Purification of Staphylocidal β -Lysin from Rabbit Serum

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A cationic protein of rabbit serum bactericidal for *Staphylococcus aureus* was purified. The specific activity per unit of protein of the purified staphylocidal preparation was approximately 37,000 times greater than that of the serum from which it was isolated. Similar techniques were used to purify serum β -lysin active against *Bacillus subtilis* approximately 24,000 times. The staphylocidal activity cannot be attributed to the same β -lysin active against *B. subtilis*, lysozyme, or antibody-complement systems. The concentrations of staphylocidal β -lysin in the sera of the five mammalian species studied did not correlate with their β -lysin activities against *B. subtilis*. The two β -lysins are similar in that both were heat-stable, sensitive to trypsin digestion, had molecular weights near 6,000, and were found in higher concentrations in serum than in plasma. Furthermore, similar techniques can be used to absorb and elute both substances in highly purified forms using cellulose asbestos filter pads and ion exchange chromatography on carboxymethyl cellulose. In contrast to the β -lysin against *B. subtilis*, the staphylocidal β -lysin was not released from blood platelets, and it was inactive in the presence of heparin, sodium citrate, sodium oxalate, ethylenediaminetetraacetic acid, acidic phospholipids, and acid pH values. A variety of proteins, including those of normal serum, preferentially inhibited the bactericidal activity of staphylocidal β -lysin but not the β -lysin against *B. subtilis*.

Heat-stable, naturally occurring bactericidal substances have been isolated from polymorphonuclear leukocytes (5, 18, 19), erythrocytes (16), and serum. A substance in human sera active against avirulent strains of staphylococci was neutralized by coagulase (4). As early as 1942, histone and protamine were shown to be bactericidal for a variety of gram-positive and gram-negative bacteria (12). Lysosomal cationic proteins other than lysozyme that are bactericidal for gram-positive as well as gram-negative bacteria have been partially separated (19). These cationic proteins differ in their specific bactericidal spectra. β -Lysin is considered to be a heat-stable constituent of normal serum and can be purified by adsorption to cellulose asbestos filter pads. It is readily separated from antibody and complement during purification and is bactericidal for a variety of gram-positive bacteria (2). It has

been unclear whether the β -lysin activity of serum against gram-positive bacteria is due to a single substance or if multiple β -lysinlike substances exist which possess similar modes of activity. The present report deals with the purification of the β -lysins active against *Bacillus subtilis* and *Staphylococcus aureus* from rabbit serum and provides evidence that two separate systems exist, one for *B. subtilis* and one for *S. aureus*.

MATERIALS AND METHODS

Organisms. Twenty-four strains of coagulase-positive *S. aureus* were used. Those numbered in the seven and eight hundred series were hospital isolates, and those designated with lower numbers were named after their phage types, except strain 1-138 which was an arbitrary designation and was obtained from this department's culture collection.

Assay procedure for staphylocidal β -lysin. The test for bactericidal activity against *S. aureus* was a modification of the test used for measuring bactericidal activity against *B. subtilis* (2), and it consisted of mixing 0.1 ml of diluted (1:4,000) 14-hr nutrient broth culture, containing 20,000 to 80,000 organisms, with 1 ml of twofold dilutions of the test sample. Dilutions were made in phosphate buffer, pH 7.5, ionic strength, 0.15. After a 2-hr incubation period at

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37 C, pour plates were prepared to determine cell viability. A control containing the phosphate buffer was used to determine the number of organisms in the inoculum. A staphylocidal β -lysin unit is defined as the amount of β -lysin that kills 99% of the organisms in the original inoculum.

