Isolation and Composition of Staphylococcal Alpha Toxin

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SUMMARY

A method is described for the preparation, on a laboratory scale, of staphylococcal alpha toxin having a purity of about 70 % and in a yield of 40 %. The method entails ammonium sulphate fractionation followed by curtain electrophoresis. The toxin so obtained contains two impurities, one of which can be removed electrophoretically, and the other ultracentrifugally. The toxin itself is a protein of molecular weight 44,000. It contains most of the usual amino acids which vary in amount from 4 residues for histidine to 44 for aspartic acid. Although electrophoretically heterogeneous its electrophoretic components produce the biological effects classically associated with alpha toxin. As by-products of purification, two other proteins were isolated in crystalline form. Their significance for pathogenicity, if any, remains to be determined.

INTRODUCTION

Alpha toxin is a substance, presumably protein, found extracellularly in cultures of *Staphylococcus aureus*. Capacity to produce it is closely associated with pathogenicity, and 'it is probably the most important and certainly the best known of the staphylococcal toxins' (Van Heyningen, 1950). Preparations of alpha toxin (*a*) are haemolytic *in vitro*, (*b*) are lethal for small laboratory animals, (*c*) produce necrotizing lesions following injection into the skin of rabbits, and (*d*) cause smooth muscle to undergo contraction and loss of function. The effects are commonly attributed to a single product of growth, but rigorous proof for this belief appears to be lacking.

Purification of alpha toxin has been carried out by Wittler & Pillemer (1948), Turpin, Relyveld, Pillet & Raynaud (1954), Butler (1959), Robinson, Thatcher & Montford (1960), and Goshi & Cluff (1960), but in no instance have the products satisfied the usual tests of homogeneity. In order to define more clearly the nature and properties of the toxin, its purification and characterization were undertaken once more. In the course of the work, two other proteins unrelated to alpha toxin were isolated in crystalline form. Their properties, so far as they are known, will also be described.

METHODS

Strain. The Wood 46 strain of *Staphylococcus aureus*, kindly supplied by Dr A. Thal, was used almost exclusively.

Measurement of haemolytic activity. Preparations of toxin were diluted in saline solution (M/13 NaCl) buffered at pH 7.0 with phosphate (M/15) containing 0.1 % (w/v) bovine serum albumin as stabilizing agent. To 1 ml. of each of a series of

dilutions increasing in steps of about 30 % was added 1 ml. of a twice-washed suspension of rabbit red blood cells. The concentration of the red cell suspension used (about 0.7 %, v/v) was adjusted so that a sample, after haemolysis with saponin and adding an equal volume of diluent, gave a Beckman absorbance at 545 m μ of 0.80. The mixtures of toxin dilutions and red cells were placed in a 37° water bath for 30 min., and then centrifuged briefly. The colour of the haemoglobin in the supernatant fluids was compared visually with that of standard haemoglobin solutions, and the dilution haemolysing 50 % of the red cells in the suspension was determined by interpolation. A unit of haemolysin is defined as that amount which liberates half the haemoglobin in the test red cell suspension under the conditions stated.

Suspensions of red cells prepared from different rabbits show wide variation in sensitivity to lysis by alpha toxin. For example, a toxin preparation tested against red cells from three different rabbits titrated at 335,000, 274,000 and 117,000 haemolytic units/ml., and a second toxin preparation tested against the same three cell suspensions gave 18,700, 14,700 and 6,500 haemolytic units/ml. An indication of the variability of red cells from different rabbits can be found in the observations of Timmerman (1937). To make all titrations comparable, a standard toxin preparation, stable when stored under ammonium sulphate, was titrated with each unknown, and the results corrected according to the deviation from the fixed value of the standard.

Protein. Protein was estimated by reading absorbance at 280 m μ in a Beckman DU spectrophotometer, with optical cells of 10 mm. light path. Because none but the crudest preparations contained a significant amount of material absorbing strongly at 260 m μ , no correction for absorbance at this wavelength was applied, and protein concentration was assumed to be directly proportional to 280 m μ absorbance.

