140. THE BIOCHEMISTRY OF THE GAS GANGRENE TOXINS

2. PARTIAL PURIFICATION OF THE TOXINS OF *CL. WELCHII*, TYPE A. SEPARATION OF α AND θ TOXINS

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THE existence of a number of toxins in the filtrates of the classical gas gangrene organism, *Cl. welchii*, type A (*perfringens*) has been recognized in recent years. Henry [1922], working with *Cl. welchii* isolated from man, distinguished between a 'haemotoxin' and a lethal 'myotoxin'. Wilsdon [1931; 1932–3] classified *Cl. welchii* into four distinct types, and Glenny *et al.* [1933] considered the toxin of type A to be a single entity. They established the parallelism of lethal, haemolytic and necrotic activities of the toxin, which they called α, to distinguish it from the β, δ and ϵ toxins of types B, C, and D. Prigge [1937] claimed to have separated by fractional precipitation with ammonium and sodium sulphates two fractions, one 'ζ' which was lethal and poorly haemolytic, and another 'α' which was haemolytic and hardly lethal. The existence of six toxins has been claimed by Weinberg *et al.* [1937], but serologically these toxins can be grouped into three antigenically distinct toxins, each showing one or more active properties [Ipsen & Davoli, 1939]. One of these three antigens, the η toxin, does not occur regularly, and so far has been found in appreciable quantity only in the filtrates of the French Lechien strain grown in a special medium; abundant antibody is, however, apparently present in Danish and British antisera [Ipsen, 1939; Weinberg & Guillaumie, 1938]. The other two, now named α and θ, apparently occur quite regularly in the toxic filtrates of *Cl. welchii*, type A, but in variable proportions. The properties of these toxins have been summarized by Ipsen & Davoli [1939] as follows:

Toxins*	Haemolysis of sheep cells	Lethality to mice	Dermal reaction
α (ζ)	+	+ +	Necrosis
θ (α)	+ + +	(+)	(Haemorrhagia)
η (η)	0	+	0

* The letters in brackets are those used by Ipsen & Davoli; it has recently been agreed by a group of English workers that this nomenclature is unfortunate, and the non-bracketed names are now used [Dalling & Stephenson, 1941].

In addition to its haemolytic, lethal and dermo-necrotic properties the α toxin is also very probably responsible for the specific reaction which takes place when toxin and normal human serum or egg-yolk solution are mixed. Its capacity to haemolyse and to react with egg-yolk solution is dependent upon the presence of free calcium ions [R. G. Macfarlane *et al.* 1941]. M. G. Macfarlane & Knight [1941] have produced evidence that indicates that an enzyme, lecithinase, which they found in toxic filtrates of *Cl. welchii*, type A, is identical with the α toxin.

The existence of an unstable haemolysin has been known since 1925. Neill [1926], working with unclassified (probably type A) *Cl. welchii* filtrates, found a haemolysin which was reversibly inactivated when the toxin was left exposed to air. The haemolytic activity could then be restored by the addition of various reducing agents. Since his haemolytic experiments were carried out in phosphate buffer the haemolytic activity of the calcium-activated α toxin was completely suppressed (see below); Neill was therefore probably estimating the activity of the θ toxin, which was first mentioned as such in print by R. G. Macfarlane *et al.* [1941]. Todd [1941] has since demonstrated the close relationship between θ toxin and the oxygen-sensitive haemolysins of streptococci, pneumococci and *Cl. tetani*. θ toxin is neutralized by high titred antistreptolysin O sera, and the antibody to θ toxin neutralizes streptolysin O. Although these oxygen-sensitive haemolysins are closely related serologically, they are not antigenically identical.

Little work has been done on the purification and separation of the toxins of *Cl. welchii*. Prigge [1937] claimed to have separated a 'ζ' toxin, with a high ratio of lethal to haemolytic activity, from an 'α' toxin with a high ratio of haemolytic to lethal activity. The separation was effected by fractional precipitation with ammonium and sodium sulphates. R. G. Macfarlane *et al.* [1941] have already pointed out that the haemolytic activity of Prigge's 'ζ' toxin was probably suppressed owing to his use of a phosphate buffer. It is also important to note that Prigge did not treat his 'ζ' and 'α' toxins with reducing agents before measuring their haemolytic activities. If a reducing substance, originally present in his crude filtrates, appeared predominantly in his 'α' fraction, then his 'ζ' fraction might still have contained large proportions of his 'α' fraction, but in a reversibly inactivated form. Ipsen *et al.* [1939] point out that the divergences in comparative titrations of various *perfringens* antitoxins with Prigge's 'ζ' and 'α' toxins do not amount to more than 30 %.

