JOURNAL OF BACTERIOLOGY, Dec., 1966
Vol. 92, No. 6
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Production, Purification, and Composition of Staphylococcal α Toxin

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Received for publication 19 August 1966

ABSTRACT

COULTER, JOHN R. (Institute of Medical and Veterinary Science, Adelaide, Australia). Production, purification, and composition of staphyloccocal α toxin. J. Bacteriol. **92**:1655–1662. 1966—Pure staphylococcal α toxin has been prepared in quantities suitable for chemical, biological, and clinical characterization. Purification was achieved by acid-methanol precipitation, chromatography on G100 Sephadex, and electrophoresis in G100 Sephadex. We recovered 25% of the crude toxin in pure form, a yield of 12 mg/liter of crude culture supernatant fluid. The pure material gave a single line on gel diffusion and on immunoelectrophoresis and gave a single symmetrical peak in the ultracentrifuge. The α toxin was highly unstable, with a half-life of 3 days at 0 C (*p*H 7.8); solutions of it could not be frozen, and we found no method to stabilize it. On standing, a thready precipitate appeared; it was inactive against rabbit red cells, was not lethal to rabbits, but was able to elicit specific anti-α antibody production in the rabbit. There is evidence that α toxin is an associating molecule, with a mean sedimentation coefficient of approximately 3.0 and a molecular weight of approximately 30,000. The lowest molecular weight, found by equilibrium ultracentrifugation, was 21,200 ± 400. The amino acid composition was determined, and the high positive charge was explained by the presence of lysine, arginine, and histidine, and by amination of the aspartic and glutamic acid residues. Histidine and arginine were shown to be N-terminal amino acids, a fact which suggests the presence of two polypeptide chains. No carbohydrate was present. The ultraviolet absorption spectrum showed a maximum at 274.5 mμ, a minimum at 251.5 mμ, and a shoulder at 292 mμ. The toxin was without proteolytic or phospholipase activity, and its highly specific action on cell membranes still remains unexplained.

From the time Ogston (23), speaking of staphylococcal infection, remarked that "general disturbances are merely due to the fluids or ptomaines that the blood carries away with it as the result of its passage through the diseased foci," interest has been shown in the exotoxins of *Staphylococcus pyogenes* and their relationship to virulence. Recently, as more gentle and effective methods of protein separation have appeared, attempts at α-toxin purification and characterization have been variously successful, but without resolution of many fundamental questions. Six groups of workers claim to have produced a substance having α-lysin activity which gave a single precipitin line on gel diffusion (7, 11, 14, 18, 19, 21). Of these, four found a single line on immunoelectrophoresis (11, 14, 18, 19), and four produced enough material to conduct ultracentrifuge studies (7, 14, 18, 19). One group (18), with rather dilute material, derived an *S* value of 1.4 giving a molecular weight of 10,000 to 15,000; a second group of these four (7) obtained an *S* value of 3.0 and, by Archibald's pseudoequilibrium method, obtained a molecular weight of 44,000, whereas the third group (19) reported an *S* value of 3.1 but no molecular weight estimation. The fourth (14) achieved a concentration of only 0.078% and was unable to derive a value for either *S* or molecular weight. Hallander (12), from gel filtration studies, postulated a molecular weight between 8,000 to 10,000 and 40,000 to 50,000. There have been several attempts to measure the isoelectric point of α toxin, notably by Butler (9) and Kitamura et al. (14), who found *p*H values of 6.4 and 6.8, respectively. However, many other workers (4, 6, 7, 18, 21) have found α toxin to be positively charged in a variety of support media at *p*H values up to 9.0; it seems unlikely

that Butler and Kitamura were in fact dealing with the same substance.