Purification of the staphylocidal substance. The substance in serum active against staphylococci can be purified by methods similar to those that have been described for β -lysin (2). Normal rabbit serum (120 ml) was allowed to flow slowly through a clarifying cellulose asbestos filter pad (Hercules filter, C-5) at 4 C. The filter pad was washed with 100 ml of 0.15 ionic strength phosphate buffer at pH 7.5 before the β -lysins were eluted with 20 ml of 1.5 M NaCl in buffer. The eluate was dialyzed against 3 liters of the buffer at 4 C. Aseptic techniques were employed throughout the procedure.

Preparation of platelet extract. An amount of 200 ml of citrated rabbit blood (0.38%) was centrifuged twice at $100 \times g$ for 15 min to remove the erythrocytes and leukocytes. The platelets were sedimented at $550 \times g$ for 15 min, washed three times in sterile physiological saline solution (PSS), and resuspended in 30 ml of PSS. This suspension was frozen and thawed five times to disrupt the platelets.

Preparation of plasma serum. Plasma serum was prepared by use of a modified procedure of Hirsh (6). Blood was collected in cold siliconized syringes and tubes and was centrifuged immediately at $550 \times g$ for 15 min at 4 C. After centrifugation, the supernatant fluid was collected and allowed to coagulate at room temperature. The fibrin clot was collapsed by applying pressure with a sterile wooden applicator stick, and the fibrin mass was removed.

Molecular weight estimation. A modification of the method of Whitaker (15) was used to estimate the molecular weights of the β -lysins. Normal rabbit serum was dialyzed against 0.15-M phosphate buffer, pH 7.5, containing 0.6 M NaCl. At lower NaCl concentrations, the β -lysin appears to attach to both Sephadex and other serum proteins. A sample (10 ml) was placed on a 2.2 by 110 cm column of Sephadex G-200, which was previously equilibrated at 4 C with the above high salt buffer. Optical densities at 280 nm were recorded, and the fractions were assayed for lysozyme and β -lysins. To avoid salt inhibition of bactericidal activity, the fractions to be assayed for β -lysin were first diluted 1:4 in sterile distilled water.

RESULTS

Staphylocidal activity of normal rabbit serum, filtered serum, and partially purified β -lysin.

Twenty-four strains of coagulase-positive *S. aureus* were tested for their susceptibility to undiluted rabbit serum, to serum filtered through a Seitz-type filter, and to β -lysin obtained from rabbit serum. In the first bactericidal tests, 5×10^5 to 1.4×10^6 staphylococci were incubated with 1 ml of undiluted sample for 2 hr at 37 C before the percentage of survival was determined and recorded (Table 1). There were considerable

differences in the susceptibility of the various strains of *S. aureus* to pooled rabbit serum. The 2-hr survival in undiluted serum varied from 0.14 to 68%. With all strains but one, filtration removed measurable staphylocidal activity from the serum. Although the majority of strains of *S. aureus* exhibited limited susceptibility to filtered serum, six strains appeared to multiply during the 2-hr incubation period. These differences between the survival of *S. aureus* in serum and in filtered serum were expected, since it had been demonstrated that filtration removed most of the β -lysin against *B. subtilis* (2).

The observation (Table 1) that the purified β -lysin was more effective in killing all strains of *S. aureus* than normal serum was unexpected because the purified β -lysin and normal serum used in these experiments had equivalent bactericidal activities against *B. subtilis*. All strains of the staphylococci tested, including those which

TABLE 1. Bactericidal activity of rabbit serum and β -lysin on coagulase positive *Staphylococcus aureus*

Strain no.	Per cent survival ^a		
	Normal serum	Filtered serum	β -Lysin
808	0.14	10	0.020
52A/79	0.94	17	0.020
816	1.9	17	0.002
3A	2.1	6.9	0.002
794	2.2	24	0.002
77	2.5	>100 ^b	0.018
823	3.0	25	0.002
804	3.3	23	0.003
7	3.6	>100 ^b	0.02
53	3.8	>100 ^b	0.01
80	5.7	20	0.001
54	6.4	>100 ^b	0.013
81	6.5	55	0.0004
789	7.4	24	0.0003
798	7.5	33	0.030
785	10	25	0.006
781	14	19	0.015
819	16	25	0.009
1-138	17	>100 ^b	0.02
801	24	30	0.012
812	29	24	0.027
75	35	>100 ^b	0.003
3C	52	76	0.10
42E	68	90	0.004

^a Percentage of organisms that survived 2 hr of incubation at 37 C. Original inoculum was 5×10^5 to 1.4×10^6 organisms/ml.