Specific haemolytic activity. On the assumption that alpha toxin is a protein, specific activity was expressed as haemolytic activity/unit absorption at 280 m μ , the latter being called for convenience an 'optical unit'.

Lethal activity. Twofold dilutions of toxin were injected intravenously into Swiss mice (20 g.). Not less than three mice were used for each dilution. The toxin was diluted in 0.85 % (w/v) sodium chloride solution containing 1 mg. gelatin/ml., and the injection volume was 0.25 ml. Mice surviving for less than 24 hr. were scored as deaths.

Starch gel electrophoresis. The method of electrophoresis and protein staining with Amido-Black as described by Smithies (1955) was used.

Ouchterlony plates. The technique described by Ouchterlony (1958) was used.

Analytical procedures. Nitrogen was estimated by a modified micro-Kjeldahl procedure; phosphorus by the method of Lohmann & Jendrassick (1926); carbohydrate by means of the anthrone reagent (Carroll, Longley & Roe, 1956); amino acids by the Piez & Morris (1960) modification of the Stein & Moore method.

RESULTS

Preparation of partially purified alpha toxin

Cultivation of organism. The medium and conditions of cultivation were, with slight modification, those devised by Dr A. Pinsky and shown by him to yield consistently satisfactory titres of alpha toxin (in 1960-61 when he was a postdoctoral fellow in this laboratory). The importance of yeast dialysis diffusate for toxin production had been demonstrated by Bramann & Norlin (1951). Medium of the following composition was used: dialysate diffusate of yeast extract 2600 ml., acid hydrolysed casein, 64 g.; glucose, 8 g.; thiamine, 0.4 mg.; nicotinic acid, 3.7 mg.; water to make volume 3200 ml. This medium was adjusted to pH 7.1, and distributed among six 2 l. Erlenmeyer flasks, and sterilization carried out for 20 min. at 123° (17 lb. steam pressure). The diffusate of yeast extract was prepared by dialysing in the cold and with occasional mixing, 1 l. of a 40 % (w/v) solution of yeast extract (Difco Laboratories, Detroit, Michigan) against 4 l. of distilled water. After 6 days the contents of the dialysis sac were discarded, and the outside solution (diffusate) now having a volume of approximately 2.6 l. was used as indicated above.

Table 1. *Purification and recovery of Staphylococcal alpha toxin*

	Volume (ml.)	Total haemolytic activity (units of haemolysin)	Specific activity (units of haemolysin per optical unit)	Recovery of activity (%)
Stage 1. Culture supernatant fluid	3000	2.75×10^8	11	100
Stage 2. Extract of crude ammonium sulphate precipitate	113	1.70×10^8	235	62
Stage 3. 0.6 saturated ammonium sulphate precipitate extracted with 0.4 saturated ammonium sulphate	102	1.68×10^8	440	61
Stage 4. Extract of stage 3 precipitated, dialysed and centrifuged	23.5	1.13×10^8	2,300	41
Stage 5. Continuous-flow electrophoresis; pooled toxic fractions	67	1.05×10^8	11,000	38
Product. First ammonium sulphate precipitate	5	0.08×10^8	13,700	39
Second ammonium sulphate precipitate	5	0.31×10^8	9,600	

The inoculum was prepared by washing the cocci of an overnight broth culture of *Staphylococcus aureus* strain Wood 46, and suspending them in 0.5 vol. of sterile saline. The flasks were inoculated with 0.3 ml. each, and incubated 15-18 hr. at 37° on a rotary shaker operating at 220 cycles/min.

Purification of toxin. The cultures were chilled, pooled, centrifuged 30 min. at 8000 rev./min. and the cocci discarded. The supernatant fluid (stage 1, Table 1)

was saturated by addition of solid ammonium sulphate, and allowed to stand overnight at 4°. All subsequent steps were carried out in the cold.