The toxin preparation of Goshi, Cluff, and Norman (11) was shown to contain 6 to 9% carbohydrate. Kumar et al. (18), from preliminary studies, assumed the presence of carbohydrate in their purified material; Kitamura and Shelton (14) showed carbohydrate but made no estimate of its concentration. Furthermore, Goshi and his colleagues showed loss of activity on treatment with α or β amylase. Bernheimer and Schwartz (7), on the other hand, showed that their 85% purified toxin gave two peaks in the ultracentrifuge, and, by density gradient ultracentrifugation, they were able to separate a material of undiminished activity with less than 1% carbohydrate. The material in the other fraction was carbohydrate. The amino acid composition of the α-toxin was determined.

With these preparations and with many others of known impurity, a variety of tests have been conducted. There seems to be general agreement that the lethal and dermonecrotoxic effect and the rabbit red cell lytic effect are due to one and the same substance, and that this substance is also lytic for sheep red cells (7, 11, 14, 18, 21, 24). One group of these workers claims (24) to have separated another fraction which is dermonecrotoxic but not lytic for rabbit red cells. Their α-toxin preparation was proteolytic for casein, whereas that of another group (7) was not. Other effects claimed are: a lytic effect on rabbit and human leukocytes (8, 11, 18); dermonecrosis of human skin (11); and, lastly, damage to human platelets (26), to rabbit kidney cells, Ehrlich ascites cells, KB cells, and monkey kidney cells in tissue culture (1, 2, 16), and to pleuropneumonia-like organisms and rabbit liver lysosomes (5, 8).

Although there is general agreement that α toxin is unstable regardless of how it is purified, the degree to which it is unstable seems to vary, and not entirely with the degree of purity. Madoff and Weinstein (21) and Kitamura et al. (14) found that their material could not be frozen, and therefore could not be lyophilized. The latter group's material lost half its activity at 4 C in 24 hr, whereas Lominski and Arbuthnott (19) found that their toxin could be stored at −20 C and Bernheimer and Schwartz's (7) preparation was fairly stable at 4 C when stored under ammonium sulfate or bovine serum albumin.

It appears that there is still doubt about the composition and properties of α toxin, and that no pure toxin has been produced suitable for definitive chemical, biological, and clinical investigations. [Bernheimer used his 85% pure material in his subsequent studies (5, 8).] This paper describes a suitable method for producing pure α toxin and describes those properties that have been revealed until now in this laboratory.

MATERIALS AND METHODS

Culture medium. The medium consisted of 1.5% Proteose Peptone (Difco B120 or Oxoid), 0.5% yeast extract (Difco B127), and 0.25% sodium lactate, and was made up to 100% with tap water; the *p*H was adjusted to 7.6. Just before use, a small amount of silicone antifoam (Imperial Chemical Industries) was added to each culture flask.

Measurement of hemolytic activity. Red cells for assay were always obtained from the same rabbit. The cells were washed three times and suspended in buffered saline so that, when diluted in the same volume of 0.5% saponin, the resulting hemoglobin solution had an absorbance of 0.8 at 545 mμ.

Doubling dilutions of toxin were made in isotonic phosphate-buffered saline (*p*H 7.4) containing 1:20,000 Merthiolate and 0.1% bovine serum albumin. An equal volume of standard red-cell suspension was added to each tube; the tubes were incubated at 37 C for 30 min and were read immediately. The first tube showing some residual cells was taken as the end point. The reciprocal of the dilution was expressed as the number of units per milliliter.

Protein estimation. Protein in column eluates and in solutions for ultracentrifugal analysis was estimated from the formula (for 1-cm cuvettes): (1.45 × absorbancy at 280 mμ) − (0.74 × absorbancy at 260 mμ) = protein concentration in milligrams per milliliter.

Antiserum. Commercial antistaphylococcal horse antiserum was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. As many as 14 precipitin lines on agar-gel diffusion against crude culture supernatant liquid were seen.

Culture. The Wood 46 strain was plated monthly onto rabbit blood-agar, and colonies showing wide zones of hemolysis were used to inoculate slopes. From these slopes, 7-ml broth subcultures were made and incubated overnight; the whole 7 ml was used to inoculate 1 liter of medium in a 5-liter Erlenmeyer flask. Two cultures were made simultaneously and incubated for 24 hr in a stream of 70% oxygen and 30% carbon dioxide. During incubation, the cultures were stirred vigorously but without froth being produced.