^b Since the bacterial count after incubation was greater than the original inoculum, it is assumed that multiplication occurred in these filtered sera.

were quite resistant to normal serum, were highly susceptible to the purified β -lysin. This difference in susceptibility to serum and the purified β -lysin is due to the interference of staphylocidal activity by other serum constituents. The staphylocidal activity of the purified preparations can be depressed by the addition of either filtered rabbit serum or sera from other species such as the guinea pig or human which contain low levels of staphylocidal β -lysin. No such inhibition exists with the purified β -lysin against *B. subtilis*.

Occurrence of serum staphylocidal substance in various mammalian sera. During preliminary experimentation, it was noted that the levels of the bactericidal activities against *B. subtilis* and *S. aureus* in various pools of sera did not correlate. This observation, along with the inhibition difference discussed, indicated that, perhaps, two distinct systems were involved. This led to the question of whether the levels of the bactericidal activities against the two organisms correlated in the sera of various mammalian species. The results (Table 2) indicate that such a correlation does not exist. It was found that the human sera contained less than 1 unit of staphylocidal β -lysin, whereas 4 units of β -lysin against *B. subtilis* were common. Rat serum was extremely active against *B. subtilis* but did not possess measurable activity against strains of *S. aureus* that were susceptible to rabbit serum. Of the five types of sera tested, only horse and rabbit sera had measurable staphylocidal β -lysin using the standard bactericidal assay system in which a β -lysin unit is based on a 99% kill of the standard inoculum. The lack of correlation supports the hypothesis that separate β -lysin systems exist.

Purification of β -lysin. Serum β -lysins against both *Staphylococcus* and *Bacillus* adhered to cellulose asbestos filter pads and were found in a purified form in eluates from these filters. Further purification of the β -lysins was performed with carboxymethyl cellulose (CM-cellulose) chromatography. After elution of the substances from the filter pad, the eluates were dialyzed overnight at 4°C against sterile 0.001 M phosphate buffer, pH 7.0. This dialyzed eluate (15 ml) was placed on a CM-cellulose column measuring 0.9×13 cm with a void volume of 10 ml which had been equilibrated with 0.001 M phosphate buffer at pH 7.0. Samples (5 ml) were collected, and their bactericidal activities were determined, as well as their protein concentrations, by Waddell's procedure (14). Approximately 85% of the total protein was removed from the column in the first 40 ml of 0.1 M phosphate buffer at pH 7.0. These fractions did not contain measurable

TABLE 2. Comparative susceptibility of *Bacillus subtilis* and *Staphylococcus aureus* to mammalian sera

Sample	β -Lysin (units/ml) ^a		
	<i>B. subtilis</i>	<i>S. aureus</i>	
		808	816
Guinea pig serum.....	<1	<1	<1
Human serum.....	4	<1	<1
Horse serum.....	8	1	1
Rabbit serum.....	16-32	2-8	2-4
Rat serum.....	256	<1	<1

^a One unit of β -lysin kills 99% of the bacteria during a 2-hr exposure.

quantities of the β -lysins. The β -lysins were eluted with 40 ml of 1.5 M NaCl in 0.15- μ phosphate buffer, pH 7.0.

