered by the intravenous route (5.2 HU). However, the 50% lethal dose of the purified cytolysin preparation by the intraperitoneal route (ca. 5,000 HU or 62.5 μg per mouse [2.2 mg/kg]) was substantially greater than the value previously reported (22) for the crude cytolysin preparation injected by the intraperitoneal route (857 HU). Thus, although both cytolysin preparations were much more lethal for mice by intravenous injection than by intraperitoneal injection, the difference for the 50% lethal doses estimated by the two routes was ca. 830-fold for the purified cytolysin preparation but only 160-fold for the crude cytolysin preparation. One possible explanation for this fivefold difference is that the contaminants in the crude preparation enhance absorption of the cytolysin from the peritoneal cavity and, thus, indirectly increase the apparent toxicity of the preparation. For example, if the contaminants compete with the cytolysin for binding sites in the peritoneal cavity, the cytolysin would reach the bloodstream faster and in larger amounts after intraperitoneal injection of the crude preparation than after injection of the purified preparation.

The purified cytolysin preparation also had vascular permeability factor activity in guinea pig skin, and the activity was similar to that previously described (22) for the crude cytolysin preparation. One blueing dose was equivalent to 25 to 50 HU (0.3 to 0.6 μg) (data not shown).

In conclusion, the pharmacological activities of the purified *V. vulnificus* toxin described in this paper (cytolytic, vascular permeability factor, and lethal activities) are consistent with the pathological features of naturally occurring and experimentally induced disease caused by the bacterium (local edema and tissue necrosis, necrotizing vasculitis, hemolytic anemia, and death). We have, therefore, begun studies with the purified toxin to examine the possible importance of the toxin in the pathogenesis of disease caused by *V. vulnificus*.

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