THE STANDARDIZATION OF STAPHYLOCOCCAL α-ANTITOXIN, WITH SPECIAL REFERENCE TO ANOMALOUS HAEMOLYSINS INCLUDING δ-LYSIN

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Several workers have encountered anomalies due to heterologous lysins during the standardization of staphylococcal α-toxin and α-antitoxin. Morgan & Graydon (1936) reported that their preparations of α-toxin often contained an additional haemolysin, which they named α_2-toxin. In some instances, α_2-toxin impeded titrations of α-toxin and α-antitoxin. Minett (1936) found that toxins prepared from certain strains of animal origin gave anomalous reactions. Smith & Price (1938*b*) described γ-haemolysin, which was apparently unrelated to any of the lysins previously investigated. More recently, δ-haemolysin has been described by Williams & Harper (1947) and Marks & Vaughan (1950).

In the first section of the present work evidence is provided that certain of these haemolysins are identical, and methods are considered for preventing their interference with titrations of α-toxin and α-antitoxin. γ-Haemolysin, which may also cause confusion in α-toxin titrations, is considered separately in the second section. In the course of the work, confirmation was obtained of the reports of Jensen (1935) and Smith & Price (1938*a*) that the reaction between staphylococcal α-toxin and α-antitoxin may fail to accord with the law of multiple proportions. The final section of this paper describes the factors responsible for this phenomenon. Only *in vitro* methods were employed, since these were the most convenient for obtaining the required quantitative information.

MATERIALS AND METHODS

For the preparation of α- and α_2-toxins, cultures were grown in air plus 20 % CO_2 at 37° C. for 3 or 4 days on the surface of nutrient agar. The medium was broken up and extracted overnight at 4° C. with 0·85 % saline. (For the production of γ-haemolysin by the strain Y 2 of Smith & Price (1938*b*) an incubation period of 5 or 6 days was employed.) The cocci were centrifuged off from the extracts, which were then stored at 0° C. after penicillin had been added to give a concentration of 20 units per ml. One α-toxin, Wood 46 H, was prepared by centrifuging a soft (0·3 %) agar culture and separating the expressed fluid.

The δ-haemolysin used in this work was prepared from alcohol extracts of cultures of strain R 1501 in the manner described by Marks & Vaughan (1950).

Simple titrations of lysins were made by the double dilution of 1 ml. amounts with 0·85 % saline, adding 0·025 ml. of a 20 % suspension of thrice-washed red blood cells and incubating in a 37° C. water-bath. Readings were made after 1 hr. incubation ('hot readings') and also, when required, after subsequent cooling to

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4° C. overnight ('cold readings'). Titres are expressed by the number of haemolytic units (H.U.) per ml. One haemolytic unit is the amount of lysin which will produce 50 % haemolysis in the conditions described. The titres recorded represent hot readings except where, in addition, cold readings are given in parentheses.

When possible, toxin-antitoxin titrations were conducted in final volumes of 0·8 ml. per tube, the reagents being diluted so that each was contained in 0·4 ml. The tables indicate the occasions when these reacting volumes were exceeded. After the reagents were mixed they were incubated for 30 min. at 37° C. before the red cells were added. The quantity of cells, duration of incubation and methods of reading were the same as for simple titrations except that lysis to any degree determined the end-point of a toxin-antitoxin titration.

When twofold dilutions of any reagent were made, mixing was performed by pipetting six times up and down a graduated 1 ml. pipette.

Rabbit cells were employed for all the titrations represented in the tables that follow.

α_2-TOXIN AND δ-HAEMOLYSIN

Morgan & Graydon (1936) noted that a staphylococcal antiserum which had been concentrated by precipitation with ammonium sulphate was less effective than the original in neutralizing certain preparations of staphylococcal toxin. It appeared that such preparations contained two components, the classical α-toxin and another. They named the latter component α_2-toxin and the former α_1, but instead of their term α_1 the name α-toxin will be retained here. Their treatment of their antiserum led to the expected increase in α-antitoxin, but apparently to a considerable decrease in the antibody to α_2-toxin. The decrease in the latter antibody was only evident when the test extract contained both α- and α_2-toxins and, in these circumstances, the influence of the α_2 component was out of proportion to its relatively small haemolytic power. When extracts containing α_2-toxin were titrated against concentrated antiserum, partial lysis was noted in several tubes before the end-point, whereas extracts containing only α-toxin gave a rapid transition between complete lysis and none. This partial neutralization characteristic of α_2-toxin was also observed when the latter was titrated in guinea-pigs' skin. α_2-Toxin appeared to be antigenic, but to a smaller degree than α-toxin, and a certain neutralizing level for α_2-toxin could not be exceeded in the serum of immunized horses.

Morgan & Graydon did not isolate α_2-toxin, and therefore could not record all of its properties. To provide evidence for the identity of α_2-toxin with δ-lysin, it will be shown below that the properties of the latter can account for all the findings of Morgan & Graydon, and titrations of both substances against fractionated sera will be compared in detail.