In this paper the partial purification of the toxins of *Cl. welchii* type A is reported, as well as the separation of the α and θ toxins.

Methods and materials

(1) *Culture medium.* The medium described by M. G. Macfarlane & Knight [1941] was used, with the exception that the peptone solutions were sterilized by passing through a 14 cm. Ford G.S. pad, rather than by steaming on three successive days.

(2) *Preparation of toxic filtrates. Cl. welchii*, type A, strain S 107, was used throughout. For purposes of inoculation into the culture medium the strain is maintained in cooked meat tubes, and the inoculations are made from fresh 16 hr. cultures. About every 3 months the activity of the culture filtrates starts to decrease; then the current cultures in the meat tubes are discarded, and new cultures are obtained by inoculating meat tubes from quiescent sealed cultures on alkaline egg medium [Robertson, 1916]. These egg tubes are inoculated from the active cultures of known toxicity and, after suitable anaerobic incubation (4 days), can be preserved in sealed tubes indefinitely. If this is done the activity of the culture filtrates can be kept at a constant level of about 90 M.L.D./ml. Cultures are grown in 2 l. volumes in round-bottomed flasks, without special anaerobic precautions. The cotton wool plugs are tied down to prevent their being pushed out by the vigorous gassing. After 6 hr. incubation in a water bath at 38° the *p*H of the cultures is about 6·0. To filter the cultures they are adjusted to *p*H 7·0 with *N* NaOH and about 20 g. kaolin per litre are stirred in; the resultant suspensions filter rapidly under gravity through pleated filter paper to give crystal clear filtrates.

(3) *Estimation of activity.* Lethal activity is determined by intravenous injection of 0·5 ml. volumes of various dilutions into the tails of 17–20 g. mice. For each determination of median lethal dose five dilutions are used, spaced about 20 % apart, and each dilution is injected into five mice. Generally the practice is to determine the number of E.U./ml. of the toxin and then to inject doses corresponding to 156, 125, 100, 80 and 64 % of 0·5 E.U. into the 5 groups of 5 mice (1 E.U. is generally equivalent to 2 M.L.D. [van Heyningen, 1941]). The percentages of deaths in each group after 48 hr. are then plotted against the corresponding doses and the median lethal dose taken as that amount of toxin which corresponds to 50 % deaths.

α Toxin is also determined by the turbidimetric method described in the preceding paper.

Haemolytic activity is determined by a method similar in principle to that described by Herbert [1941]. Five or six 1 ml. volumes of dilutions spaced about 20 % apart are incubated at 38° in 15 ml. centrifuge tubes with 1 ml. volumes of an approximately 6 % suspension of sheep red blood cells. After 30 min. incubation 5 ml. of an isotonic saline solution, containing 2–3 antitoxin units/ml., are added to each of the tubes to stop further action by the haemolysin. The unhaemolysed cells are centrifuged, and the supernatant fluids, containing varying amounts of liberated haemoglobin, are carefully poured off into test tubes. The degree of haemolysis is then compared in a colorimeter with a standard which is equivalent to 50 % haemolysis. The comparisons are made in a Bausch and Lomb colorimeter which is fitted with the blue filter provided by the makers, and with a Wratten B tricolour green filter. The percentage haemolysis is plotted against the amounts of haemolysin used, and by extrapolation the amount of haemolysin which will cause 50 % haemolysis is inferred. An arbitrary haemolytic unit (H.U.) is defined as that amount of haemolysin which will cause 50 % haemolysis in the approx. 6 % suspension of sheep red blood cells after 30 min. incubation at 38°. A typical activity curve for haemolysis is shown in Fig. 1.

