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Purification of Staphylococcal β-Hemolysin and Its Action on Staphylococcal and Streptococcal Cell Walls

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ABSTRACT

CHESBRO, WILLIAM R. (University of New Hampshire, Durham), FRED P. HEYDRICK, ROLAND MARTINEAU, AND GAIL N. PERKINS. Purification of staphylococcal β-hemolysin and its action on staphylococcal and streptococcal cell walls. J. Bacteriol. **89**:378-389. 1965.—After growth of bovine-derived strains of *Staphylococcus aureus* in a completely dialyzable medium, the β-hemolysin in the culture supernatant fluids was purified by gradient-elution chromatography on cellulose phosphate. The purified hemolysin contained two components, demonstrable by immunodiffusion or electrophoresis, but was free from α-hemolysin, coagulase, Δ-hemolysin, enterotoxins A and B, glucuronidase, hyaluronidase, lipase, muramidase, Panton-Valentine leukocidin, phosphatase, and protease. The hemolysin was heat-labile and sulfhydryl-dependent, and the preparation was leukocidal for guinea pig macrophages. When rabbit red blood cell (RBC) stroma and staphylococcal or enterococcal cell walls were treated with the purified hemolysin, it liberated mucopolysaccharides from the rabbit RBC stroma, polysaccharides and mucopolysaccharides (or mucopeptides) from the staphyloccoal cell walls, and rhamnose, glucose, an unidentified monosaccharide, *N*-acetylglucosamine, and at least two polysaccharides from the enterococcal cell walls. The hemolytic and cell-wall degradative activities had similar thermal inactivation kinetics, *p*H optima, sedimentation coefficients, and chromatographic and electrophoretic mobilities; both required Mg and were inhibited by thiol-inactivating agents. Consequently, it seems likely that both activities are expressions of the same enzyme.

β-Hemolysin production is typical of staphylococci isolated from animals (Burns and Holtman, 1960) and is particularly distinctive of strains isolated from cases of bovine mastitis (Slanetz and Bartley, 1953). Because of this almost invariant association with mastitis-producing staphylococci, the hemolysin has been purified and studied as part of a program directed toward reduction of this economically wasteful infection.

The purified preparation has been found to exhibit two activities not previously reported in connection with the β-hemolysin: (i) it is leukocidal, and (ii) it degrades a hexose-*N*-acetylhexosamine complex in the walls of staphylococci and enterococci.

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MATERIALS AND METHODS

Cultures and cultural procedures. The strain of *Staphylococcus* used in most of these studies, *Staphylococcus aureus* UNH-Donita, was maintained on slants of a medium containing 2.0% Tryptose (Difco), 1.0% Proteose Peptone (Difco), 1.0% dextrose, 0.25% NaCl, 0.25% KCl, 1.0% K_2HPO_4, 0.2% soluble starch, and 1.2% agar. After 48 hr of growth on this medium at 35 C, the slants were stored at 4 C. At 2-week intervals, the cultures were streaked on sheep-blood agar plates, and colonies showing broad zones of hemolysis were used to inoculate new slants of maintenance medium.

For hemolysin production, a completely dialyzable medium that gave good hemolysin yields without containing large amounts of nonspecific protein was prepared by dialyzing 20 g of NZ amine (Sheffield Chemical, Norwich, N.Y.), 3 g of yeast extract, 3 g of brain-heart infusion, and 3 g of veal (Difco) in 100 ml of distilled water against 1 liter of distilled water for 36 hr. Prior to autoclaving, 0.25% KCl, 0.25% NaCl, 0.15% K_2HPO_4, 0.05% $MgSO_4$, 0.25% mannitol, and 0.5% L-arginine were added to the dialysate. The

addition of mannitol was gratuitous for some strains. However, all strains gave improved yields of hemolysin in the presence of arginine.

After inoculating 50 ml of this medium in a stoppered, 1-liter flask, the flask was filled with an 80% O_2–20% CO_2 mixture and incubated at 35 C for 24 hr with shaking. This was used to inoculate 950 ml of the same medium in a 4-liter, heavy-walled flask equipped for gassing. After filling with the O_2–CO_2 mixture, the large flask was incubated at 35 C with shaking. The gas mixture was replenished after 6 and 12 hr of incubation. Growth was terminated after 24 hr by adding 5 ml of $CHCl_3$ to each flask and holding the culture at 5 C for 4 hr. The suspension was then centrifuged, and the supernatant fluid was saved for further processing.

Under these conditions, the supernatant fluids of the UNH-Donita strain showed hemolysin titers of 1:8,000 to 1:20,000.

The enterococcus used in these studies, *Streptococcus faecium* (Orla-Jensen, 1919) strain HF8AG, was maintained and grown in bulk as described elsewhere (Chesbro and Evans, 1962).

Hemolysin purification. After overnight dialysis in the cold against 3 volumes of distilled water, the culture supernatant fluid was reduced to 0.03 of its original volume by per-evaporation at 35 C. It was again dialyzed overnight in the cold and then reduced to a final volume of 30 to 50 ml. This was centrifuged at 15,000 × *g* for 15 min, and any precipitate was discarded.

A column of cellulose phosphate, standard type (Schleicher and Schuell Co., Keene, N.H.), was prepared by first washing 20 g of the ion exchanger on a Büchner funnel with 300 ml of sodium phosphate–NaCl, 0.2 M in PO_4 and 1.0 M in NaCl, adjusted to *p*H 7.4 with NaOH (Peterson and Sober, 1956). It was then rinsed with distilled water and rewashed with 300 ml of cold 1 M H_3PO_4. Finally, 14 to 16 liters of sodium phosphate, 0.001 M in PO_4, adjusted to *p*H 7.4, were passed through the funnel until the effluent *p*H reached 7.4. A 6- to 8-g amount of this washed cellulose phosphate was packed under 1 to 3 lb. of air pressure in a glass column 1.5 cm in diameter.