Marks & Vaughan (1950) showed that δ-lysin resembles the fatty acids, such as oleic acid, more than it does staphylococcal α- or β-toxin. It is soluble in ethyl alcohol but not in ether or acetone, and it is removed from solution by Seitz-filtration if the amounts filtered are not excessive. It is lytic for all species of red cells tested and distinguishable from α- and β-toxins and γ-haemolysin by its

lysis of horse cells. It is more active against human than horse cells but, although the former are often convenient, they are unsuitable for titrating δ-lysin in the presence of β-toxin, which also affects human cells. δ-Lysin is neutralized by low dilutions of normal serum, and a large part of this neutralizing power resides in the albumin fraction separated by ammonium sulphate precipitation. In the present work the globulin fraction of staphylococcal antisera (precipitated with ammonium sulphate) was found to have very little neutralizing power for δ-lysin and therapeutic antiserum consisting of enzyme-refined globulin had even less.

The source of α_2-toxin for the present work was the N.C.T.C. strain 5662 (Williams, Australia) which, according to Morgan & Graydon produces both α- and α_2-toxins. Their observation that this strain produces both α-toxin and an anomalous haemolysin little affected by concentrated antisera was confirmed. The strain was also examined with techniques which have been employed to demonstrate δ-lysin. Colonies on plasma-free blood agar plates gave wide, clearcut zones of lysis with horse and human blood, saline extracts of cultures readily lysed horse cells and also human cells, and a substance with the properties of δ-lysin could be obtained by the extraction of cultures with alcohol.

A saline extract (5662C) was prepared from strain 5662 for titration against concentrated staphylococcal antiserum. In addition to α-toxin and α_2-toxin or δ-lysin, the extract contained a small amount of β-toxin which did not interfere with the results because only hot readings were made. The titres of the extract were:

	H.U./ml.
With human cells	10 (40)
,, rabbit cells	320 (2560)
,, sheep cells	40 (640)
,, horse cells	5 (40)

The titres in parentheses represent 'cold readings'.

The concentrated antiserum KCP2296 used in the titration contained 850 units of α-antitoxin per ml. It also contained 450 units of β-antitoxin per ml., but the latter was not concerned in the reactions recorded.

The results, which appear in Table 1, show that a component of extract 5662C, lytic for rabbit cells, was almost unaffected by the concentrated antiserum. However, when native normal horse serum (inactivated at 56° C. for 30 min.), together with sufficient concentrated antiserum to keep the amounts of α-antitoxin unchanged, was titrated against the extract, the mixed sera neutralized the extract as if only α-toxin and α-antitoxin were present. Normal serum alone had little effect on the extract because its content of α-antitoxin (0·5 unit/ml.) was so small. After Seitz-filtration, extract 5662C no longer lysed human or horse cells, and its titre for rabbit cells was reduced to 160 (1280). The filtrate behaved like a pure α-toxin in its reactions with concentrated serum.

The component of extract 5662C resistant to concentrated antiserum is presumed to have been α_2-toxin. The partial haemolysis in several successive tubes, which has been described above as a characteristic of α_2-toxin, is shown in Table 1. The abolition of that lysis attributed to α_2-toxin by Seitz-filtration and by the addition of native normal serum was evidence for the identity of α_2-toxin with

δ-haemolysin since the latter behaves in the same manner. Seitz-filtration removed lysin refractory to concentrated antiserum from toxins prepared from several other strains of staphylococci.

Table 1. *The titration of mixed α- and α_2-toxins against concentrated antiserum and native serum*

Concentrated antiserum KCP 2296 (vol. in μl.)	Normal horse serum (vol. in μl.)	Total amount of α-antitoxin (units)	Percentage haemolysis by given volume of extract 5662C (in μl.)					
			12·5	25	50	100	200	400
1·176	.	1·0	—	10	40	80	100	100
0·588	.	0·5	—	20	40	80	100	100
0·294	.	0·25	—	20	60	100	100	100
0·147	.	0·125	—	30	100	100	100	100
1·130	80	1·0	—	—	—	—	—	100
0·565	40	0·5	—	—	—	—	90	100
0·282	20	0·25	—	—	—	80	100	100
0·141	10	0·125	—	—	40	100	100	100
.	80	0·04	—	80	100	100	100	100
			Volume (in μl.) of Seitz-filtered extract 5662C					
			25	50	100	200	400	800
1·176	.	1·0	—	—	—	—	—	100
0·588	.	0·5	—	—	—	—	100	100
0·294	.	0·25	—	—	—	100	100	100
0·147	.	0·125	—	—	100	100	100	100

Rabbit erythrocytes were used throughout; —, represents absence of haemolysis.

In experiments similar to those recorded in Table 1, the same results were obtained when enzyme-refined antiserum globulin was substituted for antiserum KCP 2296 or when serum albumin replaced whole normal serum.

