BIOLOGICAL ACTIVITY OF PURIFIED STAPHYLOCOCCAL ALPHA TOXIN

by

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ABSTRACT

Staphylococcal alpha toxin was purified using a stepwise procedure. First, three strains of Staphylococcus aureus were screened for alpha toxin production and the best one was selected. The second step was the selection of a growth medium and conditions of incubation that enhanced the production of alpha toxin by the selected strain. The third step was chemical separation of alpha toxin from medium components and other bacterial components. Finally, we obtained an electrophoretically purified protein that was tested for hemolytic, dermonecrotic and lethal activities as posited by Burnet's Unitarian Hypothesis. The final electrophoretically purified fraction possessed hemolytic and dermonecrotic activities but lacked lethal activity. Although the lack of a demonstrable lethal activity would appear to contradict the Unitarian Hypothesis, it is possible that either insufficient amounts of material were used to demonstrate lethality, or that alterations to alpha toxin occurred by proteases, denaturation, or the removal of necessary trace compounds thus contributing to the lack of lethal activity.

CHAPTER 1

INTRODUCTION

Klebs (in 1) first suggested that disease could be produced by diffusible substances from bacterial cultures called sepsins. His idea was supported by de Christmas who demonstrated that staphylococcus culture filtrates produce inflammatory reactions in animals. Brieger and Fraenkel showed that alcohol precipitates of such culture filtrates caused inflammation and necrotizing action on the skin at the injection site, as well as lethality in rabbits and guinea pigs. Later, Van de Velde identified a leucocidin and Kraus and Clairmont demonstrated a hemolysin in diffusible substances from staphylococci.

Interest in staphylococcal lysins waned until 1928 when, in Bundaberg, Australia, twelve of twenty-one children died within forty-eight hours after immunization against diphtheria. The Royal Commission investigated and concluded that death resulted from an overwhelming toxemia caused by staphylococci contaminating the toxin-antitoxin preparation.

After Bundaberg, most work was concentrated on the classification of staphylococcal toxins by lytic action according to the species from which erythrocytes were collected; with the lysin active against rabbit and sheep erythrocytes being designated as alpha toxin (2).

Burnet developed the Unitarian Hypothesis, which stated that the main activities of alpha toxin (lytic, dermonecrotic, lethal) were

all manifestations of a single toxic protein. By 1940, most evidence favored the Unitarian Hypothesis. For example, the three biological activities paralleled one another in culture filtrates, their chemical properties appeared similar and they could be neutralized in parallel by anti alpha toxin (3).

Bonventre (4) defined a toxin as a high molecular weight protein of microbial origin which is antigenic and causes disruption of normal physiological processes in sensitive animals. Jeljaszewicz (5) characterized alpha toxin as a protein uniting hemolytic, lethal and dermonecrotic properties and exhibiting a wide variety of other biological activities.

Alpha toxin is formed during the early logarithmic growth phase of Staphylococcus aureus and production proceeds at a constant rate until the stationary growth phase is reached (3). It has been shown to be a protein of 21,000 to 44,000 daltons, depending upon the purification procedure used (6-9). Amino acid analyses made by several groups have shown that alpha toxin contains no unusual amino acids and is lacking in cysteine, cystine and tryptophan (6, 7). Coulter (7) found histidine and arginine as the n-terminae and suggested two polypeptide chains with no disulfide bridges. Although other investigators have found different n-terminae, this may be due to the cleavage of alpha toxin by proteases produced by Staphylococcus aureus present in crude preparations.

Purified preparations of alpha toxin have been shown to contain a biologically active material with a sedimentation coefficient of

2.8S-3.1S and a biologically inactive material with a sedimentation coefficient of 12S-16S by ultracentrifugation (6, 10, 11). The inactive 12S component of alpha toxin is thought to be an aggregate of 3S segments, since 12S components can be completely reactivated by 8 M urea to yield biologically active 3S alpha toxin. The 12S components are partially reactivated by heat (100 C) and show lines of partial identity with 3S components on Ouchlerlony gel diffusion plates (3, 11). These findings can be a partial explanation of what Arrhenius observed in 1907, now termed the "Arrhenius Effect," in which crude toxin, heated to 60 C, yielded inactive precipitate which could be partially reactivated by heating to 100 C. Arbuthnott, Freer and Bernheimer (11) showed that the insoluble precipate formed at 60 C is reactivated by 8 M urea with retention of all initial biological activities. Manohar, Kumar and Lindorfer (12) showed that electrophoretically pure alpha toxin, heated to 60 C, lost all biological activities and the activities could not be restored by either 8 M urea or heat. They concluded that reactivation of alpha toxin was due to a substance distinct from alpha toxin. When the substance was absent from alpha toxin no protection would be conferred, alpha toxin would be destroyed by heat and could not be reactivated. So, heat reactivation may not be a function of alpha toxin but may be a function of a substance removed by the purification procedure. Additionally, the Arrhenius effect may be due to the presence of this structure, and it may even act as the seed crystal for the formation of molecular