The crude concentrate was allowed to enter the top of the column by gravity, and the adsorbed mixture was fractionated by gradient elution with sodium phosphate. The gradient was generated in a two-chamber system. The mixing chamber, adjacent to the column, was a flat-bottomed, cylindrical glass bottle, 4.75 cm in diameter, and initially contained 130 ml of the lower concentration of eluting buffer. The second chamber, also flat-bottomed and cylindrical, with a diameter of 7.0 cm, received 360 ml of the higher concentration of eluting buffer. A siphoning system connected the two chambers so that equal volumes of buffer simultaneously entered the column and moved between the chambers, the mixing chamber meanwhile being continuously stirred by a magnetized bar.

All operations were carried out in a cold room, and 5- or 10-ml fractions were collected automatically at a flow rate of 15 to 30 ml/hr.

Cell-wall and stroma preparation. Suspensions of washed cells from 16-hr cultures were heated at 80 C for 10 min and then disrupted in a Nossal disintegrator. Cell-wall fragments were isolated by differential centrifugation. The fragments were treated with deoxyribonuclease, ribonuclease, and trypsin, followed by repeated washing in 0.02 M phosphate buffer (*p*H 6.8), and finally frozen.

Parpart's (1942) method was used to prepare stroma of sheep and rabbit red blood cells (RBC) from whole, citrated blood.

Activity assays. The hemolysin was titrated by use of a modification of the buffer described by Jackson and Mayman (1958), consisting of 0.85% saline, 0.01 M in both K_2HPO_4 and $MgSO_4$, and adjusted to *p*H 6.8. RBC suspensions were made by washing and resuspending at 1% concentration in the same buffer. At 37 C, 1 ml of this suspension was added to 1 ml of serially diluted hemolysin, also at 37 C, and the mixture was incubated for 1 hr. It was then chilled to 5 C, and the highest dilution showing hemolysis after 4 hr was noted.

Coagulase was determined by observing the action of serial dilutions of crude and purified preparations in 0.2-ml amounts upon 0.2 ml of citrated rabbit blood after a 2-hr incubation period at 37 C.

Glucuronidase activity was determined on a 0.01 M phenolphthalein glucuronide solution prepared from the cinchonidone salt derivative (Mann Research Laboratories, Brooklyn, N.Y.), by use of the method of Fishman, Springer, and Brunetti (1948), both at *p*H 4.5 in acetate buffer and at *p*H 6.8 in saline-phosphate-magnesium buffer.

Hyaluronidase activity was determined by the method of Tolksdorf et al. (1949) with potassium hyaluronate (Calbiochem) dissolved and brought to a concentration of 0.4 mg/ml in acetate-NaCl buffer, with and without 0.01 M $MgSO_4$. Heated, acidified, human serum was used to develop the turbidity, which was read at 600 mμ.

Muramidase activity was tested by observing the lytic action of preparations on a suspension of *Micrococcus lysodeikticus* cells (Worthington Biochemicals Corp., Freehold, N.J.) in 0.02 M sodium phosphate buffer, *p*H 6.8 (Richmond, 1959*a*).

Lipase activity was determined by the egg yolk-agar plate method described by Richou, Pantaleon, and Quinchon (1960), except that the agar suspension was made in saline-phosphate-magnesium buffer with the use of Colbeck EY agar (Difco).

Immunodiffusion methods (see the following sections) were used to test for enterotoxin. Enterotoxins A and B and homologous antisera were kindly made available by Ezra Casman of the Division of Microbiology, Bureau of Biological

and Physical Sciences, Food and Drug Administration, Washington, D.C.

Phosphatase activity was determined by using *o*-carboxyphenylphosphate (Worthington Biochemical Corp., Freehold, N.J.) as substrate, and measuring the salicylic acid liberated in a Beckman DU spectrophotometer at 298 mμ (Hofstee, 1954).

Protease activity was tested by observing the clearing of heated rabbit-plasma agar plates according to the method of Christie and Wilson (1941). The test solutions were contained in stainless-steel cylinders on the agar surface.

Nuclease activity was measured by the method of Alexander, Heppel, and Hurwitz (1961).

Leukocidal activity was measured according to the technique of Woodin (1959) by use of guinea pig macrophages obtained by injection of 12% sterile casein, followed by peritoneal washing 12 hr later (Sbarra and Karnovsky, 1960). Activity of the Panton-Valentine leukocidin was measured by the slide technique described by Jackson and Little (1957) with rabbit leukocytes.

Protein was measured by the method of Lowry et al. (1951), and nucleic acids were estimated by the ratio of absorbancies at 280 and 260 mμ.

Immunodiffusion, electrophoresis, chromatography, and ultracentrifugation. Standard beta antitoxin was supplied through the courtesy of the Connaught Medical Research Laboratories, Toronto, Ontario, Canada.

Rabbit antiserum was obtained from animals receiving a series of intramuscular injections of the purified β-hemolysin containing 100 μg of protein. The hemolysin was used without detoxification, but was disinfected by saturation with $CHCl_3$. The rabbit antisera prepared in this way routinely contained 32 units of anti-β-hemolysin activity.

Bovine antiserum, kindly made available by Clara H. Bartley of the Department of Microbiology, University of New Hampshire, was obtained from animals receiving a whole cell-toxoid vaccine (Slanetz, Bartley, and Allen, 1959) containing the strains used in these studies.

Conventional gel double diffusion was performed by use of 0.8% Ionagar (Colab, Chicago Heights, Ill.) and the technique of Wilson and Pringle (1954). Slide double diffusion was performed by use of the technique described by Crowle (1958).

Electrophoretic examinations were made in a Durrum-type cell employing Schleicher and Schuell 2043a paper strips. Two buffer *p*H values were routinely employed: *p*H 7.0, obtained with a sodium phosphate buffer (ionic strength = 0.1), and *p*H 4.5, by use of an acetate-ethylenediaminetetraacetate mixture as described by Bodman (1960). A constant current of 0.1 ma/cm of width was applied for 14 to 20 hr, and the paper strips, after drying at 100 C for 20 min, were stained with 0.001% nigrosin in 2% acetic acid.