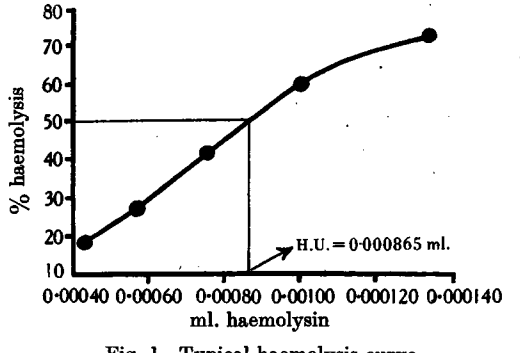

Fig. 1. Typical haemolysis curve.

The standard approx. 6 % suspension of sheep red blood cells is made as follows. The sheep's blood is defibrinated by shaking with glass beads and the red cells are centrifuged and washed twice by resuspension in volumes of isotonic (0·9 %) saline equal to double the original volume of blood. A suspension of the cells is then prepared by making up a 25 ml. volume of the packed cells to 100 ml. with isotonic saline. 0·5 ml. of this approx. 25 % suspension is laked by adding 9·5 ml. of distilled water. The resultant haemoglobin solution is then compared with a neutral grey filter, density 1·5, in a Leitz colorimeter fitted

with a green filter in the eyepiece [see Herbert, 1941]. The solution should be of such a strength that a depth of 5·8 mm. is equivalent to density 1·5; if it is too strong the original suspension is diluted accordingly with isotonic saline. The value of 5·8 corresponds to a haemoglobin solution of 0·4 %, as determined in a spectrophotometer; the approx. 25 % suspension of red blood cells is therefore standardized to contain 8 % haemoglobin. This suspension is kept in a refrigerator and diluted fourfold with isotonic saline just before use. A suspension is never kept for more than 5 days.

The 50 % haemolysis standard is prepared by laking 0·5 ml. of the approx. 6 % suspension with 6·5 ml. of distilled water.

When haemolysis by θ toxin is determined the dilutions of toxin are made in isotonic phosphate buffer *p*H 6·5. The phosphate completely suppresses the haemolysis by α toxin, which requires the presence of free calcium ions. Reversibly inactivated θ toxin is reactivated by reduction with $M/25$ thiolacetic acid; 2 ml. of the toxin are left at room temperature for 15 min. with 0·5 ml. of $M/5$ neutralized thiolacetic acid, and the dilutions in the phosphate buffer are made with this mixture.

As will be seen below, it was found that haemolysis by α toxin is of the 'hot-cold' type. In the estimation of the haemolysis by α toxin dilutions are made in isotonic saline, *p*H 6·5, and 0·005 M with respect to calcium acetate, so that the concentration of calcium in the toxin-red blood cell mixtures is 0·0025 M. After the 30 min. incubation period ice-cold isotonic antitoxin solution is added to the tubes, which are then placed in ice water for 30 min. before being centrifuged. Otherwise the procedure is the same as that described above for θ toxin. Very little liberation of haemoglobin takes place after the 30 min. period at 0°. Estimations by this method also include the activity of active θ toxin.

(4) *Carbon estimations* are carried out in the Van Slyke manometer by the wet combustion method of Van Slyke & Folch [1940]. Generally 0·5 or 1 ml. aliquots of the toxin solutions are used; the water is evaporated off by gently boiling the solutions in the combustion tube with a few pieces of quartz, and with continual agitation over a small flame. The error is not greater than 1 %. Estimations can be carried out directly on solutions which are 1/5 saturated with ammonium sulphate. In such cases an error of about +5 % is observed.

Partial purification of the toxins

The conventional media usually consist of hot-water extracts of meat, containing peptone and cooked meat, and the toxins are generally precipitated from them by 2/3 saturation with ammonium sulphate. The activity of the filtrates from these media is of the order of 3 M.L.D./mg. dry weight, which probably includes quite considerable amounts of ammonium sulphate. For the purification of the toxin of *Cl. welchii* it was necessary, therefore, to find a medium different from those which have hitherto been employed. The medium described by M. G. Macfarlane & Knight [1941], besides yielding highly active filtrates with great regularity, contains comparatively little non-dialysable or ammonium sulphate-precipitable material. The activity of the filtrates is of the order of 30 M.L.D./mg. dry weight of non-dialysable material. Thus a purification of about 10-fold can be obtained by using this medium. By the method described below the toxin is purified from M. G. Macfarlane & Knight's medium with 50 % yield and by a factor of nearly 40-fold, or, very roughly, 400-fold with respect to the non-dialysable material in the usual culture media. Precipitation of the toxins from the filtrates is very unsatisfactory since mechanical losses in the collection of the precipitate (about 1 g./l.) are comparatively large; moreover,

the amount of toxin soluble in the ammonium sulphate solution is probably appreciable in comparison with the total amount of material precipitated. Therefore it seemed desirable to concentrate and purify the toxins by absorption from the crude filtrates. This can be done as follows.