Table 2. *The titration of δ-haemolysin against concentrated antiserum and native serum*

Concentrated antiserum KCP 2296 (vol. in μl.)	Normal horse serum (vol. in μl.)	Units of α-antitoxin present	Percentage haemolysis by given volume of δ-haemolysin (in μl.)								
			1·6	3·1	6·2	12·5	25	50	100	200	400
10	.	8·5	—	—	—	20	80	95	100	100	100
5·0	.	4·2	—	—	—	40	90	100	100	100	100
2·5	.	2·1	—	—	10	50	95	100	100	100	100
1·25	.	1·0	—	—	30	60	95	100	100	100	100
0·625	.	0·5	—	—	30	60	95	100	100	100	100
.	10	0·005	—	—	—	—	—	—	—	80	100
.	5·0	0·0025	—	—	—	—	—	—	60	95	100
.	2·5	0·0012	—	—	—	—	—	60	80	100	100
.	1·25	0·0006	—	—	—	—	50	90	95	100	100
.	0·625	0·0003	—	—	—	10	60	90	95	100	100

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The chess-board titrations recorded in Table 2 show that δ-lysin reacts with concentrated and native serum in the same manner as α_2-toxin. The δ-lysin was prepared from strain R1501 by alcohol extraction; its titre for rabbit cells was 128 H.U./ml. It contained no α-toxin and so could be neutralized by normal serum without the necessity for added α-antitoxin in the form of antiserum KCP2296.

The experiments recorded in Tables 1 and 2 were repeated using a second concentrated antiserum KCP2029, which contained 400 units of α-antitoxin per ml.; similar results were obtained. The latter antiserum was then titrated first against Wood 46B, an α-toxin free from δ-lysin, and subsequently against the same toxin combined with δ-lysin. The results which are given in Table 3 demonstrate the marked effect that a small amount of δ-lysin may have when a concentrated antiserum is used in titrations. In the presence of δ-lysin the first row exhibited the partial lysis in successive tubes found by Morgan & Graydon in their titrations of α- and α_2-toxin mixtures.

Table 3. *The effect of δ-lysin on the neutralization curve of α-toxin*

Concentrated antiserum KCP2029 (vol. in μl.)	Units of α-antitoxin	Percentage haemolysis with given number of haemolytic units of α-toxin						
		2	4	8	16	32	64	128
5·0	2·0	—	—	—	—	—	—	100
2·5	1·0	—	—	—	—	—	100	100
1·25	0·5	—	—	—	—	95	100	100
0·625	0·25	—	—	—	60	100	100	100
0·312	0·125	—	—	—*	100	100	100	100
0·156	0·062	—	—	90	100	100	100	100
		Repetition of the above, incorporating given number of haemolytic units of δ-lysin						
		0·125	0·25	0·5	1	2	4	8
5·0	2·0	—	—	—	10	80	90	100
2·5	1·0	—	—	—	10	80	100	100
1·25	0·5	—	—	—	20	100	100	100
0·625	0·25	—	—	—	100	100	100	100
0·312	0·125	—	—	—	100	100	100	100
0·156	0·062	—	—	100	100	100	100	100

* Irregularities in the neutralization curve of α-toxin are considered later.

The experiments reported above show that δ-lysin may influence *in vitro* titrations in the same manner as described for α_2-toxin. It is considered that *in vivo* tests would show a similar agreement, for δ-lysin is dermonecrotic for guinea-pigs and rabbits.

The process of concentrating α-, β- and γ-antitoxins in staphylococcal antisera is accompanied by a diminution in the neutralizing power for δ-lysin or α_2-toxin, which, in contrast, is readily neutralized by the albumin fraction of normal serum. It appears therefore, that neutralization of δ-lysin or α_2-toxin is not due to antibody but resembles the inhibition by serum albumin of the lytic action of fatty acids. The data of Morgan & Graydon, which were thought to indicate that α_2-toxin was

antigenic, are open to a more concordant explanation. These workers used α- and α_2-toxin mixtures for test purposes and it is evident that either component could have determined the end-point in titrating a serum. Where their sera had a high α-antitoxin content, as determined by pure α-toxin, the neutralizing power for the mixture of α- and α_2-toxin would have been determined by the non-specific neutralization of the α_2-component, and for this reason was not increased by immunization with a toxoided α- and α_2-toxin mixture. In the second group of sera, which had a low α-antitoxin content, the neutralizing power would have been determined by the α-toxin component of the test mixture and so have been less than in the first group. When the animals of the second group were immunized, their α-antitoxin levels increased until the neutralizing power of their sera reached the fixed level of the first group representing the non-specific inhibition of α_2-toxin. The increase in neutralizing power which occurred in the second group following immunization provided the evidence, considered here to be inadequate, for the antigenicity of α_2-toxin.

The frequency with which Morgan & Graydon met extracts containing the anomalous lysin contrasts with the experience of most other workers; they found that extracts of two-thirds of the strains they examined contained α_2-toxin. Study of the properties of δ-lysin provided explanations of this circumstance. Whereas Morgan & Graydon harvested toxins after less than 48 hr. incubation—a period favourable for δ-lysin production—most workers incubate for 4 or more days by which time δ-lysin is reduced or absent. They did not follow the common practice of Seitz-filtering, which removes δ-lysin from extracts. Finally, they carried out toxin-antitoxin titrations in the small final reacting volume of 22 drops. The larger reacting volume used by most workers may obscure weak δ-lysins whose activity in the presence of concentrated antiserum is more dependent on their dilution than on the amount of antiserum present. With appropriate techniques δ-lysin production to varying degrees may be demonstrated with all α-toxigenic staphylococci isolated from lesions (Marks & Vaughan, 1950).