Table 3 records the results of a typical purification procedure of the two β -lysins. Normal serum bactericidal activities against both *B. subtilis* and *S. aureus* were almost entirely removed by filtration with little demonstrable removal of total serum protein. The specific activity, defined as β -lysin units per mg of protein, increased approximately 350 times against *B. subtilis* and approximately 5,600 times against *S. aureus* after elution and dialysis. This increase in staphylocidal β -lysin of the dialyzed eluate represents about a fivefold increase in total activity and can be explained by the removal of the inhibitory substances found in normal serum. Consequently, the relative activity of the staphylocidal β -lysin represents removal of inhibitory substances, as well as the degree of purification. This is not the case with the β -lysin against *B. subtilis* which is not inhibited by other serum constituents. The greatest degree of purification of the β -lysin against *B. subtilis* of the CM-cellulose effluent varied in other experiments from 21,000 to 38,000. The highest previously obtained purification using differential ultracentrifugation was approximately 5,000-fold (2). The original serum activity (17%) against *B. subtilis* was recovered from the CM-cellulose column. The highest relative activity of the β -lysin against *S. aureus* of the CM-cellulose fractions varied in other experiments from 27,000 to 47,000. The original serum activity (56%) was recovered in the CM-cellulose fractions. The protein concentration was greater in the fraction containing the staphylocidal β -lysin than in the fraction containing the β -lysin against *B. subtilis*. Although the peaks with the highest specific activities did not coincide, the two β -lysins were never com-

TABLE 3. Purification of the β -lysins active against *Bacillus subtilis* and *Staphylococcus aureus*

Sample	Bactericidal activity for <i>B. subtilis</i>				Bactericidal activity for <i>S. aureus</i>			
	Protein per ml	β -Lysin per ml	Specific activity ^a	Relative activity ^b	Protein per ml	β -Lysin per ml	Specific activity ^a	Relative activity ^b
	mg	units			mg	units		
Rabbit serum.....	73	16	0.22	—	73	2	0.027	—
Filtered serum.....	73	<1	—	—	73	<1	—	—
Dialyzed eluate.....	0.42	32	76.2	346	0.42	64	152	5,630
CM-cellulose effluent....	0.0015	8	5,330	24,200	0.008	8	1,000	37,000

^a Specific activity = units of β -lysin per mg of protein.^b Relative activity = specific activity of sample/specific activity of serum.

pletely separated. Numerous unsuccessful attempts were made to separate the two β -lysins by altering the ionic strengths, the pH, the ion exchange resin, the eluting buffers, and the chromatography procedure.

Comparison of the bactericidal activities of β -lysins and lysozyme in various samples. The probability that two bactericidal substances are involved, one for *B. subtilis* and one for *S. aureus*, is further strengthened by the results recorded in Table 4. These results also eliminate lysozyme as a bactericidal substance found in the purified dialyzed eluate preparations. Rabbit serum, which has significant quantities of β -lysin and lysozyme, was effective against both *B. subtilis* and *S. aureus*. All of the bactericidal activity against *B. subtilis* in rabbit serum and purified rabbit β -lysin was neutralized by guinea pig anti- β -lysin and not by normal guinea pig serum. However, both anti- β -lysin and normal guinea pig serum neutralized the bactericidal activity of these preparations against *S. aureus*. Plasma, which contains the same concentration of lysozyme as serum, was less active than serum against both *S. aureus* and *B. subtilis*. Plasma serum, which was prepared by removing the particulate elements from blood prior to coagulation, was also much less active than serum against both organisms, indicating that both substances are released from the cellular elements of the blood during the clotting process. The results with purified β -lysin from the rabbit and rat were similar to those previously discussed with rabbit and rat serum in that the rat β -lysin, which is extremely active against *B. subtilis*, was not bactericidal for *S. aureus*. Large concentrations of lysozyme, up to 5,000 μ g/ml, were not bactericidal for *S. aureus* but were bactericidal for *B. subtilis*. No β -lysin was present in the egg lysozyme because its bactericidal activity against *B. subtilis* was not altered by anti- β -lysin serum. Since there exist some subtle differences in the