Preparative electrophoresis was carried out on a horizontal bed of polyurethane foam segments, 0.5 × 1.0 × 1.6 cm each, prepared as described by Davidson (1959). Commercially available Veronal buffer, *p*H 8.6, and tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.9, (LKB Instruments, Inc., Washington, D.C.), were used. After overnight dialysis against the electrophoresis buffer containing 0.001 M mercaptoethanol, the purified hemolysin was absorbed into the segment used as the origin. The other segments were saturated with the buffer, and the system, at 4 C, was subjected to a constant current for the desired time. Each segment was then removed, its fluid content expressed by squeezing, and the amount of hemolysin and wall-degradative activity in each fraction was measured after dialysis of the fraction against the titration buffer.

Sample preparation, chromatography, and detection methods for amino acids, amino sugars, acetylated amino sugars, polyols, phosphorylated compounds, and phosphate were performed as described elsewhere (Isquith and Chesbro, 1963). Mono- and polysaccharides were detected by the technique of Partridge (1949). Monosaccharides were quantitated by the method of Jones (1958). *N*-acetylaminosugars were measured by the method of Salton (1959). Total carbohydrate was measured by the anthrone method (Neish, 1952).

Ultracentrifugation on a 5 to 20% linear sucrose gradient was performed by the modification of the method of Martin and Ames (1961) described by Bernheimer and Schwartz (1963). A 3-ml amount of the β-preparation was reduced to 1 ml by holding in dialysis tubing covered with a layer of a hygroscopic, high molecular weight, cellulose derivative (Aquacide 2, Calbiochem), and 650 μg of yeast alcohol dehydrogenase (Worthington Biochemical Corp., Freehold, N.J.) were added as a reference standard to the concentrated hemolysin preparation. The mixture was layered on 24 ml of the sucrose gradient in a polyethylene tube (25 × 75 mm) and centrifuged at 0 C for the desired length of time. The Spinco model L2 preparative ultracentrifuge, SW-25 rotor, and gradient-forming device used were kindly made available by Edward Herbst of the Department of Biochemistry, University of New Hampshire. The tube was then punctured, and the contents were permitted to flow out drop by drop; 19 fractions containing 30 drops each were collected first, followed by 51 fractions of 10 drops each (the latter fractions contained nearly 235 μliters each).

A 10-μliter amount of each fraction was assayed for β-hemolysin in the usual way, and 5 μliters were assayed for alcohol dehydrogenase by following the reduction of nicotinamide dinucleotide at 340 mμ in the presence of substrate as described by Martin and Ames (1961). The remainder of each fraction was dialyzed in the cold against 500 volumes of titration buffer three successive times. The new volume of the fraction remainder was measured, and 120 μliters were assayed for wall-degradative activity.

RESULTS

Toxin production and purification. In preliminary attempts to purify the hemolysin, we employed phase fractionation with cold alcohol and acetic acid essentially as described by Wittler and Pillimer (1948), and more recently utilized by Robinson, Thatcher, and Gagnon (1958). However, less than 5% of the β-activity of the crude culture supernatant fluid could be recovered in the precipitate, although over 70% of the α-activity was recoverable. Inspection of the data of Robinson et al. (1958) showed that they, too, recovered less than 5% of their starting activity. In contrast, Jackson (1963) reported recovering the hemolysin in good yield after Zn-alcohol and Zn-Hg precipitation: Haque and Baldwin (1963) reported good recovery after acetone precipitation.

In these and later experiments, the β-hemolysin was found to be very susceptible to denaturation. Consequently, to make a preliminary precipitation step unnecessary by avoiding the presence of large amounts of nonspecific protein in the crude supernatant fluid, a number of synthetic and semisynthetic media (Boniece, 1956; Mergenhagen, 1958; Richmond, 1959*b*) were tested as production media, but, in all of them, toxin production was less than 10% of that observed in complex media. Therefore, the complex, but completely dialyzable, production medium described in the preceding section was developed.

Culture supernatant fluids of this medium were reduced in volume and applied to columns packed with cellulose phosphate, diethylaminoethyl cellulose, or carboxymethyl cellulose. The β-hemolytic activity was only weakly retained by the anion-exchanging column, but was strongly retarded by either of the cation-exchanging columns. After preliminary trials with both cation-exchange materials, subsequent purifications were carried out with the cellulose phosphate, because it gave a slightly faster flow rate.

The fractionation of the concentrated supernatant fluid obtained by gradient elution from a cellulose phosphate column is shown in Fig. 1