Minett (1936) has also described anomalies in the neutralization of staphylococcal toxins by antisera. One of his strains, D4, has also been investigated by Smith & Price (1938*b*), who came to no definite conclusion. The same strain (N.C.T.C. 5661) was obtained for the present work. Extracts contained β-toxin and a distinct haemolysin. A typical extract gave the following titres:

	H.U./ml.
With human cells	40
,, rabbit cells	20
,, horse cells	10
,, sheep cells	40 (1280)

The activity of this extract against rabbit, horse and human cells at 37° C. was almost unaffected by concentrated or enzyme-refined antisera but was readily neutralized by normal serum or serum albumin and was removed by Seitz-filtration. It appears, therefore, that the anomalous lysin reported by Minett was also δ-lysin.

γ-HAEMOLYSIN

γ-Haemolysin has been investigated by Smith & Price (1938*b*) using the strain Y2 derived from Forssman's strain B2. Their preparation of γ-haemolysin acted on rabbit cells but was not accompanied by detectable α-toxin; its properties were to some extent obscured by the large amount of β-toxin produced simultaneously. Their strain (N.C.T.C. 5664) was examined and the existence of γ-haemolysin as a substance distinct from α-toxin, β-toxin and δ-lysin was confirmed. The extracts containing γ-lysin did not lyse horse cells, so appeared to be free from δ-lysin. It could not be established that their lysis of human cells was uninfluenced by β-toxin.

As no reference sera were available, a provisional working unit of antitoxin was established by giving to a concentrated staphylococcal antiserum the arbitrary value of 2500 units/ml. The *Lh* dose of different preparations of γ-lysin from strain 5664 contained from 12 to 16 H.U. The small unit was convenient as preparations of γ-lysin contained only 160 to 320 H.U./ml. (for rabbit cells). Normal and immune sera were then examined and the latter, which were the concentrated sera used in the work above, were found to have much the higher content of γ-antitoxin. The amount of γ-antitoxin in four different immune sera bore no relation to the amounts of α- or β-antitoxin present or to the neutralizing power for δ-lysin.

The presence in staphylococcal antisera of γ-antitoxin in concentrations far greater than those found in normal serum suggested that the material used for immunization might have contained γ-lysin or its toxoid. It seemed possible that unrecognized γ-lysin was widely distributed. Extracts were prepared from a number of strains of α-toxigenic staphylococci and examined for γ-lysin. For this purpose it was necessary to prepare an antiserum with a high ratio of α-antitoxin to γ-antitoxin so that the end-points of titrations of extracts against the serum would be determined by γ-lysin, if sufficient were present in the extract, and not by α-toxin.

Immune serum was incubated for 1 hr. at 37° C. with γ-lysin prepared from strain 5664 and kept overnight at 4° C.; the resulting precipitate was centrifuged off. The proportion of serum and lysin had been adjusted to reduce the content of γ-antitoxin in the serum to approximately a tenth of its original value. A control for the absorbed serum was made by substituting for the γ-lysin an equal volume of 50% broth-saline and treating in a similar manner. As the absorbed serum contained excess β-toxin which causes lysis of rabbit cells on cooling, no cold readings were made. The control serum finally contained 12·5 units of α-antitoxin and 25·0 units of γ-antitoxin per ml. and the absorbed serum 11·0 units of α- and 2·5 units of γ-antitoxin per ml. The immune serum used to prepare the control and absorbed sera was a mixture of five parts of refined antitoxin and one part of native normal horse serum, the latter being added to prevent interference by δ-lysin. The relatively slight loss of α-antitoxin on absorption suggested that the γ-lysin preparation contained extremely little α-toxin or toxoid.

Table 4 records the examination of twenty-three extracts made from seven

stock strains and sixteen freshly isolated strains of staphylococci. The former are first in the table. The extracts were titrated against 0·4 ml. of absorbed serum, i.e. 1 unit of γ-antitoxin and also against 0·4 ml. of control serum. In three extracts, γ-lysin could not be detected, the *Lh* dose exceeding 1 ml. In the remainder there was a difference, often considerable, in the amounts of extract combining with identical volumes of the absorbed serum and the control. As the two types of sera differed substantially only in their content of γ-antitoxin, it could be taken that the smaller amount of extract, i.e. that determined in the titration against

Table 4. *The γ-haemolysin content of extracts derived from α-toxigenic strains of staphylococci*