aggregates. Some materials have been shown to polymerize 3S alpha toxin to the 12S form (13).

Therefore, alpha toxin apparently exists in at least three states: 3S biologically active toxin of 21,000-44,000 daltons; 12S biologically inactive toxin of 240,000-330,000 daltons; and biologically inactive toxin of varying degrees of molecular aggregation. In fact, Bernheimer (14) has shown that as much as 30 percent of pure 3S alpha toxin may be in the polymerized 12S form.

Isoelectric point determinations have been made by numerous investigators (8, 15, 16), with Wadstrom (17) observing that alpha toxin could be separated into at least four components having different isoelectric points and Arbuthnott et al. (13) noting six components: alpha A - 8.55 $\pm$ 0.12; alpha B - 9.15 $\pm$ 0.07; alpha C - 7.36 $\pm$ 0.03; alpha D - 6.28 $\pm$ 0.11; and two minor components with points of 4.32 $\pm$ 0.25 and 10.01 $\pm$ 0.05. Alpha A and B have been shown to undergo reversible interconversion and alpha A apparently comprises at least 80-90 percent of all hemolytic activity (15-17). Wadstrom (17) suggested that multiple forms of alpha toxin may differ only in conformation. He took individually focused peaks, refocused the peaks, and found the same multiple forms as in his primary isolation. According to Arbuthnott et al. (11) the multiple forms could also be accounted for by simple molecular aggregation, the formation of ligands or, most importantly, due to the presence of trace level impurities. Substances have been shown to be co-isolated with alpha toxin that may influence its biochemical reactivity and physical conformation (13, 18).

Dalen (19) analyzed heat precipitated culture filtrates at different pH values and observed differences in protein patterns on gel electrophoresis, referring to those proteins as pH dependent co-precipitates, because several of the proteins did not precipitate when heated in the absence of alpha toxin.

With the development of more sophisticated equipment and techniques, highly purified preparations of alpha toxin have been obtained. These preparations seem to possess all the biological activities attributed to crude preparations (6), although trace level impurities may be present in the preparations making verification of the Unitarian Hypothesis difficult.

Genetic verification of the Unitarian Hypothesis has not been clear either. Non-hemolytic mutants were isolated and tested for dermonecrotic and lethal activities as well as the presence of material with immunological cross reactivity with alpha toxin (20, 21). The results suggest that a single mutational event can alter hemolytic, dermonecrotic and lethal activities, whereas mutants still produce an immunologically cross reacting material. One mutant deficient in hemolysin but retaining dermonecrotoxin and lethal toxin was isolated, suggesting that the mutant organism produced a protein altered only in primary structure affecting the specificity of a single site on the molecule. The investigators postulated that two genetic loci are involved in alpha toxin production and divided the mutants into two groups. Group one mutants possess a structural gene mutation resulting in a lack of hemolytic activity only, whereas group two mutants have no

biological activity. Although the above results seem to be supportive of the Unitarian Hypothesis, only mutants without hemolytic activity were selected so that mutants possessing hemolytic activity but deficient in dermonecrotic or lethal characteristics would not be detected.

Alpha toxin exerts its hemolytic activity by a two step process in which it binds to erthrocyte membranes (22) and then potassium ions are released, followed by rapid hemoglobin leakage through the damaged membrane.

The lethal effect of alpha toxin results from central nervous system involvement as alpha toxin has been found in appreciable amounts in the brain shortly after intravenous injection. In addition to its occurrence in the brain, the presence of the toxin causes severe disturbances in the thalamic reticular system and in the disappearance of brain bioelectric activity (23, 24). Lethality in mice, according to LD_{50} determinations, is approximately 0.70 µg/mouse.