The precipitate was recovered by stirring into the saturated culture supernatant fluid 15 g. powdered cellulose and filtering through a large coarse sintered-glass filter. The filtrate was discarded, the precipitate emulsified in 40 ml. 0.03 M-borate buffer (pH 8.6) and stirred for a few minutes. Following centrifugation for 10 min. at 10,000 rev./min. the supernatant fluid was decanted, and the precipitate extracted three more times with 40 ml. portions of the same buffer. The four extracts were combined (stage 2) and the residue discarded.

The pooled extract was made approximately 0.6 saturated with ammonium sulphate by addition of 4.2 g. solid ammonium sulphate for each 10 ml., and stirred for 25 min. Following centrifugation for 10 min. at 10,000 rev./min., the supernatant fluid was discarded and the precipitate stored overnight. The precipitate was extracted five times with 20 ml. portions of 0.4 saturated ammonium sulphate, allowing for each extraction 30 min. with intermittent stirring. The extracts were pooled (stage 3).

The extract was brought to 0.6 saturation by dissolving 1.4 g. ammonium sulphate for each 10 ml. After standing for 25 min. with occasional stirring, the mixture was centrifuged, and the clear supernatant fluid discarded. The precipitate was dissolved in 3 ml. 0.03 M-borate buffer (pH 8.3) and with the aid of additional small amounts of buffer totalling 2.5 ml., was quantitatively transferred to a cellophan sac. The solution was dialysed overnight against 6 l. distilled water. When, after dialysis, the volume exceeded 25 ml., it was reduced by pervaporation to 15–25 ml. The small amount of precipitate which formed during dialysis was removed by centrifugation and discarded (stage 4).

After adding 0.3 ml. of 0.04 % (w/v) phenol red, as a marker, the solution was fractionated by means of a Spinco Model CF curtain electrophoresis apparatus (Beckman Instruments, Inc., Belmont, California) according to the following conditions: buffer: 0.06 M-borate (pH 8.3) containing M/5000 ethylenediaminetetraacetic acid (EDTA); paper: that supplied by Spinco; buffer level at feeder curtain: 6.5 cm.; Feed-tab cut above drip-point no. 21; sample feed rate: about 1 ml./hr.; drip-point collection rate: about 0.5 ml./tube/hr.; temperature 3 mm. from curtain 8°; potential difference: 400 V. between electrodes; current: 13 mA.; duration of run: 20–30 hr.

The distribution of protein as measured by light absorption at 280 m μ , and of toxic (haemolytic) activity of the 32 fractions resulting from continuous flow electrophoresis are shown in Fig. 1. It can be seen that the alpha toxin activity coincides with the major protein peak. As a routine, the alpha toxin was recovered in concentrated form by pooling appropriate fractions and dialysing against 75 % saturated neutral ammonium sulphate. In the present instance, the five fractions obtained from drip-points no. 17 to no. 21 were pooled (stage 5) and then dialysed against two changes of 60 % saturated neutral ammonium sulphate, 200 ml. each. After 7 days, the silky precipitate which had formed was separated by centrifugation and suspended in 5 ml. of 60 % saturated neutral ammonium sulphate solution (first precipitate). The supernatant fluid was dialysed overnight against 200 ml. of 80 % saturated neutral ammonium sulphate. The precipitate which formed was separated and suspended in 5 ml. of 80 % saturated neutral ammo-

nium sulphate (second precipitate). The two precipitates are designated 'partially purified alpha toxin'. The recovery and degree of purification at each stage are shown in Table 1.

Fig. 1. Continuous-flow electrophoretic pattern of stage 4 material showing distribution of alpha toxin as haemolytic activity (solid circles) and of protein as extinction at 280 $m\mu$ (open circles).

Analysis of partially purified alpha toxin spectrophotometrically, ultracentrifugally, by starch-gel electrophoresis, and immunologically

The product obtained by the foregoing procedure, dialysed free from ammonium sulphate, yielded a faintly yellowish solution whose ultra violet (u.v.)-absorption curve resembled those usually given by proteins. It showed a maximum at 277 $m\mu$, a minimum at 247 $m\mu$, and a slight but definite shoulder at 290 $m\mu$.