TABLE 4. Comparison of the bactericidal activities of the β -lysins and lysozyme against *Staphylococcus aureus* and *Bacillus subtilis*

Sample	<i>S. aureus</i> (units/ml)	<i>B. subtilis</i> (units/ml)	Lysozyme (μ g/ml ^a)
Rabbit serum.....	2-8	32	2
Rabbit plasma.....	0	1	2
Plasma serum.....	0	4	2
β -Lysin (rabbit).....	32-64	32	0
β -Lysin (rat).....	0	256	0
Lysozyme (egg).....	0	128 ^b	5,000
Lysozyme (rabbit spleen)...	0	2 ^b	136
Platelet extract (rabbit)...	0	32	0
PMN extract (rabbit)....	0	64 ^b	3,000

^a Lysozyme concentrations were determined by the method of Jollès (8).^b Bactericidal activity of lysozyme and of the polymorphonuclear leukocyte extract was not due to β -lysin since anti- β -lysin did not neutralize this activity.

lysozymes from various sources (9), lysozyme from rabbit spleens was isolated and assayed. Both the bactericidal results and anti- β -lysin neutralization results were essentially the same as obtained with egg lysozyme. Aqueous extracts of platelets, which have been demonstrated to be the source of serum β -lysin (3, 7) against *B. subtilis*, were not lethal for *S. aureus*. Aqueous extracts of peritoneal polymorphonuclear leukocytes, which were rich in lysozyme, had no measurable activity against *S. aureus*. The bactericidal activity against *B. subtilis* of the polymorphonuclear leukocyte extracts was attributed mainly to the high lysozyme content, since it was not neutralized by anti- β -lysin serum and it was highly active against *Micrococcus lysodeikticus* cell walls.

Further differentiation of staphylocidal β -lysin from lysozyme is provided in Fig. 1. In this study, viable *S. aureus* strain 808 cells were incubated

FIG. 1. Effect of β -lysin and lysozyme on the viable count and turbidity of *Staphylococcus aureus*.

at 37 C in solutions of lysozyme (1,000 μ g/ml), staphylocidal β -lysin (32 units/ml), and PSS. At various times, the viable count and optical density at 650 nm were determined. Immediately following the combination of the staphylococci and lysozyme, the optical density increased to approximately twice that of a suspension in PSS due to flocculation. The optical density of this mixture diminished rapidly during the first 0.5 hr but did not drop as low as the PSS control at any time. No death was observed during these turbidity changes. The viable counts of the lysozyme-treated staphylococci correlated at all times with those of the PSS control. On the other hand, both the viable count and the optical density of the staphylococci decreased in β -lysin. In unpublished studies on the mechanism of killing of *B. subtilis* by β -lysin, it was demonstrated that β -lysin does not dissolve the cell wall, but following death by β -lysin, enzymes are released that result in autolysis of the cell. This may also be the case with staphylocidal β -lysin, since the decrease in viable count preceded the drop in optical density.

*Other properties distinguishing staphylocidal β -lysin from the β -lysin active against *B. subtilis*.* The activity of β -lysin assayed in the presence of acidic phospholipids, anticoagulants, and proteins is given in Table 5. The β -lysin against *B. subtilis* was inhibited by cephalin, lecithin, and phosphatidylserine, as reported by Joos and Hall (10), but not by heparin, sodium citrate, sodium oxalate, ethylenediaminetetraacetate (EDTA), or solutions of protein. The staphylo-

cidal β -lysin was inhibited by all of these agents. One-half of the purified *S. aureus* β -lysin (16 units/ml) was destroyed after 30 min in 0.1 M acetate or phosphate buffer between pH 4 and 7. The β -lysin against *B. subtilis* was not altered by these conditions. Neither of the β -lysins was destroyed in alkaline solutions up to pH 10. Both β -lysins studied in this communication were inactive in solutions containing 1.5 M NaCl, but they were not simultaneously inactivated as ionic strength was increased. For example, the β -lysin against *S. aureus* was approximately one-half as active at an ionic strength of 0.5 as at 0.15 μ , whereas the β -lysin against *B. subtilis* was only one-eighth as active at 0.5 μ . In addition, the staphylocidal β -lysin maintained one-eighth of its activity at 0.75 μ , whereas the β -lysin against *B. subtilis* had no demonstrable activity at 0.6 μ .