Dermonecrotic properties of alpha toxin probably result from either prolonged vasospasm (25, 26) or by a combination of increased vascular permeability, degranulation of platelets and rupture of lysosomes.

In order to test the Unitarian Hypothesis, we propose to obtain a pure preparation of alpha toxin by chemically separating it from the myriad of extracellular metabolites produced by Staphylococcus aureus during culture.

By selecting a medium containing the necessary components for enhanced toxin production and only small dialyzable molecules, a high

yield of relatively clean toxin can be produced which can be more easily separated from other extracellular products. Then, by using the relatively rapid hemolytic assay, one can screen for the presence of alpha toxin.

After separation at the isoelectric point, determination of purity and molecular weight will be made by SDS-polyacrylamide gel electrophoresis.

Finally, when purity is verified, the biological properties attributed to alpha toxin in the Unitarian Hypothesis will be tested using the hemolytic assay, intraperitoneal injections for lethality, and intradermal injections for dermonecrosis.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains

Staphylococcus aureus, Wood 46, SSCC, and Wood S.U.I. strains were obtained from the stock culture collection of the Department of Microbiology and Medical Technology, The University of Arizona.

Culture Media

Tryptic soy broth (Difco), Casamino acids (19), Coulter medium (7), and Coulter dialysate were used in this study.

Coulter dialysate was prepared in the following manner: 30 g proteose peptone, 10 g yeast extract and 5 ml sodium lactate were made up to one liter with distilled water and dialyzed against one liter of distilled water at 4 C overnight. The dialysate was used for the cultivation of bacteria.

Cultural Conditions

Starter bacterial cultures were grown in test tubes containing Brain-Heart Infusion Broth for 24 hours at 37 C with lyophilized inocula. These cultures were used as inoculum for one liter flasks containing 250 ml of culture media. The culture was incubated for 18 hours at 37 C with shaking at 60 RPM.

Eighteen hour bacterial cultures were centrifuged at 12,000 x g at 4 C for 20 min. and supernatant fluid filtered through a 0.22 μ Gelman filter to obtain culture filtrate.

Hemolytic Assay

The culture filtrate and all other alpha toxin preparations were assayed in the following manner. A serial two-fold dilution scheme using a 0.5 ml sample and 0.5 ml NaCl (0.154 M) was made, to which 0.5 ml of a 2 percent washed rabbit erythrocyte suspension was added. The tubes were incubated in a 37 C water bath for 30 minutes. The titer (one hemolytic unit) of the toxin was read visually as the reciprocal of the highest dilution of toxin showing complete hemolysis of erythrocytes.

Rabbit Erythrocytes

Erythrocytes were collected from the same rabbit each time by bleeding from the ear artery with the addition of heparin (10U/ml). The cells were washed twice in saline (.154 M) and made to a 2 percent (V/V) solution in saline.

Heat Precipitated Fraction

The culture filtrate was adjusted to pH 5.0 with 1.0 M HCl and heated to 60 C for five minutes in a water bath (19).

Concentration Procedure

The heat precipitated fraction was centrifuged at 20,000 x g for 20 minutes at 4 C and the pellet material resuspended in borate

buffer (0.03 M, pH 8.6). This material was washed two times in the same buffer and concentrated to 1/10 the volume using an Amicon Stirred Cell with a PM 30 filter.

Protein Determination

Protein determinations were made using Eagle's Modification of the Lowry Method (27) using crystalline bovine serum albumin (Sigma) as the standard and read at 750 nm on a Beckman Acta III Spectrophotometer.

Preparative Electrophoresis

Two mg of the washed, concentrated, heat precipitated protein were dissolved in borate buffer (0.03 M, pH 8.6) containing 8 M urea (Mallinckrodt) and applied to each sample chamber (7.5 cm x 0.5 cm) of disc gels. The gels were made with 7.5 percent acrylamide, borate buffer (0.15 M, pH 8.9) and polymerized with TEMED (N,N,N^1N^1-tetramethyl-ethylene-deamine) and ammonium persulfate (Canalco). The reservoirs contained borate buffer (0.15 M, pH 8.6) to create a conduction gap between the sample chamber and the reservoir and gel. Electrophoresis was made at a constant voltage of 25 V for two hours (19).