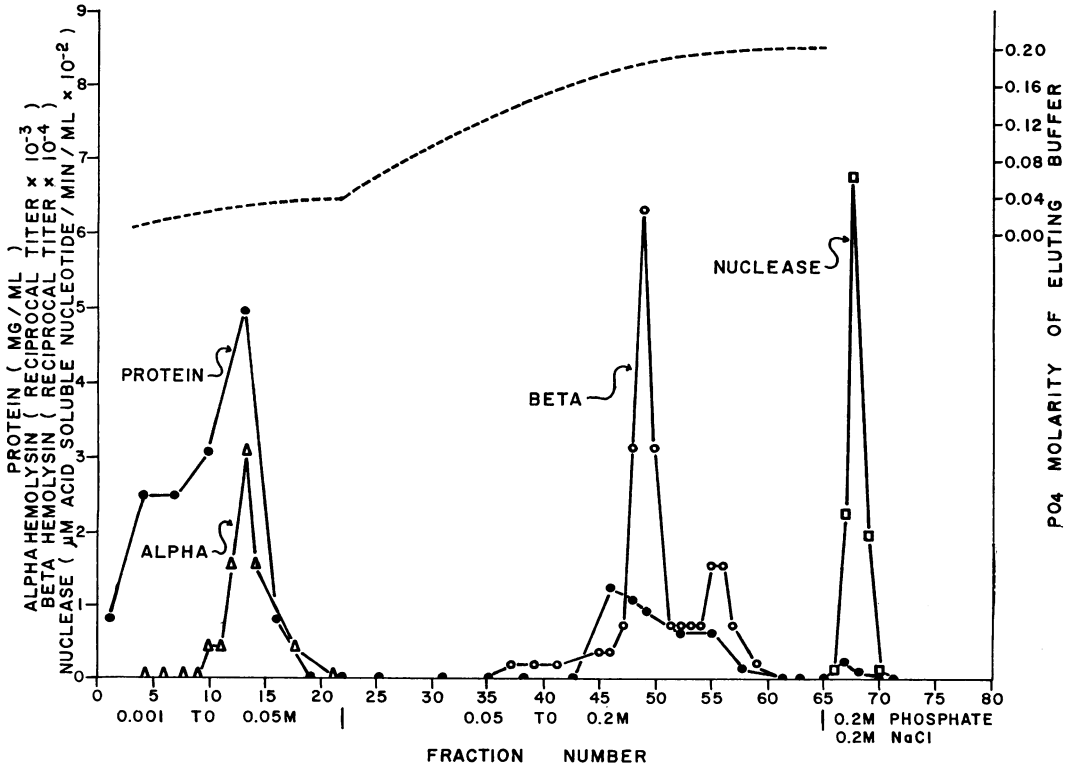

FIG. 1. *Fractionation of the nondialyzable, exocellular proteins of Staphylococcus aureus UNH Donita by sodium ion-gradient elution chromatography at pH 7.4 with a column (1.4 × 15 cm) of cellulose phosphate, and collection of 10-ml fractions at a flow rate of 25 ml/hr.*

In the peak-activity fraction, the specific activity of the hemolysin was 150-fold that of the concentrated culture supernatant fluid, and it was cleanly resolved from the α-hemolysin and the nuclease.

In the peak fraction, 0.00625 ml of toxin neutralized one standard unit of antitoxin, and examination by gel double diffusion against both rabbit and bovine antisera yielded a major and minor line of precipitation.

A major and a minor component were also distinguishable by electrophoresis, the former accounting for 85% of the fraction's protein content. Both components migrated cathodically at *p*H 7.0, the major component showing the greater excursion.

The ratio of absorbancies at 280 and 260 mμ indicated that the purified preparation contained at most 3% nucleic acid.

The preparation at this stage showed marked instability: its activity was lost upon standing at room temperature, when subjected to protracted agitation, or when filtered through sintered glass, Selas, or membrane filters.

Further attempts at purification resulted in drastic losses of activity and were abandoned.

The general lability of the hemolysin was suggestive of a protein dependent upon thiol groups for activity. Several thiol-inactivating agents were tested, and the hemolysin was found to be sensitive to *p*-chloromercuribenzoate and iodoacetate; a 0.001 M solution of the former caused 50% inhibition of the hemolysin, and the same concentration of the latter caused 100% inhibition. A number of other thiol-inactivating reagents were tested, but were themselves strongly hemolytic at concentrations at which they might be expected to inactivate the hemolysin.

When the purified hemolysin was treated with trypsin (1.0 mg/ml), its activity was completely resistant to digestion for over 24 hr; under the same conditions, the activities of the α-hemolysin and the nuclease were destroyed in 120 min. The effect of trypsin on the β-activity of crude culture supernatant fluids was also tested, and here, too, the activity proved resistant.

However, the trypsinized hemolysin was found to be no longer strongly retarded on the cellulose phosphate column, displacing instead in the same elution region as α-hemolysin. Trypsin treatment, although leaving hemolytic activity intact, apparently cleaved basic residues from the hemolysin.

Chymotrypsin, pepsin, and papain rapidly destroyed the activity of the hemolysin.

A number of small protein peaks emerged between the α- and β-peaks during gradient elution with three of the five staphylococcal strains thus far examined (although not with the Donita strain illustrated in Fig. 1), and hemolytic activity was associated with some of these additional peaks in two of the three strains. A hemolysin was found which hemolyzed rabbit RBC at 37 C and sheep RBC after a "hot-cold" sequence. It was thus dissimilar to any of the classically described hemolysins, but was similar in activity to the "anionic-beta hemolysin" reported by Haque and Baldwin (1963). On the basis of protein and activity measurements, there was only 5% as much of this hemolysin as there was β-hemolysin in the culture supernatant fluid.

The occasional occurrence of such lesser hemolytic activities in culture supernatant fluids is the probable basis of reports of multiple β-hemolysins, such as Thaysen's (1948), who noted two antigenically distinguishable β-hemolysins from an animal-derived strain of *S. aureus*.

Activity tests on the purified hemolysin. The finding of Jackson and Mayman (1958) that the hemolysin requires Mg as a cofactor was confirmed. In the absence of added Mg, the hemolysin's activity was reduced over 95%.

The purified preparation was tested for activities known to be associated with the exocellular products of staphylococci, partly to determine its separation from these activities, and partly to determine whether β-lysis was the sole property of the hemolysin.

The concentration of protein tested was 50 μg/ml (approximately 3,000 hemolytic units), except in the gel double-diffusion test for enterotoxin, when 100 μg of hemolysin protein were used in the antigen well.

The only activity other than β-hemolysis that the purified preparation exhibited in these tests was a leukocidal effect on guinea pig macrophages. Under the standard conditions of the test, a hemolysin dilution containing 1.7 μg of protein caused complete inhibition of the macrophages' ability to reduce phenol-iodo-2,6-dichlorophenol; Giemsa-stained smears of the mixture showed the leukocytes to be swollen and distorted, or disintegrated.

The activity-test results were negative for α-hemolysin, coagulase, enterotoxin, glucuronidase, hyaluronidase, lipase, muramidase, nuclease, Panton-Valentine leukocidin, phosphatase, and protease.