Purification. After being centrifuged and then cooled to 0 C, the clear supernatant fluid (usually in the amount of 1,800 ml) was treated in the following manner. The *p*H was adjusted to 4.0 with glacial acetic acid (about 30 ml), and methanol, previously chilled to −30 C, was added to a final concentration of 25% (about 600 ml). The precipitate which formed was allowed to settle for several hours, and was then centrifuged; the supernatant fluid was discarded. The precipitate was extracted four times with 10-ml changes of 0.15 M sodium acetate and then was discarded. The 40-ml extract was fractionated on a 5.5 × 64 cm column of Sephadex G100. The column was jacketed, and both the column and the fractions (fraction volume = 13 ml) were kept at 4 C. The fractions containing α toxin were combined.

The combined fractions totaling about 160 ml

were concentrated to 12 ml in an L.K.B. ultrafiltration apparatus (L.K.B.-Produkter AB, Stockholm, Sweden). This solution was then fractionated by column electrophoresis with Sephadex G100 as an inert support. The column (Fig. 1) measured 4.2 × 69 cm. A buffer, 0.025 M sodium borate and 0.05 M potassium dihydrogen phosphate (*p*H 7.8), was used. The bottom of the column was closed with a coarse glass sinter which provided electrical continuity with the surrounding buffer. Buffer was pumped between the electrical compartments in both directions so that the *p*H did not vary throughout the separation by more than 0.1. Electrical leakage through the pump was prevented by running the buffer in fine, nonwettable plastic tubes and by breaking the streams with air bubbles. A center tube enhanced cooling by giving the Sephadex an annular cross section. The whole apparatus was surrounded by a cooling jacket; the temperature did not rise above 5 C in the outer buffer vessel and not above 8 C in the Sephadex throughout the separation process, which was continued for 96 hr at 260 v. The lower end of the column terminated in a ground joint; at the end of electrophoresis, a collecting chamber was attached and the contents eluted to a fraction collector.

The fractions containing α toxin were combined and concentrated by ultrafiltration.

RESULTS AND DISCUSSION

Wood 46 strain. Wood 46 is an unusual strain, in that phage type 42D is more often associated with bovine than human infections. It was coagulase-negative when tested by the slide technique, but was coagulase-positive according to the tube test. When plated onto rabbit blood-agar, colonies were surrounded either by wide or by very narrow hemolytic zones. Initially, attempts were made to produce α toxin in continuous culture by use of the same medium. Gradually, however, the proportion of "nontoxin" producers in the culture increased until, after about 2 weeks, an equilibrium consisting of 95% "nontoxin" producers and 5% toxin producers was established. The α-toxin titer always reflected the proportion of α-toxin producers in the culture. Kjems (15) has also drawn attention to two substrains of the Wood 46 strain.