Examination in an analytical ultracentrifuge of a solution containing 7.5 optical units of partially purified alpha toxin/ml. 0.03M-borate buffer (pH 8.3) revealed: (a) a major component accounting for 85 % of the area of the pattern and having an S_{20} value of 3 Svedberg units; (b) a minor component accounting for 15 % and having an S_{20} value of 12 Svedberg units (Fig. 2). By means of a separation cell, a portion of the major component was isolated free from the minor component. Its specific haemolytic activity was, within experimental error, the same as that of the starting material. These observations indicate that the major component was toxin.

Starch-gel electrophoresis of 0.5 optical unit of partially purified toxin, followed by protein staining with Amido-Black revealed two bands on the cathode side of the origin (Fig. 2b). The heavy-staining band was estimated to account for roughly 90 % of the total protein; the light-staining band for 10 %. The position of the heavy-staining band coincided exactly with one previously identified as alpha toxin from among many bands present in electrophoretic patterns of unfractionated crude mixtures of staphylococcal extracellular proteins (Bernheimer & Schwartz, 1961). The 12S component, which was subsequently isolated, yielded no stainable band when subjected to starch gel electrophoresis.

An Ouchterlony plate (Fig. 4b) in which one optical unit of partially purified alpha toxin was diffused against commercial antitoxin known to contain a variety of antibodies against staphylococcal proteins, revealed only two antigens: one

expressed by a heavy line of precipitate and identifiable with a pure alpha toxin line (Fig. 4c); a second which was expressed as a faint line and presumably the same material as the 10 % component demonstrable by starch gel electrophoresis.

Fractionation and analysis of partially purified alpha toxin by means of zone electrophoresis in a density gradient

The foregoing observations are interpreted as indicating that the toxin preparations obtained by the procedure described have a purity of 70–85 %. They were studied further by means of zone electrophoresis in a sucrose density gradient, a technique shown not only to be capable of considerable resolving power but one which is also useful preparatively (Bernheimer, 1962).

Fig. 2

Fig. 3

Fig. 2. Sedimentation pattern of a 0.5 % (w/v) solution of partially purified alpha toxin in 0.03 M-borate buffer (pH 8.3) recorded after 20 min. at 59,780 rev./min. in the model E Spinco centrifuge at 20°.

Fig. 3. Representation of starch gel electrophoretic patterns. Arrow indicates origin; anode is to the right of origin. *a*, Crude extracellular proteins prepared as described by Bernheimer & Schwartz (1961). The complex of bands on the anode side of the origin is not shown but can be seen in Fig. 1. of the paper cited. *b*, 0.5 optical unit of partially purified alpha toxin. *c*, 0.5 optical unit of alpha₀ toxin. *d*, 0.5 optical unit of A-protein. *e*, 0.5 optical unit of X-protein.

Ninety-six optical units of partially purified alpha toxin were fractionated in the apparatus designed by Svensson (1960), using the general conditions described earlier (Bernheimer, 1962). The distribution among the fractions, of toxin as haemolytic activity, and of protein as optical units, is shown in Fig. 5. Seventy-seven % of the input haemolytic activity was recovered. Aside from the small protein peak at

Fraction 260 and that at Fraction 110 which appears to represent a small amount of toxin adsorbed to a finely divided precipitate, the protein between Fractions 160 and 240 is divisible into four zones, namely, from right to left in Fig. 5, α_a , α_b , α_c and α_d . The mean specific haemolytic activities of the fractions of these zones were 15000, 14,700, 9500 and 250, respectively. (Dialysis of the fractions of α_d against 0.03M-borate containing M/5000 EDTA followed by ammonium sulphate precipitation was accompanied by an 18-fold increase in activity; from this it is concluded that an appreciable part of the protein of the α_d zone consisted of inactive toxin capable of activation).