Since staphylococcal leukocidin is considered to be inert toward the guinea pig macrophage (Gladstone and van Heyningen, 1957), this leukocidal capacity is apparently an intrinsic property of the β-hemolysin, and is not due to

contamination with leukocidin. To determine whether the leukocidin was present, the purified hemolysin was tested for ability to distort or disrupt rabbit lymphocytes, a characteristic of the leukocidin, and was found not to possess such ability. The Δ-hemolysin has also been implicated as a leukocide by Jackson and Little (1957), but 50 μg of the purified β-hemolysin protein (approximately 3,000 hemolytic units) showed no hemolytic activity for rabbit RBC and was active upon sheet RBC only after a hot-cold sequence. Consequently, it was free from the Δ-hemolysin.

An in vivo test of the β-hemolysin's activity was made by intradermal injection in rabbits. The hemolysin was injected singly or in combination with a dilution of the chromatographic fraction containing peak α-hemolysin activity. The diluent in all cases was supplemented with 0.01 M $MgSO_4$, and a total volume of 1 ml was injected. The results (average of two rabbits) are shown in Table 1.

When injected singly, the β-hemolysin was almost without effect; injected with the α-hemolysin, it definitely accelerated the rate of necrotization.

Action of the purified β-hemolysin on staphylococcal and streptococcal walls. S. aureus and *S. faecium* purified cell-wall material (20 mg each, dry weight) was treated with 0.5 mg of the hemolysin in 3 ml of 0.02 M phosphate buffer, *p*H 6.8, which was 0.005 M in mercaptoethanol and 0.01 M in $MgSO_4$. The mixture was incubated for 8 hr at 37 C along with controls containing either wall material or hemolysin.

At the end of the incubation period, the mixtures were centrifuged at 25,000 × *g* at 4 C, and the supernatant fluids were decanted. These were placed on a Dowex 50 column in the H^+ form. The column was first eluted with distilled water, the eluate containing neutral and acidic compounds, and then eluted with 0.1 M piperidine (Buchanan, 1957) to displace basic compounds. The different eluates were concentrated in vacuo and examined by chromatography on Whatman no. 4 paper by use of a butanol-pyridine-water mixture (8:8:4) for development and on Whatman no. 1 paper with a butanol-acetic acid-water mixture (12:3:5) for development.

The chromatograms were examined for ninhydrin-positive compounds, phosphorylated compounds and phosphate, hexosamines, *N*-acetylhexosamines, carbohydrates, polyols, and materials quenching at 260 mμ.

In the supernatant fluid of the hemolysin-treated staphylococcal cell walls, two spots reacting with carbohydrate spray reagents were detected with values relative to glucose (R_G) of 0.15 and 0.58 in the butanol-pyridine-water solvent, and a spot reacting with both carbohydrate spray reagents and ninhydrin was detected with an R_G value of 0.75 in the same solvent. Because of their low mobilities, it is presumed that all three compounds are polymers, the former two being polysaccharides and the latter being a mucopolysaccharide or mucopeptide.

In the supernatant fluid of the hemolysin-treated streptococcal cell walls, the following compounds were detected: rhamnose, glucose, a monosaccharide with an R_G of 1.17 in the butanol-pyridine-water solvent, *N*-acetylglucosamine, and two spots reacting with carbohydrate spray reagents, with R_G values of 0.12 and 0.25 in the butanol-pyridine-water solvent—presumably polysaccharides in view of their low mobilities.

These compounds were unique to the supernatant fluids of wall-hemolysin mixtures and did not appear on chromatograms of either wall materials or hemolysin supernatant fluids incubated separately.

No detectable polyols, phosphorylated compounds, phosphate, or free amino acids were liberated from the wall material of either genus.

TABLE 1. *Dermonecrotic activity of α- and β-hemolysins injected intradermally into rabbits singly and in combination*

Postinjection time of observation	Toxin effect at site of injection		
	α-hemolysin (500 hemolytic units)	β-hemolysin (200 μg)	α- and β-hemolysin
hr			
2	Erythrogenic circle, 15 to 18 mm in diameter	No effect	Necrotic circle, 22 mm in diameter
4	Necrotic circle, 22 to 30 mm in diameter	No effect	Necrotic circle, 26 to 38 mm in diameter
24	Necrotic circle, 27 to 40 mm in diameter	Slight erythrogenesis at injection site	Necrotic circle, 30 to 40 mm in diameter

The ability of the hemolysin to degrade stroma of sheep and rabbit RBC was tested under similar conditions. The supernatant fluid of the sheep stroma-hemolysin mixture contained no detectable amounts of compounds similar to those released from bacterial cell walls. The supernatant fluid of the rabbit stroma-hemolysin mixture, however, contained a small amount of material which reacted both with the carbohydrate spray reagent and ninhydrin, and exhibited low mobility in the butanol-pyridine-water solvent. Thus, presumably, it was a mucopolysaccharide.

Comparison of the hemolytic and cell-wall degradative activities of the purified hemolysin. To characterize the cell-wall degradative activity of the purified preparation, a standard reaction mixture containing 7.5 mg (dry weight) of *S. faecium* cell-wall material per ml was used. The walls were suspended in 0.001 M phosphate buffer, containing 0.01 M $MgSO_4$, at *p*H 6.8, except in those experiments in which the effect of omitting Mg, changing *p*H, or changing the concentration of wall material was studied.

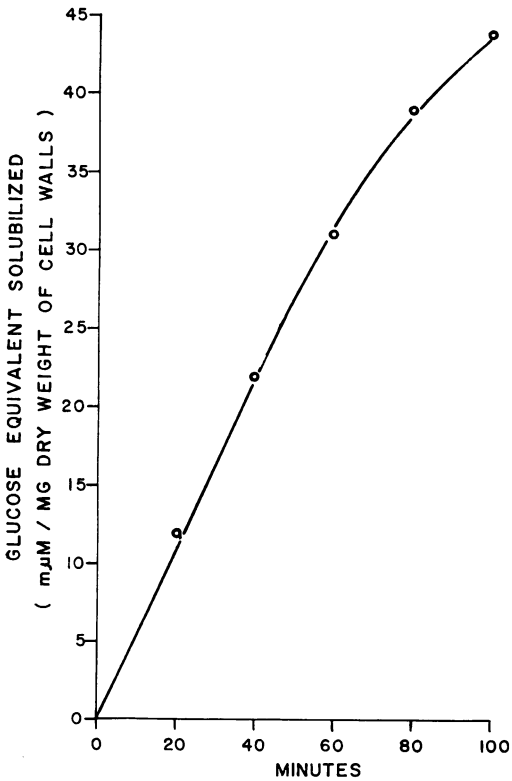

FIG. 2. *Release of soluble carbohydrate from cell walls of Streptococcus faecium by purified staphylococcal β-hemolysin at 37 C. The reaction mixture contained 7.5 mg (dry weight) of cell walls and 20 μg of hemolysin protein per ml in 0.001 M phosphate buffer, 0.01 M in $MgSO_4$, at pH 6.8.*