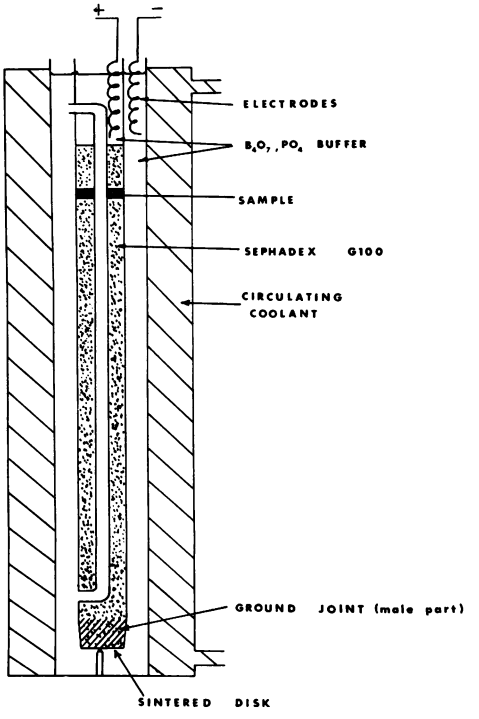

FIG. 1. *Electrophoretic column with Sephadex G100 as an inert support medium.*

Culture. Adequate aeration without frothing was particularly important, and, if this was achieved, a high titer of relatively clean α toxin was obtained. Increasing the ratio of depth to surface or inadequate stirring resulted in slower growth, so that longer incubation was required to achieve the same bacterial density. Even then, a lower titer and a greater number of contaminating proteins resulted. Too vigorous stirring or absence of antifoam, with frothing, caused a poor yield of toxin, even though the bacterial density was unimpaired. It has been shown by Kapral, Keough, and Taubler (13) that the rate of α-toxin production in vivo is a function of bacterial cell multiplication. It would appear that any method of culture used for the preparation of α toxin should achieve its highest titer with the attainment of greatest cell concentration. Most workers have used a 48- to 96-hr incubation period and have begun their purification with a highly impure toxin. The present method, although not preventing some variation in yield even under apparently identical conditions, gave culture supernatant fluid titers of 256 to 1,024.

Purification. The first two steps were essentially those of Wittler and Pillemer (28), except that 25% methanol was used for precipitation; 15% methanol was found to leave behind a considerable amount of α toxin. When extracting the precipitate, it was found that further extraction would elute more toxin but of lower titer. Usually, one-third to one-half of the toxin in the crude supernatant liquid was present in the first 40 ml extracted. On chromatographic separation in Sephadex, all the α toxin was confined to a center peak (Fig. 2); on gel diffusion plates, this material revealed one strong and several fainter lines. Hallander (12) obtained a similar fractionation on G100 and showed that the center peak also contained leucocidin, enterotoxin, and fibrinolysin. The third peak contained nucleic acids, shown by absorbance at 260 mμ. At this stage, difficulties

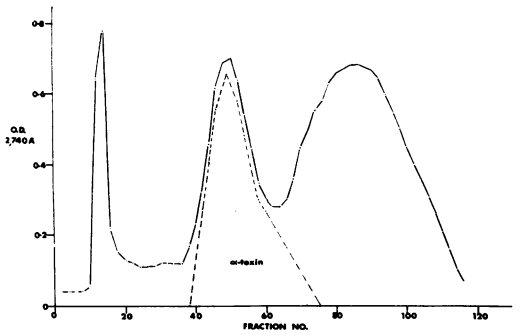

FIG. 2. *Elution curve for extracts of acid-methanol precipitate, from Sephadex G100 in 0.15 M sodium acetate buffer.*

were encountered in concentrating the toxin. Freezing, and therefore also freeze-drying, resulted in marked loss of activity, as did also precipitation with ammonium sulfate, ethyl alcohol, and methanol, or *p*H adjustment to 4.3. Ultrafiltration resulted in some loss, which was indicated by the appearance of a fine threadlike precipitate.

A variety of support media for the separation of α toxin by electrophoresis have been used, and all reports have commented on the strong positive charge of α toxin even up to high *p*H values, as indicated by the fact that α toxin always is the component nearest the cathode after separation. Of all the techniques used so far, density gradient electrophoresis seemed the most useful on a preparative scale, but the apparatus was found difficult to set up and was subject to convective disturbances during the separation. The apparatus described above was developed to overcome this difficulty. Sephadex G100 was used as a support medium, because the materials being fractionated were chromatographically homogeneous with respect to this medium. Any separation which occurred electrophoretically would therefore not be disturbed when the column was eluted. We made use of the fact that this apparatus can be used as an electrophoretic column of infinite length. By adjustment of the buffer levels, an upward flow of buffer with a downward movement of α toxin by electrophoresis was achieved. In this way, all contaminating proteins were washed from the top of the column in the exchanging buffer while α toxin was retained. Figure 3 shows a typical elution curve for this column. The yield of pure α toxin was 12 mg per liter of culture supernatant fluid, and this material had an activity of 10,000 units per mg of protein. Approximately 25% of the crude toxin was recovered in pure form.