Strain of staphylococcus providing extract	*Lh* dose for absorbed serum* (ml.)	*Lh* dose for control serum† (ml.)	Observed titre for rabbit cells	Calculated titre for rabbit cells of γ-haemolysin fraction
R 3611	0·06	0·5	800	200
R 1672	0·25	> 1·0	200	50
R 1501	0·5	> 1·0	400	25
Wood 46	> 1·0	> 1·0	400	< 12·5
30/30 Sweden	1·0	> 1·0	400	12·5
Barrs Canada	> 1·0	> 1·0	1600	< 12·5
24 Canada	> 1·0	> 1·0	400	< 12·5
01	0·06	0·5	400	200
F 5	0·06	0·5	400	200
06	0·5	> 1·0	100	25
015	0·12	> 1·0	200	100
017	0·25	> 1·0	100	50
029	0·25	> 1·0	200	50
045	0·12	1·0	400	100
047	0·12	1·0	800	100
056	0·25	> 1·0	200	50
072	0·12	1·0	400	100
085	0·12	1·0	200	100
090	0·25	1·0	400	50
AB	0·06	0·5	800	200
Baldwin	0·5	> 1·0	100	25
SD	0·25	> 1·0	200	50
X	0·06	0·5	400	200

* 0·4 ml. of absorbed serum was used, containing 1·0 unit of γ-antitoxin and 4·4 units of α-antitoxin.

† The control consisted of 0·4 ml. of unabsorbed serum, containing 10 units of γ-antitoxin and 5 units of α-antitoxin.

absorbed serum, represented the *Lh* dose of γ-lysin. It was not necessary to discover whether α-toxin or γ-lysin determined the end-point of the titrations against the control serum. The titres of γ-lysin, where it could be demonstrated in extracts, were estimated approximately by allowing 12·5 H.U. to the *Lh* dose. These calculated titres are compared in Table 4 with the actual titres of the extract for rabbit cells. The difference between the titres of each extract indicate the activity of the α-toxin present. Intervals of 100 % were used in the titrations represented in Table 4.

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The results provide evidence of the production of considerable amounts of γ-haemolysin by many strains of α-toxigenic staphylococci. However, the stock strains Wood 46, 30/30 Sweden, Barrs Canada and 24 Canada produced little or none. It is possible that in the latter cases prolonged maintenance in the laboratory had depressed γ-lysin production, or perhaps certain stock strains had been selected for survival because they did not produce anomalous lysins.

Fresh extracts were prepared from cultures of the five strains shown to produce most γ-lysin and each was titrated, together with a Wood 46 extract as a control, against four different immune sera, using intervals of 10 %. The Wood 46 extract gave values corresponding to the α-antitoxin content of each serum. In contrast, the *Lh* values for the other five extracts corresponded to the γ-antitoxin content of the sera except in certain of the titrations against the two sera with the highest ratio of γ- to α-antitoxin in which α-toxin *Lh* values were obtained. It appears, therefore, as foreseen by Smith & Price, that erroneous estimates of α-toxin or α-antitoxin, due to the presence of γ-lysin, may easily arise when extracts and sera of imperfectly known constitution are employed.

Some of the properties of γ-lysin were examined to determine whether it should be grouped with α- and β-toxins or with δ-lysin. Chess-board titrations of γ-lysin against concentrated or enzyme-refined sera resembled those of α-toxin. The neutralization of γ-lysin by sera has a specific character unlike that of δ-lysin. When the final red-cell concentration is varied between 0·25 and 2·0 % the fraction of the available cells lysed by any given dilution of γ-lysin is approximately constant. In this respect γ-lysin resembles α- and β-toxin and differs from δ-lysin, which lyses a smaller fraction of the cells when their concentration is increased (Marks & Vaughan, 1950). Attempts to extract γ-lysin from cultures of strain 5664 with the technique used to prepare δ-lysin were unsuccessful. Strain 5664 did not give reactions which could be considered characteristic in plasma-free blood agar plates made with horse, human, rabbit or sheep blood. Incubation of the inoculated plates in air plus CO_2 for 48 hr. at 37° C. gave rise to lysis of rabbit cells and a small zone of partial lysis of human cells. No effect was produced on horse or sheep cells except for a discoloured zone due to β-toxin with the latter species.

DEVIATION OF α-TOXIN-ANTITOXIN REACTIONS FROM THE LAW OF MULTIPLE PROPORTIONS

Employing the technique of skin tests in rabbits, Jensen (1935) found the *Lr*/100 dose of an α-toxin to be 15 % higher, and the *Lr*/300 dose 38 % higher than the amounts calculated from the *Lr*/3 dose which contained 300 M.R.D. Larger deviations were reported by Smith & Price (1938*a*), who found the *Lh*/40 dose to be 2·4 times and the *Lh*/100 dose 4·7 times, the amounts calculated from the *Lh* dose. All their titrations below the *Lh* level deviated to some degree from the calculated, the discrepancy increasing as the level of the test was lowered. They did not observe the same phenomenon in β-toxin-antitoxin titrations.

The results of chess-board titrations of α-toxin and antitoxin confirmed the findings of Smith & Price and, by analogy, those of Jensen. If the law of multiple

proportions had operated the neutralization curve would have been a simple diagonal, but the titration represented by Table 5 has two departures from the diagonal when hot readings are considered. In this experiment the law appears to hold in titrations above the *Lh* level as the previous workers found.

Similar types of neutralization curve were obtained with toxins prepared from nine other strains of staphylococci. Three different antisera gave concordant results.