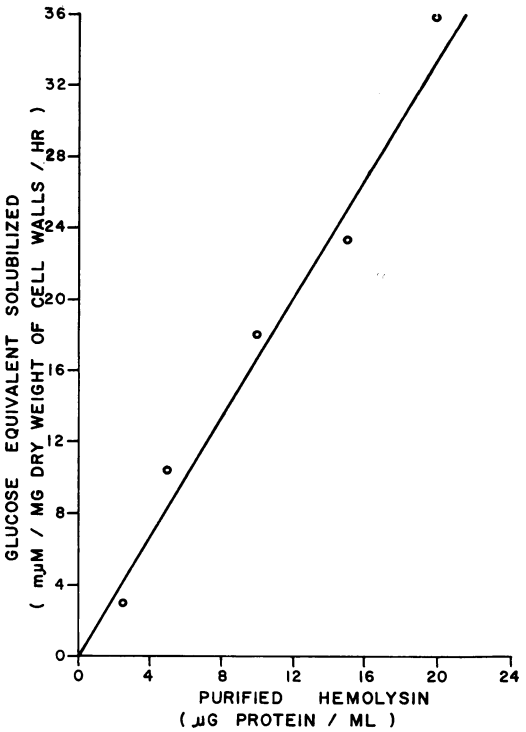

FIG. 3. *Effect of increasing hemolysin concentration upon the rate of release of soluble carbohydrate from cell walls of Streptococcus faecium by purified staphylococcal β-hemolysin at 37 C. In addition to the purified hemolysin, reaction mixtures contained 7.5 mg (dry weight) of cell walls per ml in 0.001 M phosphate buffer, 0.01 M in $MgSO_4$, at pH 6.8.*

The addition of 50 μliters of acetic acid per ml of reaction mixture, followed by chilling to 4 C, was found to be an effective way to terminate the reaction and avoided the use of stronger mineral or organic acids—such as $HClO_4$ or trichloroacetic acid—which might be extractive of wall components, particularly teichoic acid. After termination of the reaction, the mixture, at 4 C, was centrifuged at 10,000 × *g* for 20 min, and the supernatant fluid was analyzed for released, soluble carbohydrate by the anthrone method.

In each experiment, control mixtures containing hemolysin autoclaved for 15 min were included, as well as control mixtures lacking hemolysin.

The release of soluble carbohydrate from enterococcal cell walls in a reaction mixture containing 20 μg of hemolysin protein per ml is

shown in Fig. 2. The release of carbohydrate-reacting material was nearly linear for 1 hr at 37 C. Figures 3 and 4 show the effect upon the rate of carbohydrate solubilization of varying hemolysin and wall concentrations. The rate was proportional to the hemolysin concentration and showed substrate (i.e., wall material) saturation.

The effect of *p*H upon the wall-degradative and β-hemolytic activities was determined over the *p*H range 4.0 to 9.0 (Fig. 5).

Both the hemolytic and cell-wall degradative activities had *p*H optima between 6.5 and 7.0 and responded in the same way to increasing alkalinity. The wall-degradative activity, however, decreased less rapidly with increasing acidity than did the hemolytic activity. Since the expression of the preparation's attack upon the erythrocytes depends upon its osmotic characteristics (Jackson and Mayman, 1958), which in turn are complex functions of the erythrocyte's ionic environment, and the expression of the preparation's attack on the wall fragments depends directly on the enzymatic susceptibility of the substrate in the fragments, the responses of the two activities to *p*H changes seem in reasonable agreement. However, the observed differences constitute the strongest evidence that the two activities do not reside in the same molecular species.

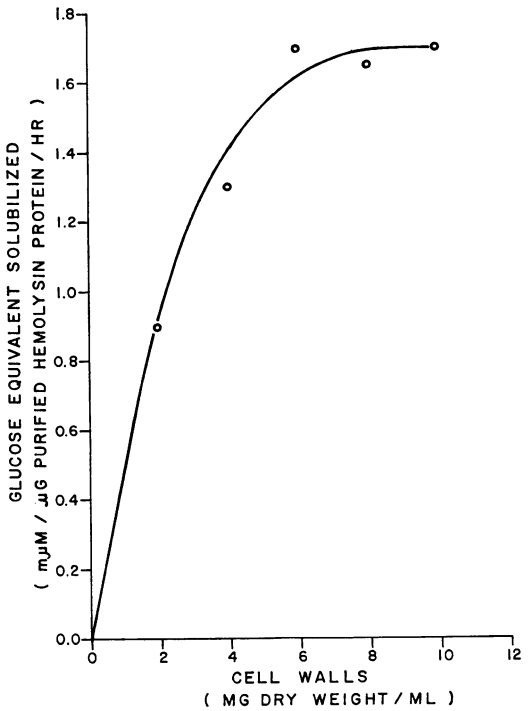

FIG. 4. *Effect of increasing cell-wall concentration upon the rate of release of soluble carbohydrate from cell walls of Streptococcus faecium by purified staphylococcal β-hemolysin at 37 C. In addition to the cell walls, reaction mixtures contained 20 μg of hemolysin protein per ml in 0.001 M phosphate buffer, 0.01 M in $MgSO_4$, at pH 6.8.*