Molecular characteristics of staphylocidal β -lysin. In enzyme inactivation studies, it was observed that 32 units of staphylocidal β -lysin in dialyzed eluate was completely inactivated during 1 hr of incubation with two times crystallized trypsin (0.5 mg/ml) at 37 C, pH 7.5. This inactivation was not due to the previously de-

TABLE 5. Effect of acidic phospholipids, anticoagulants, and proteins on β -lysin activity

Treatment	β -Lysin (units/ml)	
	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>
None.....	16	32
Cephalin (17 mg/ml).....	0	0
Lecithin (50 mg/ml).....	0	0
Phosphatidylserine (50 mg/ml).....	0	0
Heparin (400 units/ml).....	16	0
Sodium citrate (3.8 mg/ml)...	16	0
Sodium oxalate (1.3 mg/ml)...	16	0
EDTA (2 mg/ml).....	16	0
Bovine plasma albumin (50 mg/ml).....	16	0
Peptone (50 mg/ml).....	16	0
Filtered rabbit serum (diluted 1:2).....	16	2 ^a
Guinea pig serum (diluted 1:2).....	16	<2 ^b
Human serum (diluted 1:2)...	16	<2 ^b

^a The activity of the purified staphylocidal β -lysin in the presence of filtered rabbit serum is equivalent to that of normal rabbit serum. All other agents used for treatment were not bactericidal in the absence of β -lysin.

^b Since equal volumes of sera were added to β -lysin the lowest concentration that could be measured was 2 units.

scribed inactivation observed with higher concentrations of proteins because incubation of β -lysin with equal concentrations of bovine serum albumin at these conditions did not inhibit the bactericidal activity. And, unlike the immediate nonspecific protein inactivation, the inactivation by trypsin became more pronounced with time. The concentration of trypsin used had no effect on the viability of the organisms. The staphylocidal β -lysin was not sensitive to 100 μ g of deoxyribonuclease II per ml or to 100 μ g of ribonuclease per ml; it did not absorb significantly at 260 nm as compared to its absorbance at 280 nm, indicating that it is not a nucleic acid. In addition, it failed to yield a positive Molisch test. Purified staphylocidal β -lysin was completely resistant to inactivation at 56 C for 30 min. The staphylocidal β -lysin is probably a cationic molecule because it was inactivated by strongly acidic molecules, and because it attached to CM-cellulose and bentonite, cation exchange resins.

The molecular weights of the staphylocidal β -lysin and the β -lysin active against *B. subtilis* were estimated by use of Whitaker's techniques with Sephadex G-200 (15). In triplicate experiments, the molecular weight of both β -lysins varied from 5,500 to 6,000. A high NaCl concentration (0.6 M) was required to minimize adsorption of these bactericidal agents to the Sephadex and to other serum constituents. Both β -lysins came off in the same effluent volume. Figure 2 shows the results when 10 ml of fresh rabbit serum was placed on the column after dialysis against the high salt buffer. The classical three protein peaks were obtained with a small fourth peak containing both β -lysins. The lysozyme was detected by the reduction of turbidity of a suspension of *M. lysodeikticus* over a 1-hr period at 37 C. The β -lysins were detected by the standard assay against *B. subtilis* and *S. aureus* strain 808 and are plotted as the percentage of mortality

in the β -lysins diluted 1:4 with distilled water. The molecular characteristics of the β -lysins against staphylococci and bacilli are similar in that both are heat-stable cationic proteins with similar net charges and molecular weights.