Table 5. *The titration of α-toxin against α-antitoxin (using 100 % intervals)*

Units of α-antitoxin	Percentage haemolysis with given volume (in μl.) of α-toxin Wood 46B									
	1·6	3·1	6·2	12·5	25	50	100	200	400	800
4·0	.	.	.	.	.	.	—	—	—	100
2·0	.	.	.	.	.	.	—	—	100	100
1·0	.	.	.	.	—	—	—	100	100	.
0·5	.	.	.	.	—	—	95(100)	100	100	.
0·25	.	.	.	—	—	60(100)	100	100	.	.
0·125	.	.	.	—	— (80)	100	100	100	.	.
0·0625	.	.	—	— (10)	90(100)	100	100	.	.	.
0·0312	.	.	—	50(100)	100	100	100	.	.	.
0·0156	—	—	— (90)	90(100)	100	100	.	.	.	.
0·0078	—	—(40)	40(100)	95(100)	100	100	.	.	.	.

Where hot and cold readings differ, the latter are given in parentheses.

There appeared to be two possible explanations for the observed form of the neutralization curve. The first was that the extracts used contained several, or perhaps a large number, of lysins which, reacting with a corresponding series of antitoxins, gave a composite neutralization curve, the irregularities being due to the supersession of titrations which became diluted out. No evidence was found to support this hypothesis as a general explanation, but the work on γ-lysin described earlier showed that it could operate on occasions. If an antiserum with a high ratio of α- to γ-antitoxin were titrated against an extract containing both α-toxin and γ-lysin with the α-toxin in slight or moderate excess, a break in the neutralization curve could occur when the γ-lysin component was diluted out. The second and more probable explanation of the irregularity in the neutralization curves was that it was due to loss of potency of α-toxin on dilution. The constraints imposed by the technique would lead to the step-like appearance of irregularity.

To investigate the effect of dilution on the potency of α-toxin, a chess-board titration was made using 20 % intervals and the results, which appear in Table 6, may be compared with those in Table 5, where 100 % intervals were used. The titrations in Table 6 are divided into two sections, (*a*) and (*b*), the former above the *Lh* level and the latter below. Table 6(*b*) clearly indicates that a gradual loss of potency took place, increasing in successive dilutions with an accompanying decrease in percentage lysis until lysis failed to occur in the expected tube. However, a factor other than dilution was found to operate. Titrations at 20 % intervals carried out above the *Lh* level (Table 6*a*) revealed a loss of potency which had not been detected by titrations at 100 % intervals at similar *Lh* levels (Table 5). A control for the titrations in Table 6*a* was made by performing the

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dilution for the lowest row of the chess-board in one step. The control titration gave the end-point calculated from the 5 *Lh* dose and thus showed no loss of potency. It appeared from these experiments that the manipulation of toxin incurred during numerous successive dilutions contributed to its loss of potency.

Table 6. *The titration of α-toxin against α-antitoxin (using 20 % intervals)*

α-Antitoxin diluted in given order	Percentage lysis with α-toxin diluted in given order									
	10	9	8	7	6	5	4	3	2	1
	(*a*) Above *Lh* level. Dilution 1 ≡ 5 units antitoxin									
1	.	.	.	.	—	—	—	—	100	100
2	.	.	.	.	—	—	—	90(100)	100	100
3	.	.	.	.	—	—	50(100)	100	100	100
4	.	.	.	—	—	—(40)	100	100	100	.
5	.	.	—	—	—	100	100	100	.	.
6	.	—	—	—	90(100)	100	100	.	.	.
7	—	—	—	40(100)	100	100	.	.	.	.
8	—	—	—(60)	100	.	.	.	.	.	.
	(*b*) Below *Lh* level. Dilution 1 ≡ 0·5 units antitoxin									
1	.	.	.	—	—	—	—	— (10)	10(100)	95(100)
2	.	.	.	—	—	—	—	— (80)	90(100)	100
3	.	.	—	—	—	—	— (70)	40(100)	95(100)	.
4	.	.	—	—	—	— (40)	10 (90)	90(100)	100	.
5	.	—	—	—	—	— (90)	60(100)	100	.	.
6	.	—	—	—	— (70)	20(100)	90(100)	100	.	.
7	—	—	—	— (30)	10 (90)	60(100)	95(100)	.	.	.
8	—	—	—(10)	— (80)	40(100)	90(100)	100	.	.	.
9	—	—	—(70)	10 (90)	60(100)	95(100)	100	.	.	.
10	—	—(30)	—(90)	40(100)	80(100)	100	100	.	.	.

The following experiment was performed to investigate the effect of manipulation of α-toxin. α-Toxin Wood 46H was titrated at the calculated 5 *Lh*, *Lh*, *Lh*/5 and *Lh*/25 levels against corresponding fivefold dilutions of α-antitoxin, the dilutions of toxin being made with minimal disturbance. The test doses of toxin were kept constant and the amounts of antitoxin were varied with 20 % intervals. The results of these titrations appear in Table 7(*a*) and constitute the controls. The test titrations (Table 7(*b*)) were performed in the same way except that the toxin, after first having been diluted for titration at each level, was sucked vigorously twenty times up and down a 1 ml. pipette having a narrow orifice. The volume of each dilution treated in this manner was 4·0 ml. The experiments were extended to investigate the effect of broth on the loss of potency of toxin, for it appeared that broth might be responsible for the relative stability of low dilutions of toxin. The two sets of titrations described above were therefore repeated using infusion broth as a diluent of the toxin instead of saline. The results appear in sections *c* and *d* of Table 7. The dilutions of antitoxin were made up to 0·6 ml. with saline. The volume of toxin, whether diluted in broth or saline, was kept constant at 0·4 ml.