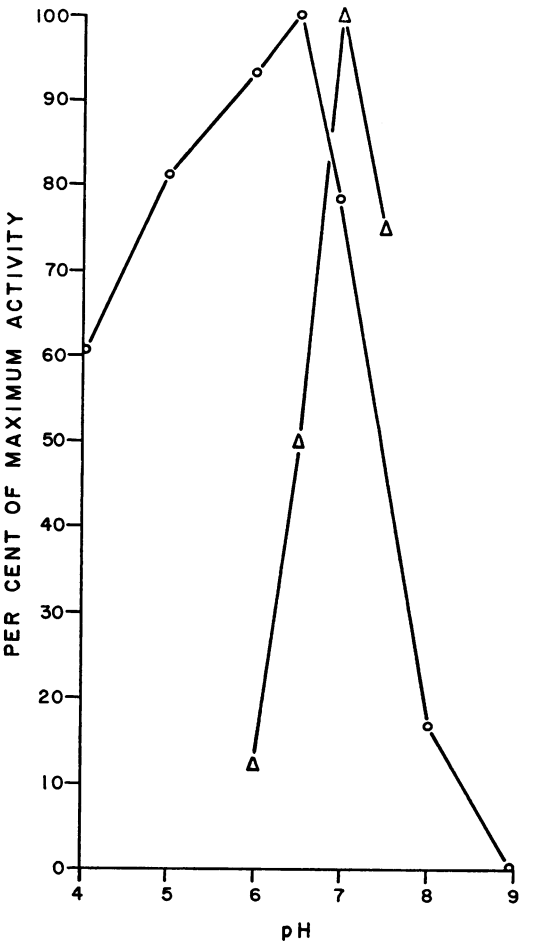

FIG. 5. *Comparison of the effect of pH upon the hemolytic (△) and cell-wall degradative (○) activities of purified staphylococcal β-hemolysin at 37 C. The reaction mixtures were buffered with 0.001 M acetate over the pH range 4.1 to 6.5, with 0.001 M phosphate buffer over the range 5.5 to 7.5, and with 0.001 M Tris over the range 7.0 to 9.0. All solutions were 0.01 M in $MgSO_4$. Hemolytic activity was measured in isotonic saline.*

Fractions 40 through 80 of the toxin fractionation (Fig. 1) were then assayed for wall-degradative activity. The degradative activity of the fractions closely paralleled their hemolytic activity.

The thermal sensitivity of the two activities was compared by heating 200-μliter amounts of the purified hemolysin in 0.001 M phosphate buffer, containing 0.01 M $MgSO_4$, at *p*H 6.8, in sealed glass tubing 4 mm in diameter. The samples were heated for 5 min at 60, 75, 90, and 105 C, then assayed for residual hemolytic and wall-degradative activity. The resultant inactivation curves are shown in Fig. 6.

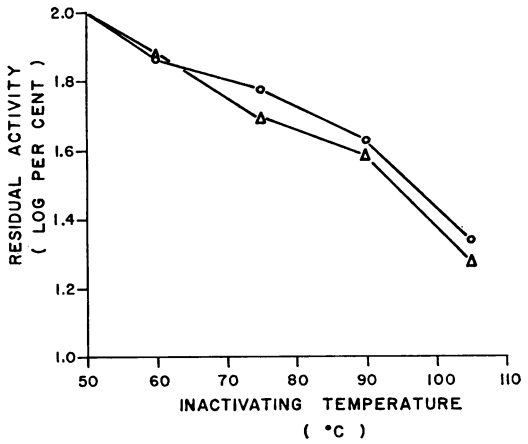

FIG. 6. *Comparison of the thermal lability of the hemolytic (△) and cell-wall degradative (○) activities of purified staphylococcal β-hemolysin. The hemolysin was held for 5 min at the indicated temperature.*

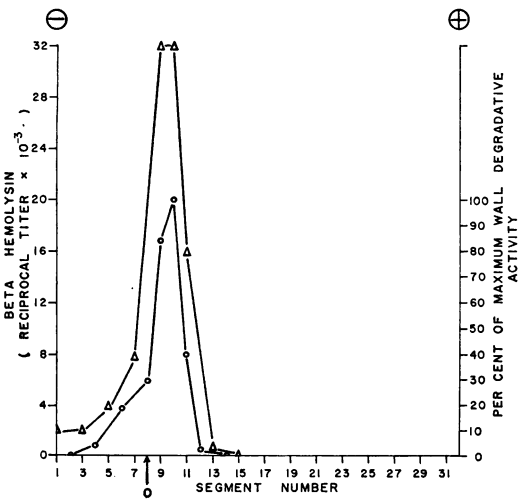

FIG. 7. *Distribution of the hemolytic (△) and cell-wall degradative (○) activities of purified staphylococcal β-hemolysin after electrophoresis on polyurethane foam segments for 16 hr at 4 C. A current of 2.5 ma/cm was applied to the segments, which were each 1 cm wide and saturated with Tris buffer, pH 8.9, that had a conductivity of 3 mmho.*

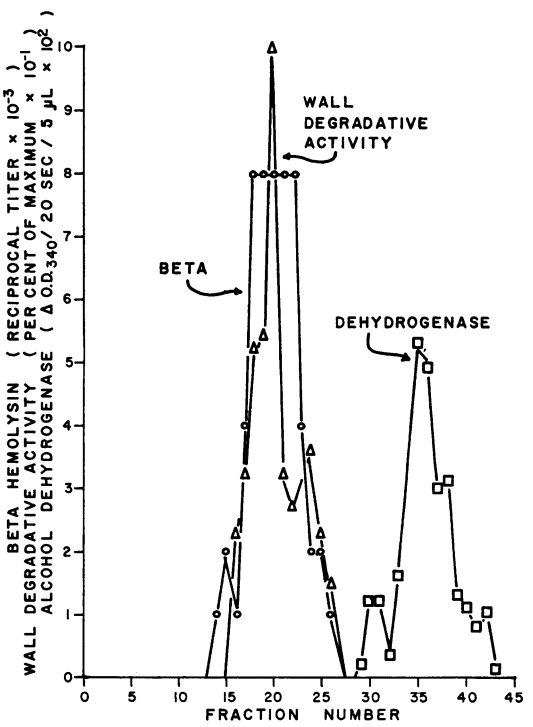

FIG. 8. *Distribution of the hemolytic and cell-wall degradative activities of purified staphylococcal β-hemolysin, and of yeast alcohol dehydrogenase, after centrifugation at 24,000 rev/min for 20 hr on a 5 to 20% linear sucrose gradient.*

The thermal labilities of the two activities were quite similar. Both activities were relatively heat-labile, being over 50% diminished in 5 min at 75 C.