Tests of purity. On agar-gel diffusion, a single line was given, and, when rabbit red cells were incorporated in the agar, it was seen that this line corresponded with the edge of the lysed cells (Fig. 4). On immunoelectrophoresis, a single line appeared on the cathode side of the origin (Fig. 5). In another immunoelectrophoretic preparation, a layer of agar with rabbit red cells was superimposed, and the edge of the zone of lysis again corresponded with the precipitin line. The proof that the precipitin line is in fact caused by the interaction of α toxin and antitoxin is necessary, and yet it does not appear to have been sought by previous authors.

In an ultracentrifuge (Spinco model E), a single symmetrical peak (Fig. 6) was given, and this peak sedimented with an $S_{20,w}{}^0$ value of 2.8. This was derived from rates for the 1% and 0.5% solutions of $S_{20,w}{}^{0.5\%} = 2.8_0$ and $S_{20,w}{}^{1\%} = 2.7_3$, assuming a partial specific volume of 0.75 and a linear relationship between S and concentration. Assuming the molecule to be spherical, $S_{20,w}{}^0 = 2.8_7$ corresponds with a molecular weight of 30,000.

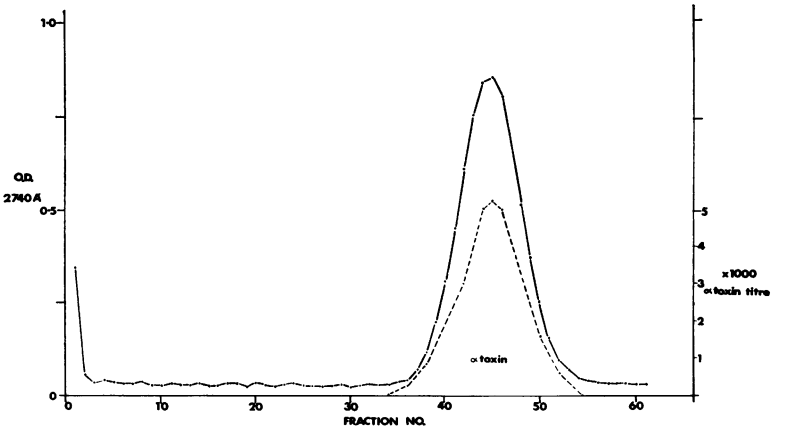

FIG. 3. *After 96 hr at 260 v, α toxin was eluted from the electrophoresis column as a single symmetrical peak.*

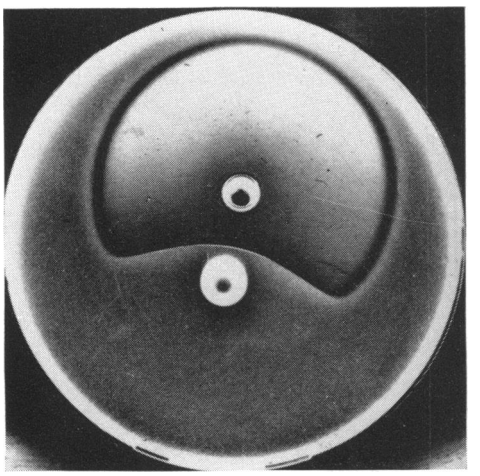

FIG. 4. *On gel diffusion in agar with rabbit red cells, a single precipitin line was seen, and this line corresponded with the edge of the zone of lysed cells.*

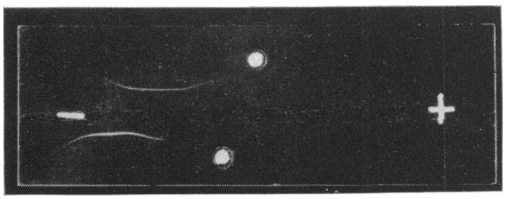

FIG. 5. *Immunoelectrophoresis in barbitone buffer (pH 8.6). Electrophoresis run 1.5 hr at 90 v.*

The method of Atassi and Gandhi (3) gave a molecular weight of 29,600. Molecular weight measurement by short-column (1 mm) equilibrium centrifugation gave a value of 21,200 ± 400. The discrepancy in these results is discussed below.