Fig. 4. Precipitin reactions in Ouchterlony plate after 5 days. Centre well: 0.1 ml. antitoxic horse serum. *a*, 0.5 mg. crude staphylococcal extracellular proteins prepared as described by Bernheimer & Schwartz (1961); *b*, one optical unit of partially purified alpha toxin (stage 5 material); *c*, one optical unit of α_a toxin; *d*, 0.5 optical unit of α_b toxin; *e*, 0.5 optical unit of α_c toxin; *f*, 0.5 optical unit of α_d toxin.

Starch gel electrophoretic patterns done on samples containing 0.5 optical unit revealed a single heavily-stained alpha toxin band at the same locus for all four varieties of toxin. In addition, α_a and α_b showed a very faint band on the anode side of the origin. The latter was not consistently demonstrable and its origin is obscure. α_b , α_c and α_d showed a faint but distinct band (beta of Fig. 3*b*) slightly to the right of the heavy band; the pattern for α_a can be seen in fig. 3*c*.

The results of agar diffusion analysis (Fig. 4) were in the main consistent with

those obtained from starch gel electrophoresis. Alpha_a showed a single heavy line of precipitate. Alpha_b, alpha_c and alpha_d produced a similar line giving a reaction of identity with that of alpha_a, but alpha_c showed a second, fainter line which in time tended to merge with the heavy line.

Besides having similar specific haemolytic activities, alpha_a, alpha_b and alpha_c are potent lethal agents (Table 2). The ratio of haemolytic to lethal potency of culture supernatant fluid, alpha_a, alpha_b and alpha_d are, within experimental error, identical, but that of alpha_c is somewhat out of line with the others. The fractions of alpha toxin, when injected into the skin of rabbits, induced the formation of necrotic lesions identical in appearance with the classical description (Elek, 1959) of dermonecrosis associated with alpha toxin. About 1 µg. of partially purified alpha toxin contained in 0.1 ml. gelatin saline produced in 72 hr. a lesion 20 mm. in diameter, as did also the same weight of alpha_b. Alpha_a in the same amount, produced a lesion of about half this diameter.

Table 2. *Comparison of haemolytic and lethal activities of staphylococcal toxin fractions*

Toxin preparation	Mouse LD 50 dose/ml.	Units of haemolysin
		Mouse LD 50 dose
Alpha _a	5700	22
Alpha _b	2600	22
Alpha _c	1620	41
Alpha _d	2400	19
Culture supernate (strain Wood 46)	108	15
Culture supernate (strain 4423)	20	17

The foregoing results are interpreted as meaning that four species of molecules are demonstrable in partially purified alpha toxin when it is subjected to analysis by density gradient electrophoresis. They are alpha toxins, which exhibit different electrophoretic mobilities, but they are antigenically identical, and they are identical, or nearly so, in their biological (lethal, haemolytic, dermonecrotic) activity. However, a fifth species of molecule was detectable by starch gel electrophoresis and was antigenically distinct from toxin. Its relationship, if any, to alpha toxin is unknown.

Further examination of alpha_a. Because the component designated alpha_a appeared to be a homogeneous substance, except for a trace of contaminating protein, and because it had properties identical with those classically associated with alpha toxin, it was examined further. It was prepared by electrophoretically fractionating partially purified toxin in a sucrose density gradient, combining appropriate fractions, dialysing the pool against 0.03M-borate buffer to remove sucrose, and precipitating with 75 % saturated neutral ammonium sulphate. It was stored under ammonium sulphate, and portions were dialysed free from salt as needed. A solution having an absorbance at 280 mµ of 1.00 contained 109 µg. N/ml., 13,000 haemolytic units/ml., and 600 mouse LD 50 per ml.

One of the features characteristic of alpha toxin as distinct from other staphylococcal haemolysins is the sensitivity of rabbit, and to a less extent of sheep

erythrocytes to its action, and the relative resistance of red cells of certain other animal species (Elek, 1959). In Table 8 are shown the results obtained when the same preparation of alpha_a toxin was tested against suspensions of diverse types of red cell made up to contain the same concentration of haemoglobin. It can be seen that the variation in the sensitivity of the red cells to toxin was very great indeed, and in general was in agreement with the experience of earlier workers.