A sample of the purified preparation was subjected to electrophoresis on polyurethane foam segments. The identical distribution of the two activities after 16 hr is shown in Fig. 7. The activities also showed identical migrations in *p*H 8.6 Veronal buffer, but moved cathodically at this *p*H. The isoelectric point of the hemolysin thus lies between *p*H 8.6 and 8.9.

The hemolysin preparation, with yeast alcohol dehydrogenase added as a reference protein, was subjected to sucrose-gradient ultracentrifugation. After fractionation of the sucrose column and assay of the β-hemolytic and alcohol dehydrogenase activity of each fraction, the fractions were exhaustively dialyzed and then assayed for wall-degradative activity. The distribution of the three activities in the uppermost 45 fractions is shown in Fig. 8.

The alcohol dehydrogenase sedimented 1.55 cm from the center of the applied protein layer,

and the degradative and hemolytic activities sedimented at 0.83 cm. Assuming the same partial specific volumes for both the reference protein and the β-hemolysin, and by use of the method of Martin and Ames (1961), an approximate molecular weight of 59,000 can be calculated for the hemolysin.

When the thiol inhibitors described in an earlier section, which inhibited the hemolytic activity of the purified preparation, were tested for their effect on the degradative activity, this activity was no longer detectable in the purified preparation after treatment with either 0.001 M *p*-chloromercuribenzoate or iodoacetate.

The carbohydrate-releasing capacity of the preparation, like its hemolytic capacity, was strongly dependent upon the addition of Mg to the reaction mixture; less than 15% remained when Mg was omitted.

To assess what proportion of the total wall-carbohydrate material could be solubilized, a reaction mixture containing 20 μg of hemolysin protein per ml and the usual amount of wall material was incubated at 37 C for 2 hr. A control mixture containing autoclaved hemolysin was incubated concurrently. After centrifugation, the carbohydrate in samples of the supernatant fluids and the deposited wall materials was determined by the anthrone method. The balance of each supernatant fluid was concentrated in vacuo, and its content of free glucose, rhamnose, and *N*-acetylglucosamine was determined chromatographically by use of a homologous standard for each of the monosaccharides.

The hemolysin preparation released the equivalent of 52 mμmoles of glucose per mg (dry weight) of wall material. Since there were approximately 1.9 μmoles of glucose equivalent per mg (dry weight) of wall material present in the control mixture, about 2.7% of the total carbohydrate content of the wall material was released.

The preparation liberated 8 mμmoles of rhamnose, 9 mμmoles of *N*-acetylglucosamine, and 15 mμmoles of glucose per mg (dry weight) of wall material, nearly a 1:1:2 proportion.

The glucose equivalent of the unidentified monosaccharide was 7 mμmoles liberated per mg (dry weight) of wall material. Thus, there were 13 mμmoles of glucose equivalent per mg (dry weight) of wall material liberated in polysaccharides, as determined by difference from the total carbohydrate released.

DISCUSSION

The correspondence between the β-hemolytic and the cell-wall degradative capacity of the purified preparation in chromatographic and electrophoretic mobility, sedimentation coefficient, *p*H optimum, Mg requirement, sensitivity to thiol inhibitors, and heat lability makes it likely that both are activities of the same exocellular enzyme.

Other exocellular enzymes produced by *S. aureus* known to be active against cell-wall material, the virolysin (Ralston et al., 1961) and the enzyme studied by Richmond (1959*a*), cause lysis of *M. lysodeikticus*, a capability lacking in the purified β-hemolysin preparation. The wall-degradative activity of the hemolysin preparation is therefore unlikely to be related to the activity of either of these two enzymes.

The polymeric nature of the degradation products from the staphylococcal cell walls hinders identification of the components acted upon, but the products obtained from the enterococcal cell walls correspond to constituents of the fraction VIII obtained by Ikawa (1961) from the cell walls of *S. faecalis*; a comparison of the amount of cell-wall carbohydrate liberated by the hemolysin preparation in the present experiments with the amount reported by Ikawa to be present in fraction VIII indicates that the hemolysin released about 10% of the carbohydrate in the fraction.

In addition to glucose, rhamnose, and amino sugar, fraction VIII contained ribitol, phosphate, and D-alanine (the latter three compounds presumably existed as ribitol teichoic acid prior to hydrolysis of the fraction). Since none of these latter compounds was released by the hemolysin, the hexose-*N*-acetylglucosamine polymer (or polymers), which is its substrate, and the phosphopolyol polymer must be distinct structures, although possibly cross-linked. Such cross-linkage, either by hindering the hemolysin's attack or permitting retention of much of the degradation product by the wall fragments through unattacked bonds, could account for the apparently small percentage of substrate rendered soluble by the hemolysin.

The diverse character of the end products and the ability of the hemolysin to attack what must be differing substrates in such taxonomically separated genera as *Staphylococcus* and *Streptococcus* indicate that it is relatively nonspecific. Consequently, it seems likely that its hemolytic and leukocidal activities derive from an ability to attack similar substrates in erythrocytes and leukocytes.

The failure to detect degradation products after treatment of sheep RBC stroma with the purified hemolysin suggests that the action of the hemolysin on the sheep RBC wall produces a

weakened structure, but is too limited to yield much soluble degradation product. Detection of degradation products after treatment of rabbit RBC stroma with the purified hemolysin, despite the preparation's complete lack of hemolytic activity toward rabbit RBC, indicates, on the other hand, that in these cells the attacked structure is not crucial to their osmotic stability.

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