Properties. The pure toxin was highly unstable and was almost completely destroyed by freezing; it could not therefore be lyophilized. When kept in borate-phosphate buffer at 0 C, it had a half-life of 3 days, and the inactive toxin appeared as a precipitate which was easily removed by centrifugation. This precipitate, although not lytic to rabbit red cells and not lethal for rabbits, was yet able to stimulate antibody production in rabbits. When suspended in buffer and injected intravenously, 1 mg of precipitate, corresponding to 10,000 units, produced no immediate effect. Similarly, twice this amount given intramuscularly appeared to be harmless. Serum from this rabbit 1 week later gave a single line on gel diffusion against crude or purified α toxin. This confirms a similar finding by Kitamura and Shelton (14) and suggests a method for raising monospecific anti-

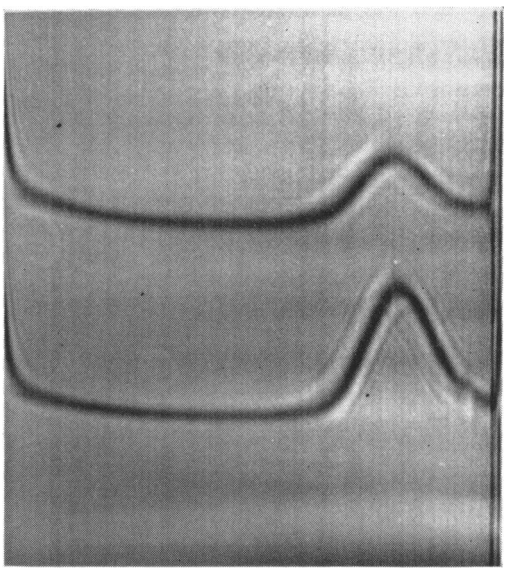

FIG. 6. *Sedimentation patterns of 0.5% and 1% solutions of α toxin (after 32 min at 59,780 rev/min in borate-phosphate buffer) in the Spinco model E ultracentrifuge.*

body in human volunteers in the investigation of the virulence role of α toxin.

Lominski, Arbuthnott, and Spence (19) commented on the gradual appearance of 16*S* particles in their toxin. Bernheimer and Schwartz (7) obtained an *S* value of 3.0 but a molecular weight of 44,000 by a pseudoequilibrium method. Bernheimer and Schwartz's figures could be reconciled with each other by postulating a linear rather than a spherical molecule; but the molecular weight of 21,200, found by equilibrium centrifugation in the present study, cannot be reconciled with an *S* value of 2.9. A time of 3 days separated the sedimentation and the equilibrium estimations; before the latter occurred, the accumulated precipitate was separated by centrifugation. It appears that pure α toxin exists in several polymeric forms in rapid equilibrium and has a gradual drift toward inactivation, precipitation, and higher molecular weight. It is known that insulin and α chymotrypsin, although they give single symmetrical peaks in an ultracentrifuge, exist as equilibrium polymeric mixtures. This concept is supported by the separation of four zones of lytic activity by density gradient electrophoresis by Bernheimer and Schwartz (7). The zones differed in lytic activity but were otherwise identical; the lytic activity of the less active zones could be made to match the more active by precipitation and redissolving. In the present studies, some gel diffusion preparations showed bands well outside the area of in-

teraction with antiserum (Fig. 7), a fact which could be explained by differential diffusion of molecules of different sizes. This partial equilibrium concept would also explain how α toxin, despite its lability, was purified at all. During separation in Sephadex, larger molecular weight forms run ahead of smaller ones with consequent dissociation, whereas the smaller molecules tail behind with a tendency to polymerize, increase their speed, and catch up. It is therefore suggested that an $S_{20,w}{}^0$ of 2.9 and a molecular weight of 30,000 might be taken as average values for an associating system.