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If no destruction of α-toxin had been caused by manipulation or dilution in the experiments represented in Table 7, titrations at all *Lh* levels would have been expected to give similar end-points, viz. lysis with all amounts of antitoxin but the greatest. However, section *a* shows that saline dilutions made with minimal disturbance led to a loss of 20 % of the toxin at the *Lh*/5 level and 36 % at the *Lh*/25 level. When saline dilutions were agitated (section *b*) the loss of toxin was much greater except at the 5 *Lh* level where the test toxin contained 62·5 % broth. Dilution and agitation had relatively little effect on the toxin when broth was used as the diluent (sections *c* and *d*).

Table 7. *The influence of agitation on the potency of α-toxin solutions*

Treatment of α-toxin Wood 46H*	Level of testing (calc.)	Percentage lysis with given vol. (in ml.) of appropriate dilution of α-antitoxin							
		0·10	0·13	0·16	0·2	0·25	0·32	0·4	0·5
(*a*) Diluted with saline	5 *Lh*	.	.	.	100	100	100	90	—
	Lh	.	.	.	100	100	95	70	—
Not agitated	*Lh*/5	.	100	100	95	60	30	—	—
	Lh/25	.	60	50	40	20	—	—	—
(*b*) Diluted with saline	5 *Lh*	.	.	.	100	100	100	90	—
	Lh	100	100	90	20	—	—	—	—
Agitated	*Lh*/5	40	10	—	—	—	—	—	—
	Lh/25	—	—	—	—	—	—	—	—
(*c*) Diluted with broth	5 *Lh*	.	.	.	100	100	100	90	—
	Lh	.	.	.	100	100	100	80	—
Not agitated	*Lh*/5	.	.	.	90	80	40	—	—
	Lh/25	.	.	.	40	20	10	—	—
(*d*) Diluted with broth	5 *Lh*	.	.	.	100	100	100	90	—
	Lh	.	.	.	100	100	95	40	—
Agitated	*Lh*/5	.	.	.	90	80	20	—	—
	Lh/25	.	.	.	20	10	Trace	—	—

* *Lh* dose 0·050 ml.

To determine the protective actions of various concentrations of broth, α-toxin Wood 46H was diluted 1 in 8 with different proportions of broth and saline. An *Lh* dose of toxin was contained in 0·4 ml. of each mixture. The several mixtures were agitated and then 0·4 ml. doses tested against varying quantities of α-antitoxin; the results are given in Table 8, which also records the effect of different amounts of agitation on toxin Wood 46H diluted 1 in 8 with saline. The reacting volume in these titrations was made up to 1·0 ml. with saline so that the concentration of broth in the final test mixtures never exceeded 40 %. Broth in excess of this concentration was found to reduce the activity of α-toxin. These experiments showed that the presence of 25 % or more broth afforded α-toxin considerable protection against destruction by agitation. In a preparation containing 12·5 % broth, loss of toxin increased with the amount of agitation but not in simple proportion. Other experiments showed that the addition of broth to saline dilutions after agitation had no enhancing effect on the toxin, thus demonstrating that broth does not act by causing dissociation of the toxin-antitoxin compound.

Agitation of dilute solutions of α-antitoxin in the manner described had no appreciable effect on their potency. It was estimated that in the double dilution of an α-toxin to reach a testing level of *Lh*/25 approximately half the loss of potency

Table 8. *The influence of agitation on the potency of α-toxin with different concentrations of broth, and with different amounts of agitation*

Concentration of broth in test dose of toxin (*Lh* dose) (%)	Amount of agitation of test toxin (pipettings)	Percentage lysis with given number of units of α-antitoxin									Titre of agitated test toxin*
		0·25	0·32	0·4	0·5	0·62	0·8	1·0	1·25	1·55	
100	None (control)	.	.	.	100	100	100	80	—	—	320
100	20	.	.	.	100	100	90	50	—	—	320
75	20	.	.	.	100	100	95	40	—	—	320
50	20	.	.	.	100	100	95	40	—	—	320
25	20	.	.	.	100	100	80	—	—	—	320
12·5	20	100	100	80	—	—	—	—	—	—	160
12·5	0	.	.	.	100	100	95	70	—	—	.
12·5	5	.	.	100	100	95	40	—	—	—	.
12·5	10	.	.	95	40	—	—	—	—	—	.
12·5	20	80	30	—	—	—	—	—	—	—	.
12·5	40	20	—	—	—	—	—	—	—	—	.

* End-point tubes had progressively less lysis as broth concentration diminished.

was due to dilution *per se* and half to the agitation caused by the dilution technique. When broth was present in the toxin at a concentration of 50 % or more, the loss of potency due to agitation was averted and that due to dilution approximately halved.