Table 3. *Sensitivity to alpha toxin of red blood cells from different animals*

Animal	Specific haemolytic activity using species of cells indicated	μg. toxin required to cause 50 % haemolysis
Rabbit	14,900	0.048
Mouse	2,400	0.29
Sheep	580	1.2
Human	99	7.2
Horse	62	11.5
Guinea pig	62	11.5

Fig. 5

Fig. 6

Fig. 5. Density gradient electrophoretic pattern of partially purified alpha toxin. Solid circles: protein as extinction at 280 mμ; open circles, alpha toxin as haemolytic activity. Arrow indicates starting zone. Anode to left of starting zone. Buffer: 0.03M-borate (pH 8.3) containing M/5000 EDTA. Sucrose gradient: 0–50 % (w/v); 800 V and 21–18 mA.; 21 hr. Sample consisted of 2.24 ml. borate buffer containing 96 optical units of partially purified toxin and 170 mg. glycine.

Fig. 6. Separation of 12S component (small peak) from staphylococcal alpha toxin (large peak) by ultracentrifugation in sucrose density gradient.

Examination of 0.6 % (w/v) solution of alpha_a in a Spinco Model E ultracentrifuge (Beckman Instruments, Inc., Belmont, California) revealed a pattern essentially identical with that of Fig. 2. It was evident, therefore, that alpha_a was contaminated with the 12S component that had been detected earlier in partially purified toxin.

Separation of the 12S component from alpha toxin. Zonal ultracentrifugation in a sucrose density gradient was carried out according to conditions similar to those

described by Martin & Ames (1961). One ml. of a 1 % (w/v) solution of partially purified toxin was layered on a 27 ml. linear gradient of 5–40 % sucrose contained in a 25 by 75 mm. polyethylene tube. After centrifugation for 20 hr. at 25,000 rev./min, a hole was punched in the bottom of the tube and 27 fractions of approximately one ml. each were collected and estimated for protein. As shown in Fig. 6 the 12S component separated from the alpha toxin. The fractions comprising the 12S peak possessed no haemolytic activity while those of the alpha toxin peak had the expected activity of 12,000 to 13,000 haemolytic units/optical unit. One hundred μ g. of the 12S component, or roughly 100 times as much as the LD 50 of alpha toxin, did not kill when injected intravenously into mice. Electrophoresis, in starch gel, of as much as 500 μ g. of the isolated 12S component, followed by protein staining, revealed no band. A solution of 12S material containing 1 mg./ml. had a 280 m μ . absorbance of 0.60 but the u.v. absorption spectrum showed no well-defined peak at 280 m μ . nor elsewhere. It is concluded from these findings that the 12S substance is probably not a simple protein and that it is unrelated to alpha toxin.

Characterization of 12S-free alpha toxin. For further characterization, partially purified alpha toxin free from 12S component rather than the alpha_a component was used. This choice was made because alpha_a represents only about one-fifth of the active material of partially purified toxin and it may not be representative, in all respects, of the whole. 12S-free toxin was prepared by ultracentrifugation in sucrose density gradients as described in the preceding paragraph. The fractions comprising the major peak were pooled and dialysed overnight in the cold against borate buffer to remove sucrose. The toxin was then salted out by dialysing the solution, in two steps, against 20 vol. of neutral 85 % saturated ammonium sulphate. The precipitate was stored under ammonium sulphate and dialysed free from ammonium sulphate as needed, against borate buffer.

Ammonium sulphate precipitated alpha toxin consisted microscopically of very small granules which were slightly birefringent but which exhibited no uniformity of shape. When the ammonium sulphate precipitated toxin was dissolved in borate buffer (pH 8.3) and dialysed in the cold against the same buffer to a final toxin concentration of 2 % (w/v), there developed a precipitate having a silky sheen. The particles comprising the precipitate were nearly invisible when examined by ordinary light microscopy, but were seen by dark field to consist of feathery lenticular units that were relatively uniform in size and shape. After washing in saturated ammonium sulphate solution and dissolving in buffer, the resulting solution had the same specific activity as had been found earlier for highly purified toxin preparations.