Ultraviolet absorption spectrum. Pure α toxin exhibited a typical protein ultraviolet absorption spectrum (Fig. 8), with a minimum at 251.5 mμ, a maximum at 274.5 mμ, and a shoulder at 292 mμ. This compares with Bernheimer and Schwartz's figures of 247, 277, and 290 for material containing 15% carbohydrate (7).

Carbohydrate. Pure α toxin was tested for the presence of carbohydrate by the anthrone reaction. It was found that carbohydrate, if present, forms less than 1% of the toxin by weight.

Amino acid analysis. We submitted two toxin samples to acid hydrolysis and amino acid analysis. Table 1 lists the amino acid frequency with the lowest molecular weight of 21,000 as the monomer. These figures compare well with those given by Bernheimer and Schwartz. There seems no doubt that the α toxin prepared in this laboratory is the same as theirs. The strong positive charge carried by α toxin and the presence of a large amount of ammonia suggest that many glutamic and aspartic acids are present as glutamine and aspargine. The N-terminal amino acid(s) was investigated by the dansyl reaction of W. R. Gray and B. S. Hartley (Biochem. J. **89**:59P, 1963) as modified by C. Hann (*unpublished data*); arginine and histidine were revealed. Any other amino acid forming

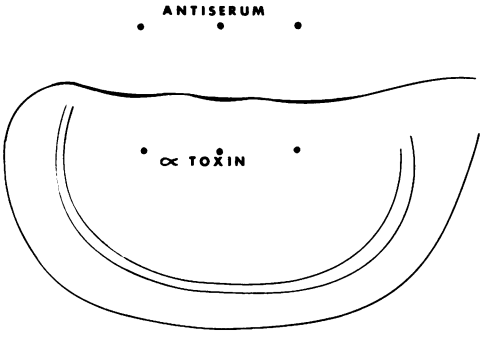

FIG. 7. *Gel diffusion precipitation between pure α toxin and antiserum, showing lines of precipitation in agar outside the zone of interaction.*

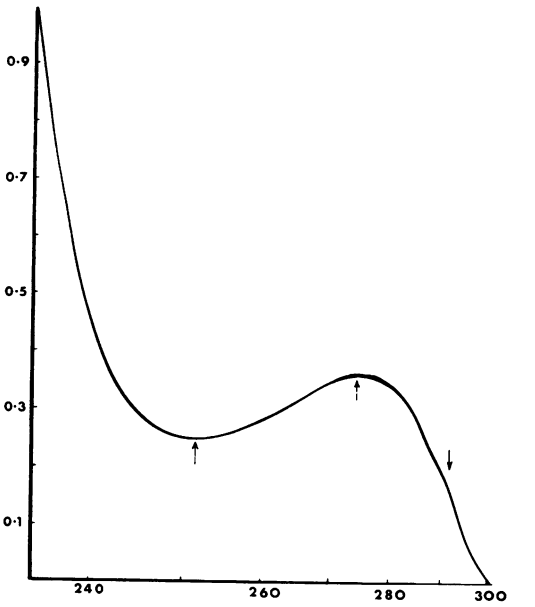

FIG. 8. *Absorption spectrum of pure α toxin. Arrows indicate the minimum (251.5 mμ), the maximum (274.5 mμ), and a shoulder (292 mμ).*