Titrations of weak α-toxins suggested that some dissociation of the toxin-antitoxin compound could occur at low test levels. In Table 6 (*b*) the titrations in rows 8 and 10 show that successive tubes free from lysis after 1 hr. at 37° C. exhibited lysis after standing overnight at 4° C. A trace of unneutralized lysin would account for this behaviour in a single tube but not in successive tubes. Dissociation would tend to counteract the effect of loss of potency at low test levels, but it appears impossible to do more than estimate the resultant of the agencies and minimize the effect of dissociation by adhering to hot readings.

Dilution and agitation had much less effect on β-toxin than on α-toxin, but owing to the necessity for cold readings the effect of dissociation was difficult to estimate. Broth had an unexpected effect for, in addition to protecting β-toxin against the effect of agitation, it appeared to encourage the dissociation of the β-toxin-antitoxin compound at the *Lh*/25 test level and below. It has already been reported above that broth does not promote the dissociation of α-toxin from combined antitoxin.

γ-Haemolysin reacted to dilution and agitation in the same way as α-toxin. *The effect of broth was not studied. δ-Lysin could not be tested with the same technique as it does not react with* antisera in a specific manner, but simple titrations showed a definite loss of potency after the agitation by means of twenty pipettings described above. Broth reduces the activity of δ-lysin.

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DISCUSSION

The tests used to determine end-points of toxin-antitoxin titrations are relatively non-specific when they depend on haemolysis or skin necrosis or on a lethal action without characteristic pharmacological effects. Detection of agents capable of interference with titrations would be facilitated by the provision of a number of standard antitoxic sera prepared with antigens from different strains. A refined antitoxin may be required to demonstrate that the neutralization of a toxin is entirely dependent on antibody and not partially or wholly non-specific. Provisions of this kind would have clarified earlier the status and incidence of α_2-toxin, γ-haemolysin and the other reported anomalies and would probably prove of benefit in the investigation of other toxin-antitoxin systems of doubtful homogeneity.

Since sufficient evidence appears available to identify δ-haemolysin with α_2-toxin, it is suggested that the former name be preferred in spite of lack of priority, for α-toxin is a quite unrelated substance. As γ-haemolysin has properties in common with α- and β-toxins, the name γ-toxin appears appropriate.

δ-Lysin must be considered when titrations involving α-, β- or γ-toxin are undertaken. Its removal from test toxins by Seitz-filtration will usually be possible, but, if not, its concentration may be kept to the minimum by incubating cultures for 4 days or more. Strains may be selected which produce little δ-lysin. Sufficient native serum or serum albumin may be incorporated in tests to neutralize δ-lysin when the latter cannot be excluded.

Experience during this work has suggested the likelihood of erroneous estimates of α-antitoxin when a wide range of extracts is tested by the haemolytic method against sera having a high ratio of α- to γ-antitoxin. It appears advisable to define the γ-antitoxin content of standard antitoxic sera and to use test extracts containing little γ-toxin when α-antitoxin is being titrated. From the work of Smith & Price (1938*b*) it appears unlikely that *in vivo* titrations of α-toxin would be invalidated by γ-toxin. If, however, the γ-toxin and antitoxin content of the reagents are known to be in suitable proportions, the convenient haemolytic method need not be abandoned.

The experiments above have shown that, while agitation has little effect on preparations of α-toxin containing a high percentage of broth, a considerable loss of toxin occurs when the broth concentration is below 25%. These findings are consistent with the results of previous workers. Whereas Casman (1938) improved the yield of α-toxin in broth cultures by gassing with oxygen plus CO_2, Gladstone (1938) using a broth-free medium of known constitution found gassing very detrimental to α-toxin production. As in the present work, Gladstone also found that gassing, e.g. with nitrogen, destroyed both the haemolytic power of preformed α-toxin and its combining power for antitoxin. Later, Casman (1940) obtained better yields of α-toxin by rocking broth cultures—a less violent procedure.

Loss of toxin due to dilution and manipulation may be minimized by gentle treatment and by the use of broth as a diluent, but cannot be exactly controlled.

Such a loss is without effect on the comparison of an unknown serum with a standard serum if they are titrated in parallel. However, in the simple titration of toxins, where only indicator effects (e.g. M.H.D.) are estimated, the physical treatment of the toxins and the nature of the diluent become important factors in achieving reproducible results.

SUMMARY

1. Evidence is submitted that an anomalous rabbit cell lysin reported by Minett (1936), α_2-toxin and δ-haemolysin are identical. The last name is preferred.

2. Precautions are necessary to avoid interference by δ-haemolysin in titrations of α-toxin and α-antitoxin.

3. The existence of γ-haemolysin is confirmed; its wide distribution and possible confusion with α-toxin are noted. The name γ-toxin is thought more suitable than γ-haemolysin.

4. Multiple standard antisera are desirable for the detection of anomalous toxins. At least one should be of refined globulin.

5. Dilution and manipulation inactivate staphylococcal toxins and their effect needs consideration in quantitative work. Infusion broth increases the stability of diluted α- and β-toxins.

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