Solutions of toxin buffered at pH 8.3 retained full activity for several weeks when stored at -15° but showed significant loss when stored for more than 2 weeks at 4° . The toxin could be kept for at least several months under ammonium sulphate at 4° without loss. Highly dilute solutions underwent rapid loss of activity but they could be effectively stabilized by gelatin or bovine serum albumin at 1 mg./ml. Dialysis of 0.1 % (w/v) solution against cold distilled water was accompanied by marked loss of activity and the loss was not prevented by M/5000 EDTA.

Ultracentrifugal analysis of a 0.6 % (w/v) solution of toxin revealed a single component having a sedimentation constant of 3.0 Svedberg units. The molecular weight, as determined by the Archibald method (Schachman, 1959) was 44,000;

this is an average of 42,930 at the meniscus and 45,150 at the bottom of the cell. The toxin contained less than 0.025% P and less than 1% carbohydrate (as glucose); its amino acid composition is shown in Table 4.

Absence of proteolytic activity. It has been suggested that alpha toxin is a proteolytic enzyme (Robinson *et al.* 1960). By the casein digestion method of Kunitz (1947) the amount of trichloroacetic acid-soluble 280 m μ -absorbing material liberated from 10 mg. casein by 100 μ g. of partially purified alpha toxin, or of the alpha_a component, was negligible. Under the same conditions one-fiftieth this weight of crystalline trypsin liberated a measurable amount of acid soluble 280 m μ -absorbing material. Similarly, partially purified alpha toxin in a final concentration of 10 μ g./ml. neither liquefied 1% gelatin nor clotted milk. It is concluded that alpha toxin does not cause significant proteolysis of the substrates tested.

Table 4. *Amino acid composition of staphylococcal alpha toxin*

Constituent	mg./16 mg. N	Residues*
Cysteic acid	0	.
Taurine	0	.
Urea	0	.
Methionine sulfoxides	1.77	.
Hydroxyproline	0	.
Aspartic acid	13.2	44
Glutamine and asparagine	0	.
Threonine	6.15	23
Serine	5.22	22
Glutamic acid	7.15	21
Citrulline	0	.
Proline	1.91	7
Glycine	3.90	23
Alanine	2.52	12
Cystine	0	.
α -Amino- <i>n</i> -butyric acid	0	.
Valine	3.22	12
Methionine	1.92	10
Isoleucine	3.78	13
Leucine	4.34	15
Tyrosine	3.84	9
Phenylalanine	3.62	10
Ornithine	0	.
Ammonia	2.76	71
Lysine	7.68	23
Histidine	1.87	4
Arginine	3.89	10
Tryptophan	?	.
? Ethanolamine	0.21	.
Unidentified peak no. 1	0.176†	.
Unidentified peak no. 2	0.252†	.
Unidentified peak no. 3	0.292†	.

* Residues calculated for molecular weight 44,000; † Uncorrected leucine equivalents.

A- and X-proteins. As by-products of the purification of alpha toxin two proteins designated A and X were obtained in crystalline form. A was isolated on several occasions by dialysing against cold 60–70% saturated neutral ammonium sulphate the fractions comprising the small protein peak to the right of alpha toxin (Fig. 1). It consisted of cottony aggregates of fine needles. Protein X was isolated in two

instances only, both times by dialysing the protein of or near Fraction 11 (Fig. 1) against cold 55 % saturated acid ammonium sulphate; it consisted of strongly birefringent needles. Each protein gave rise to a characteristic band on starch gel electrophoresis (Fig. 3*d* and 3*e*), protein A to a band on the cathode side of the origin, and protein X to a very faint band on the anode side. Both showed u.v.-absorption spectra characteristic of proteins. In an Ouchterlony plate with a commercial staphylococcal antitoxic serum, protein A yielded in 3 weeks a single faint band of precipitate. Intravenous injection into mice of about 100 μ g. of either protein was followed by no obvious manifestation of illness. Neither protein has thus far been identified with any of the known extracellular products of staphylococcal growth.