TABLE 1. *Amino acid composition of staphylococcal α toxin*

Constituent	Sample 1		Sample 2	
	Concn[a]	No. of residues	Concn[a]	No. of residues
Lysine	0.166	14	0.114	13
Histidine	0.024	2	0.018	2
Ammonia	Very high		0.468	52
Arginine	0.046	4	0.035	4
Aspartic acid (asparagine)	0.330	27	0.221	25
Threonine	0.165	14	0.111	12
Serine	0.134	11	0.091	10
Glutamic acid (glutamine)	0.147	12	0.101	11
Proline	0.063	5	0.048	5
Glycine	0.156	13	0.109	12
Alanine	0.085	7	0.060	7
Valine	0.105	9	0.076	8
Methionine	Trace		Trace	
Methionine sulfoxide	0.026	3	0.009	2
Methionine sulfone	0.006		0.002	
Isoleucine	0.104	8	0.071	8
Leucine	0.093	8	0.062	7
Tyrosine	0.058	5	0.047	5
Phenylalanine	0.065	5	0.045	5
Cysteic acid	0.005	?	0.004	?
Tryptophan	?	?	?	?

[a] Concentration is in micromoles of amino acid per milliliter of solution.

more than 5% of the total N-terminal amino acids would have been shown by the technique used. This suggests that the α-toxin molecule consists of two polypeptide chains with arginine and histidine in the N-terminal positions. Noll (22), in a recent publication, has drawn attention to the N-terminal residues of 38 purified proteins. He reported that neither arginine nor histidine ever occurs in this position. If the instability of α toxin and perhaps of other proteins possessing these N-terminal amino acids is related to this structure, the two observations are reconciled. It might then also be postulated that the unusual biological properties of α toxin are related to the N-terminal end of the peptide chains. The unusual N-terminal acids could also result from activation of an inactive precursor by the removal of a portion of longer peptide chains. This would explain the safe manufacture of so powerful a cell membrane lysin inside the staphylococcal cell.

Enzyme activity. Of the trichloroacetic acid-insoluble material in a crude commercial egg albumin, 5% was solubilized by incubation with α toxin for 0.5 hr at 37 C. The method was that used by Bernheimer and Schwartz (7). Casein remained unaffected, and the method was found unsuitable for gelatin. When purified ovalbumin was used, no proteolytic activity could be detected.

Phospholipase activity was tested by the method of MacFarlane and Knight (20) on crude egg lecithin, and on the total phospholipids extracted from rabbit red-cell envelopes by the method of Dawson, Hemington, and Lindsay (10). No phospholipase A, C, or D activity could be detected. How α toxin acts on subcellular membranes remains a matter for speculation.

This preparation of pure α toxin, simply and in quantity, has cleared the way for an investigation of four important questions. What is the target or substrate present in the wide variety of membranes on which α toxin has an effect, and what changes are wrought in this substrate? What is the structure of α toxin and how is its structure related to its activity? What part does α toxin play in the virulence of staphylococci? Here the use of the inactive precipitated toxin to elicit a specific antibody response should be valuable. Knowing answers to these questions, then how can we use this knowledge to modify staphylococcal disease? Work is currently in progress to investigate the first two questions.

ADDENDUM IN PROOF

Since submission of this paper for publication, repeated attempts at disulfide bond reduction by mercaptoethanol and sulfite with chromatographic fractionation on C.M.-cellulose have failed to show separation of two peptide chains. Furthermore, the α toxin so treated shows an arginine amino terminal residue only. It may perhaps be relevant to the structure of the active center of bacterial exotoxins that Gerwing et al. (J. Bacteriol. **91**:484, 1966) have recently shown that *C. botulinum* type B toxin also has an arginine amino terminus.

ACKNOWLEDGMENTS

We thank S. Fisher, formerly at Kanematsu Institute, Sydney, Australia, for the Wood 46 strain, J. Coates and J. Fennell of the Physical and Inorganic Chemistry Department, University of Adelaide, for ultracentrifuge studies, A. Inglis of the C.S.I.R.O. Wool Research Laboratories, Melbourne, for amino acid analyses, and, from this Institute, C. Hann for modification of the dansyl coupling method of N-terminal amino acid estimation, J. Higgins for valuable technical assistance, and E. Hackett for encouraging support.

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