Method I.

1000 ml. toxic filtrate.
75 ml. *M*/5 phosphate buffer *p*H 6·5.
650 ml. acetone.
15 ml. *M* calcium acetate.

The filtrate is cooled to 0° in a jar immersed in ice and salt, *M*/5 phosphate buffer *p*H 6·5 is added and then acetone at −10° is slowly poured in, with constant stirring, until its concentration is 40 %. The temperature must not rise above 2–3°, as the toxin is otherwise destroyed. *M* calcium acetate is then slowly added with stirring. The resultant precipitate of calcium phosphate adsorbs the toxin. After standing at 0° for at least 15 min. the precipitate is centrifuged and eluted twice with successive 50 ml. volumes of 1/5 saturated ammonium sulphate solution in water, and the two eluates are combined. The eluate is light yellow and contains 50–60 % of the toxin, purified about 10-fold.

This procedure is recommended only for small quantities of toxin since it is difficult and tedious to maintain a low enough temperature when working with large volumes.

Unless acetone is used the toxins are not adsorbed on the calcium phosphate, but if the filtrate is first dialysed the concentration of acetone can be lowered to 20 %; this concentration does not injure the toxins and consequently the adsorption can be carried out at room temperature.

For large volumes of filtrate the following procedure is therefore used.

Method II. The filtrate is dialysed overnight (16 hr.) in wide cellophane tubing ('Visking' sausage casing, 40 mm. wide when flat) against running tap water. Adsorption is then carried out with the following proportions of materials:

1000 ml. dialysed filtrate.
75 ml. *M*/5 phosphate buffer *p*H 6·5.
250 ml. acetone.
15 ml. *M* calcium acetate.

The acetone is added slowly at room temperature, with constant stirring. After the calcium acetate has been added the adsorption is allowed to proceed for 15 min. and the calcium phosphate is then collected. When large volumes are used it is most convenient to use a Sharples centrifuge which can be operated continuously. The rotating cylinder of the centrifuge must have holes in the top so arranged that only the 'supernatant' fluid is ejected; the precipitate is spun on to the wall of the cylinder, which is lined with hardened filter paper. The liquid enters the rotating cylinder from the bottom through a 3·5 mm. delivery jet, under a head of about 75 cm., and under these conditions passes through the centrifuge at the rate of about 1 l. per min. The calcium phosphate precipitate is small in relation to the total volume of fluid and settles in a thin layer on the filter paper. The rapid flow through the 3·5 mm. delivery jet ensures that the precipitate is distributed over the total height of the filter paper liner, and does not clog the lower part of the cylinder. The 'supernatant' still contains a little precipitate. After the fluid has passed through the centrifuge once via the 3·5 mm. delivery jet, it is sent through again, without stopping the centrifuge, via a 1·5 mm. jet. In this way the precipitate is quantitatively collected at an

overall rate of flow of about 250 ml./min. It is easy to deal with 10 l. or more in this manner. After centrifuging, the filter paper liner is carefully withdrawn from the cylinder and spread out on a sheet of glass. The precipitate is then scraped off with a table knife and suspended in a volume of 1/5 saturated ammonium sulphate solution equal to 1/20 the original volume of filtrate. The precipitate is finely suspended and spun down in an ordinary centrifuge, and the elution repeated. The two eluates are combined to give a light yellow solution containing 70–80 % of the toxin, purified over 10-fold.

The toxin in these eluates can be further purified and concentrated by a second adsorption and elution. The eluate is dialysed for 6 hr. against running tap water, and then, since the volume is conveniently small, subjected to the procedure in method I. It is more convenient at this stage to concentrate 5-fold rather than 10-fold; a further 3-fold purification is effected and the yield is again 70–80 %. It is important not to wash the calcium phosphate precipitates with water, since this would remove quite a large proportion of the toxins.