Preparation of Material for Assay

After preparative electrophoresis, the precipitate remaining in the sample chamber was removed by aspiration. The first three mm of the gel were sliced from the slab and placed in a tube containing borate buffer (0.03 M, pH 8.3) and 8 M urea to elute any toxin

penetrating the gel. The aspirated precipitate was combined with the eluted sample and dialyzed against NaCl (0.154 M, pH 7.2) (200 times the volume) for 24 hours at 4 C.

Analytical Electrophoresis

Polyacrylamide gel electrophoresis was done on the electrophoretically purified fraction of alpha toxin using a Canalco Disc Electrophoresis Apparatus and a Buchler Power Supply.

Acrylamide gels containing 10 percent acrylamide (Bio-Rad) were made with Swank and Munkres buffer (0.1 M Tris-phosphate, 0.1 percent SDS, pH 6.8) containing 3 M urea and polymerized with 0.075 percent TEMED and 0.07 percent ammonium persulfate in 7.5 x 0.5 cm tubes.

Swank and Munkres buffer was also used for the reservoir solutions while the sample buffer (0.02 M Tris-phosphate, 2 percent SDS, 20 percent sucrose) contained 8 M urea and 0.02 percent bromphenol blue as a tracking dye.

The anode was placed at the bottom and current was applied at 4 ma/gel.

The following protein standards were used for molecular weight determinations: (Sigma) human albumin, 67,000 daltons; ovalbumin, 46,000 daltons; horse myoglobin, 17,500 daltons; and ribonuclease A, 13,500 daltons. Ten micrograms of each standard and twenty micrograms of sample were used in a total of 25 µl and were treated in sample buffer plus 8 M urea for ten minutes at 60 C prior to application to sample chamber.

The gels were stained with Comassie Blue R-250 (Bio-Rad) for six hours at room temperature and destained with a solution containing 10 percent acetic acid and 10 percent isopropanol overnight at room temperature.

Animal Studies

Dermonecrotic Activity

Crude filtered, heat precipitated and dialyzed, electrophoretically purified alpha toxin were injected intradermally in 0.1 ml volumes in multiple sites into two New Zealand White rabbits per group. Skin reactions were measured at 72 hours.

Lethal Activity

Crude filtered and heat precipitated alpha toxin were screened for lethality by injecting undilute samples intraperitoneally into three Swiss Webster mice per group and observed at 24 hours for lethality. Electrophoretically purified alpha toxin was diluted twofold in saline and five dilutions of alpha toxin were injected intraperitoneally in 0.5 ml volumes into five Swiss Webster mice per group and observed at 24 hours for lethality. A flow diagram of the purification steps in shown in Fig. 1.