DISCUSSION

The foregoing results indicate that staphylococcal alpha toxin is a protein and that its physical and chemical properties do not differ uniquely from those of many other proteins. Assuming a nitrogen content of 16 %, the amino acids and related compounds recovered from acid hydrolysed toxin accounted for 82 % of the weight of the toxin. The apparent absence of cystine is notable. Using the same figure for nitrogen content, 1 mg. of pure toxin is calculated to have 19,000 haemolytic units, and since cultures contain as much as 1600 haemolytic units/ml., (most or all of the activity appearing to be alpha) it follows that 1 l. of culture can contain as much as 84 mg. toxin, i.e. about 2 % of the dry weight of the cocci that produced it. The concentration of toxins in bacterial filtrates in general has been estimated to be from 5 to 20 mg./l. at most (van Heyningen, 1950). Many of the earlier reports on the purification of alpha toxin do not contain sufficient detail about methods to allow repetition of the work, and in most instances no indication has been given of the yields obtained relative to the amounts of crude toxin started with. The present paper describes methods which permit the preparation of toxin of about 70 % purity, in yields of about 40 %, and without the need for industrial facilities, and procedures are described whereby further purification can be achieved.

Three kinds of preparations were studied: (a) 'partially purified toxin', (b) an electrophoretic fraction of (a) designated α_{a_2} ; (c) ultracentrifugally purified toxin derived from (a) designated 12S-free toxin. Although none of these preparations proved to be completely homogeneous when examined by a sufficient number of physical methods, their state of heterogeneity is operationally definable. The partially purified toxin contained two minor components which appear to be unrelated to toxin: a protein designated beta and demonstrable immunologically and by starch gel electrophoresis, and a substance designated 12S that was demonstrated ultracentrifugally. Together they account for most of the impurity. Aside from these substances, alpha toxin itself seems to be heterogeneous inasmuch as it is partially resolvable into several components by means of density gradient electrophoresis. So far as these have been studied, the electrophoretically separable components do not differ from each other in any very striking fashion as regards their biological properties, but further study of this point would be desirable. The results support the original and subsequently much discussed suggestion of Burnet (1929) that lethal, haemolytic and dermonecrotic effects are caused by a single toxin. The slowest moving electrophoretic fraction, α_{a_2} , was physically isolated and

although it was free from the beta impurity it still contained 12S material. Similarly, ultracentrifugally pure material can be obtained but contaminated with beta.

Aside from its gross biological effects, virtually nothing is known of the mode of action of staphylococcal alpha toxin. Contrary to an earlier suggestion, it does not appear to be a proteolytic enzyme. In view of the rapidity with which it brings about cell damage and in view of its remarkable lytic action on red blood cells, as distinct from diphtheria, tetanus and botulinum toxins which have neither of these properties, perhaps the best hypothesis is that it alters or disrupts cell membranes.

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Following completion of the manuscript of this paper, there appeared several publications concerned partly or wholly with the purification of staphylococcal alpha toxin. Madoff & Weinstein (1962) obtained by metal-ion precipitation, column chromatography and curtain electrophoresis, a highly active, unstable product which yielded a single line in Ouchterlony plates, and two bands when examined by means of starch gel electrophoresis. Kumar & Lindorfer (1962) and Kumar, Loken, Kenyon & Lindorfer (1962) obtained by means of curtain electrophoresis alone, alpha toxin in a purified state. There are, however, several discrepancies between the results of the latter authors and ours. Their product contained significant amounts of carbohydrate and had a sedimentation constant at 18.6° of 1.4, while our alpha toxin is free from carbohydrate and has a sedimentation constant at 20° of 3. Data on the kinetics of haemolysis induced by partially purified alpha toxin were reported by Lominski & Arbuthnott (1962).

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