Typical results of a double adsorption and elution are shown in Table 1.

Table 1. *Purification of toxin by adsorption and elution*

Preparation	Volume ml.	E.U./ml.	mg. C/ml.	E.U./mg. C	Overall yield %	Overall purification	Test dose E.U.
Crude filtrate	2500	45	17	2·6	—	—	4·43
Dialysed filtrate	3000	37·6	1·09	34·4	100	—	—
1st eluate	250	324	0·76	427	72	12·4-fold	—
2nd eluate	50	1215	0·91	1336	54	38·8-fold	3·95

The degree of purification is with reference to the dialysed filtrate.

The decrease of the toxin in the test dose probably indicates that a small degree of toxoid formation has taken place during the purification.

The solution containing 1215 E.U./ml. is light yellow, and, in 1/5 saturated ammonium sulphate solution, retains its activity for at least 10 days at 0°. After it has been dialysed the toxin solution loses its activity rapidly. The most convenient way to keep the toxin is to dialyse the eluate overnight, and then to dry it from the frozen state. This procedure does not cause any loss in activity, and results in a light white product which is easily soluble in water or isotonic saline. The carbon content of the final product is about 50 %, and the M.L.D. is about 0·75 μg.

The second eluate contains about 2 mg. of non-dialysable material per ml., and does not give a precipitate with trichloroacetic acid, or when saturated with solid ammonium sulphate. On dialysis against a saturated solution of ammonium sulphate, however, a precipitate is obtained. The possibilities of further purification along these lines are being investigated.

Separation of α and θ toxins

It was found that under suitable conditions θ toxin can be quantitatively adsorbed from a mixture of α and θ toxins. If 2 ml. undiluted sheep red blood cells are mixed with 8 ml. of fresh toxic filtrate and left for 5 min. at 0°, the supernatant, after the red cells have been centrifuged, will show no θ activity, as measured in isotonic phosphate buffer, even after reduction with thiolacetic acid. If the centrifuged red cells are resuspended in isotonic saline at 38° they are lysed almost immediately. The α toxin content of the supernatant, as measured by the turbidimetric method, is unchanged, or slightly diminished,

but the haemolytic activity when measured in calcium saline is high. If the filtrate is left with the red cells for longer than 5 min. more α toxin will be adsorbed, but the adsorption of the α toxin apparently does not begin until all the θ toxin has been adsorbed. The adsorption of θ toxin can also be carried out with smaller amounts of red cells, but then the time of adsorption must be increased in proportion. Fig. 2 shows the effect of time on the adsorption of θ

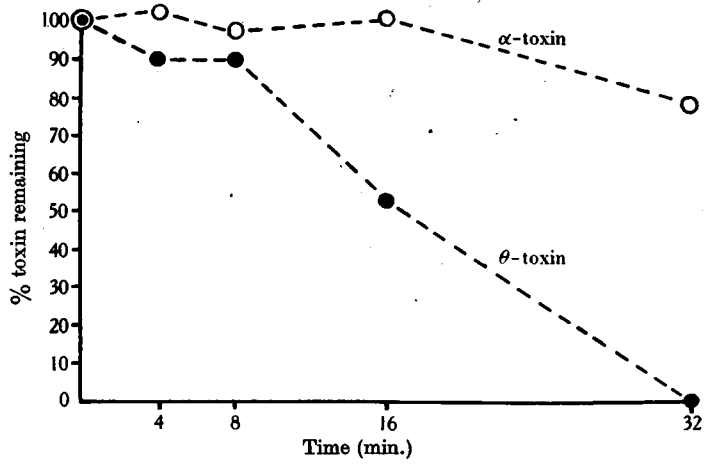

Fig. 2. Effect of time on adsorption of α and θ toxins by red blood cells.

and α toxins when 2 ml. amounts of fresh filtrate containing 48·5 E.U./ml. were left with 0·1 ml. amounts of undiluted washed sheep red blood cells at 0°; α toxin was determined by the turbidimetric method, and θ toxin was determined in phosphate buffer after reduction with thiolacetic acid. During adsorption a very slight degree of haemolysis may take place, in which case a small amount of liberated haemoglobin will appear in the supernatant. θ toxin can also be adsorbed from diluted (60 E.U./ml.) solutions of the partially purified toxins. The θ toxin content of the purified fractions has not yet been determined quantitatively, nor has the adsorption of θ toxin from high concentrations of toxins been studied. The haemolytic activity of the θ-free preparations is completely inhibited by low concentrations of Glenny's α antitoxin, and unaffected by high concentrations of his θ antitoxin.