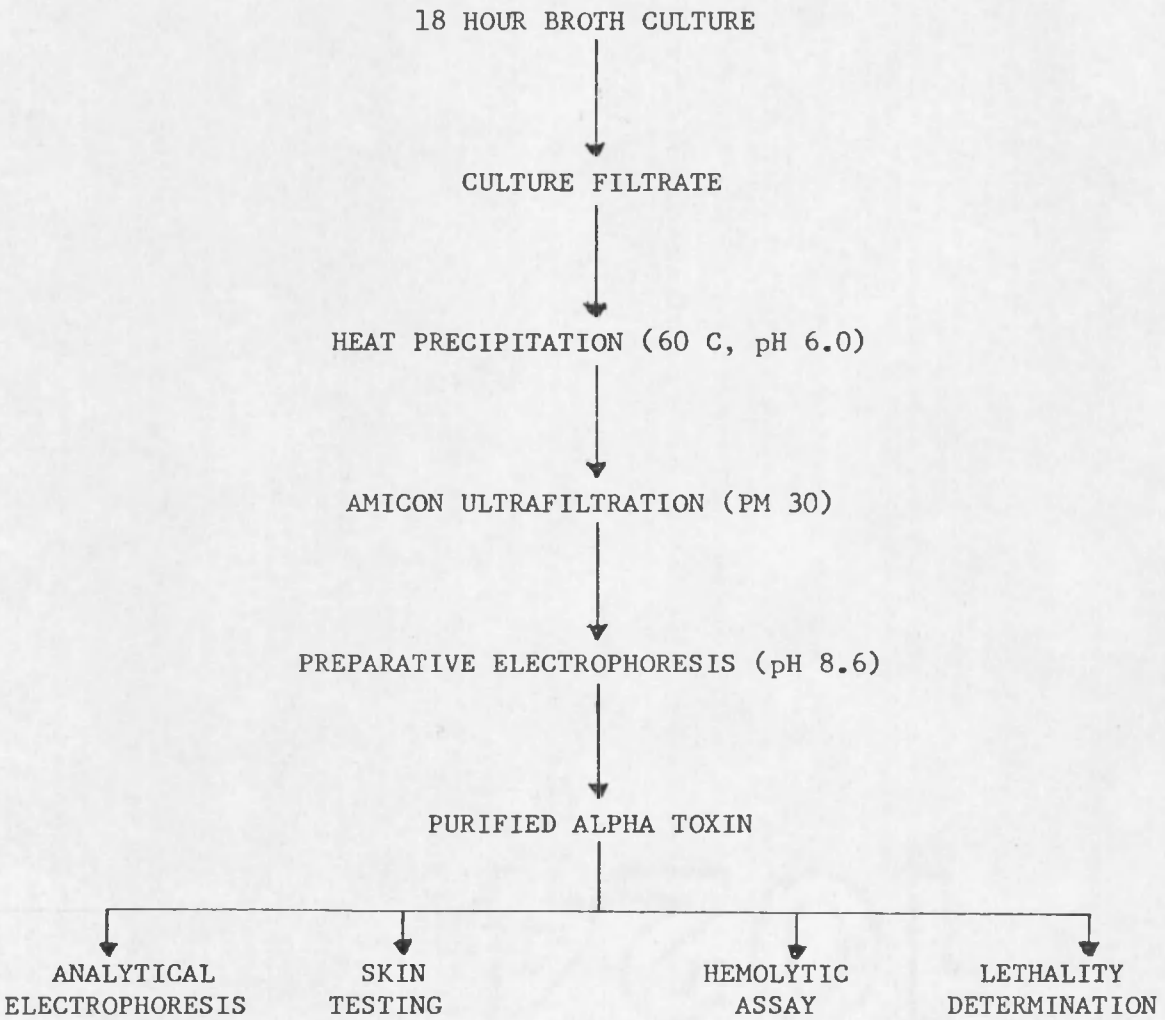

Fig. 1. Flow Diagram of Purification and Test Procedure for Staphylococcal Alpha Toxin

CHAPTER 3

RESULTS

Strain Selection

Wood 46, Wood S.U.I., and SSCC strains of Staphylococcus aureus were screened for alpha toxin production. Strains were grown in Tryptic soy broth (Difco) for 18 hours at 37 C and filtrate was assayed using the hemolytic assay. Table 1 shows that Wood 46 was found to produce the highest yield of alpha toxin.

Selection of Cultural Conditions

Wood 46 was grown in Coulter medium (7), Coulter medium dialysate, and Casamino acids in air and CO_2 to determine which medium and gaseous condition resulted in the highest yield of alpha toxin. Table 2 indicates that Coulter medium and Coulter dialysate were equally good in promoting alpha toxin production. Coulter dialysate was selected for use in the remainder of these studies because it contains only dialyzable components that can be readily removed from larger bacterial metabolites.

Purification of Toxin

As noted in Table 3, purification of alpha toxin increased specific activity (HU/ml protein) from 37.05 in the crude filtrate fraction to 109.4 in the heat precipitated fraction and to 556.5 in the electrophoretically purified fraction of alpha toxin.

Table 1. Alpha Toxin Production by Three Strains of Staphylococcus aureus

Strain	Hemolytic Units (HU)/ml[a]
Wood 46	16
Wood S.U.I.	4
SSCC	0

[a]The reciprocal of the highest dilution giving complete hemolysis of a 2 percent solution of rabbit erythrocytes.

Table 2. Staphylococcal Alpha Toxin Production under Different Conditions of Incubation and in Different Media[a]

Cultural Conditions	HU/ml
Air	
Coulter Medium	256
Coulter Dialysate	256
Casamino Acids	16
CO_2	
Coulter Medium	128
Coulter Dialysate	128
Casamino Acids	4

[a]Cultural conditions used to determine maximum amount of alpha toxin produced by Wood 46 after 18 hours at 37 C with shaking at 60 RPM.

Table 3. Hemolytic Activity of Various Staphylococcal Alpha Toxin Fractions

Fraction	Hemolytic Units[a]	Protein[b]	Specific Activity[c]
Crude Filtrate	256	6.91	37.05
Crude Filtrate (5 x concentrate)	512	14.26	35.91
Heat Precipitate (10 x concentration)	256	2.34	109.4
Electrophoretic Fraction	128	0.23	556.5

[a]Expressed as the reciprocal of the highest dilution giving complete hemolysis of a 2 percent solution of rabbit erythrocytes.