DISCUSSION

The staphylocidal substance found in rabbit serum is one of a group of small basic proteins which possess bactericidal activity. This group of proteins includes the histones (1, 12), the lysosomal cationic proteins, and the serum β -lysins (10). Although these cationic proteins have similar molecular characteristics, they appear to differ significantly in their bactericidal spectra, as demonstrated by Zeya and Spitznagel in the case of lysosomal cationic proteins (20) and in this study of the serum β -lysins. Although the mechanism of bactericidal activity of this group of basic proteins is unknown, their individual site of action appears to be specific and varies for different basic proteins. This is demonstrated by the different antibacterial spectra of these agents. This study has been limited to the bactericidal activity of β -lysin against *S. aureus* and *B. subtilis*. It has not been excluded that these β -lysins may be active against other species of bacteria and the possibility exists that other β -lysins may be present in the purified preparations. Study on the antimicrobial spectrum of each rabbit β -lysin has been hindered by the fact that the purified preparations contained both types of β -lysin. In contrast to the prior belief that one β -lysin was involved for serum bactericidal activity, this study indicates that resolution of the β -lysins in the purified preparations will have to precede further elucidation of the bactericidal spectrum of each β -lysin.

Staphylocidal β -lysin has been demonstrated to vary in the five different mammalian species examined. Only horse and rabbit sera had sufficient quantities of β -lysin to kill 99% of a standard bacterial inoculum. Mackie and Finkelstein (11) and Myrvik (13) found bactericidal activity against staphylococci in various species, including high levels in the rat. Mackie and Finkelstein used assay systems that measured a difference in percentage of kill rather than β -lysin units that are dependent upon a 99% kill. Myrvik used a strain of *S. albus* for his test organism. The possibility exists that the substance he was measuring in rat serum was something other than the β -lysin active against *S. aureus*. Mackie and Finkelstein stated that two strains of *S. aureus* and one strain of *S. albus* were killed by rabbit and rat sera; one strain of *S. aureus* was non-susceptible to rat serum. This difference may be

FIG. 2. Separation of bactericidal substances of serum by Sephadex G-200 gel filtration.

due to coagulase production by the strains involved, as suggested by Yotis and Ekstedt (17), who demonstrated that coagulase-positive strains were much less susceptible to the lethal effects of serum. All strains used in the present study were coagulase-positive.

Plasma serum contained much less staphylocidal β -lysin than the serum collected following coagulation in the presence of the particulate elements of blood; this phenomenon indicated that staphylocidal β -lysin might be released from cellular elements during coagulation of whole blood. The observation that no staphylocidal activity was found in aqueous extracts of platelets or polymorphonuclear leukocytes was unexpected. Platelets are rich in β -lysin against *B. subtilis*, and the leukocytes are rich in bactericidal lysozomal cationic proteins. These negative results may be due to the absence of staphylocidal β -lysin or a consequence of the described protein inhibition of this β -lysin. Consequently, the origin of serum staphylocidal β -lysin is still open to speculation.

Due to the separation of β -lysin against *S. aureus* from lysozyme and because of the characteristics it possesses that differ from those of the substance active against *B. subtilis*, it is felt that it is a distinct bactericidal agent which was not previously purified nor were its properties defined to the extent provided by this study.

As a host defensive substance acting *in vivo*, the staphylocidal β -lysin may play a relatively minor role because of its inhibition by proteins, its low concentration in plasma, and its low concentration in the sera of most species. It is possible that staphylocidal β -lysin will be shown to be of importance in conferring a significant degree of natural immunity on those hosts possessing this compound. It may be of importance in helping to overcome localized staphylococcal infections and may possibly be demonstrated to be highly active alone or in conjunction with other substances at *in vivo* sites of infection. It is conceivable that this system may be used to elucidate the mechanisms by which extracellular and intracellular cationic proteins and polypeptides inhibit or kill bacteria.

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