[b]Expressed as mg/ml.

[c]Expressed as hemolytic units per mg protein.

Any attempt at concentrating alpha toxin caused a loss of yield, probably as a result of molecular aggregation or destruction by proteolytic enzymes.

When the electrophoretically purified fraction was subjected to analytical electrophoresis, one band corresponding to 35,000 daltons was present.

Biological Activities

Crude filtered, heat precipitated and electrophoretically purified fractions of alpha toxin were evaluated for biological activities, as shown in Table 4. Undilute crude filtrates and heat precipitated fractions possess the biological characteristics attributed to alpha toxin in the Unitarian Hypothesis, although the more highly purified electrophoretic fraction lacks the lethal activity.

Table 4. Biological Activity of Staphylococcal Alpha Toxin Fractions

Fraction	Specific Activity[a]	Dermonecrosis[b]	Lethality[c]
Crude Filtrate (CF)	37.05	25 mm ± 2	Lethal
Heat Precipitate (HP	109.4	32 mm ± 2	Lethal
Electrophoretic (EP)	556.5	32 mm ± 2	Not Lethal

[a]Expressed as hemolytic units per mg protein.

[b]Mean of skin reactions measured 72 hours after injection of 0.1 ml of fraction intradermally.

[c]Determined 24 hours after injection of 0.5 ml of fraction intraperitoneally. CF and HP fractions were lethal for all animals per group. EP fraction was not lethal at any dilution for all animals per group.

CHAPTER 4

DISCUSSION

Although an electrophoretically purified protein possessing hemolytic and dermonecrotic properties but lacking lethal activity would appear to disagree with the Unitarian Hypothesis, the unusual behavior of alpha toxin in pure and impure forms must be considered. Its interaction with co-precipitates or trace level impurities, reactivity with proteases and alterations of molecular structure may be important.

The three primary biological activities of alpha toxin were maintained during the purification process until preparative electrophoresis was done, at which point lethal activity could no longer be demonstrated.

The loss of lethal activity in the electrophoretically purified fraction could be due to a number of possibilities, the first being the injection of alpha toxin in amounts insufficient to provoke a lethal response. This possibility would appear to be unlikely primarily because the specific activity of electrophoretically purified alpha toxin as measured by the hemolytic assay is at least five to fifteen-fold higher than less active fractions that did result in a lethal response. Additionally, electrophoretically purified alpha toxin was injected in protein quantities 100-fold higher than should have been necessary to demonstrate lethality.

Second, a possible explanation of the absence of lethal activity might be that co-precipitating compounds (19) or trace level impurities (12, 13, 18) which have been shown to influence its biochemical reactivity and physical conformation were removed during purification and resulted in the loss of lethality only by an as yet unidentified mechanism. It has been reported (12) that electrophoretically purified alpha toxin could irreversibly lose biological activity when an essential but unidentified factor was removed.

A third possible explanation of the removal of lethality during purification might be that proteases produced by Staphylococcus aureus and present in the heat precipitated fraction could have selectively cleaved alpha toxin during preparative electrophoresis with cleavage affecting predominantly the lethal moeity of alpha toxin. If cleavage did occur, the cleaved portion would have been relatively small as purified alpha toxin was determined in this study to be approximately 35,000 daltons, consistent with other reports (6-9).

More simply, a fourth possibility which could explain the loss of lethal activity during purification of alpha toxin is the possibility that during the electrophoretic process molecular alteration or partial denaturation occurred to alpha toxin, affecting the lethal site of the molecule. With the occurrence of denaturation, the overall size of alpha toxin would not change, but its biological reactivity might. Alterations of alpha toxin structure have been shown to affect selectively one or more of its biological activities (20, 21).

Finally, it is possible that the pure preparation of alpha toxin obtained in this study possessing only hemolytic and dermonecrotic properties was an actual separation of biological activities, indicating that more than one molecular species may be responsible for the characteristics attributed to alpha toxin. However, in order to determine whether or not the Unitarian Hypothesis is correct or incorrect, more studies would need to be undertaken to elucidate the nature of interaction of alpha toxin with trace impurities as well as interaction with proteolytic enzymes produced by Staphylococcus aureus.

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