The conditions outlined above for the adsorption of θ toxin hold only for fresh filtrates. When 8 ml. of a filtrate which had stood for 10 days at 0° were treated with 2 ml. of undiluted washed sheep red blood cells for 5 min. at 0°, only about 70 % of the θ toxin was adsorbed. The θ activity of the fresh filtrate was 471 H.U./ml., and after reduction with thiolacetic acid it was the same, viz. 480 H.U./ml. The θ activity of the 10-day-old filtrate was 23 H.U./ml., but on reduction it was restored to 473 H.U./ml. Reduction of the stale filtrate and consequent reactivation of the θ toxin did not bring about a greater adsorption of θ toxin. Nor was more than 70 % adsorbed when the proportion of filtrate to red blood cells was changed from 8 : 2 to 5 : 5, or when the adsorption time was increased from 5 min. to 30 min.

This incomplete adsorption of θ toxin from stale filtrates, as compared with the quantitative adsorption from fresh filtrates, is inexplicable. It is apparently

not due to conversion of θ toxin into toxoid, since its activity could be completely restored by reduction; nor can it be due to the reversible inactivation of θ toxin since the reactivated toxin was not adsorbed to a greater extent than the inactivated toxin. An extended investigation of the adsorption is obviously desirable.

It was found that α toxin is a 'hot-cold' haemolysin. This interesting type of haemolysis has been known for some time in connexion with the staphylococcus β haemolysin [see Glenny & Stevens, 1935; Roy, 1937, and Smith & Price, 1938]. Ipsen *et al.* [1939], working with a toxin-antitoxin mixture, found that no haemolysis took place after 2 hr. at 37°, but that haemolysis did take place during a subsequent 18 hr. period at room temperature. The toxin was one of Prigge's, with a low ratio of haemolysin to lethal toxin, and presumably contained a high ratio of α toxin, or θ toxin in reversibly inactivated state. These workers state that the effect observed could not be compared with the 'hot-cold' lysis of staphylococcus β haemolysin since it was not observed with the toxin alone. They ascribe the effect to a probable dissociation of the toxin-antitoxin complex at low temperature.

When a suitable concentration of the α and θ toxins in calcium saline was incubated for 30 min. at 38° with a 6 % suspension of sheep red blood cells there was no apparent haemolysis; if 5 ml. of isotonic antitoxin solution at 38° were added there was still no haemolysis, but if 5 ml. of antitoxin solution at room temperature, or at 0°, were added, complete haemolysis took place very rapidly. Complete haemolysis also took place when the incubated mixture of toxin and red blood cells was cooled in ice. α toxin which has been freed of θ toxin by adsorption exhibits the same phenomenon, but if the concentration of α is sufficiently high complete haemolysis will take place during the incubation period. θ toxin is an ordinary 'hot' haemolysin; haemolysis by solutions of toxic filtrate in phosphate buffer, in which α is inactive, is not of the 'hot-cold' variety.

The lysis which takes place during the cold period is apparently independent of further action by the α toxin. Two equal amounts of α toxin were incubated with the red blood cell suspension for 30 min. at 38°; to one mixture were added 5 ml. of an isotonic solution containing 3 antitoxin units/ml., and to the other 5 ml. of ordinary isotonic saline. After 1 hr. at 0° the degree of haemolysis was estimated and found to be the same in both tubes.

In the estimation of α toxin it is therefore necessary to cool the toxin-red blood cell mixtures before the degree of haemolysis is determined. The 30 min. period at 0°, mentioned in the section on methods and materials, was chosen because very little further haemolysis takes place after this time.

Table 2 shows typical values obtained in the determinations of haemolysis by the α and θ toxins of *Cl. welchii*, type A, strain S 107. The preparation in sections 5 and 9, the θ-free filtrate, was obtained by treating 8 ml. of fresh filtrate with 2 ml. undiluted washed sheep red blood cells for 5 min. at 0°. After the red cells had been centrifuged the supernatant had no θ activity and the α toxin content, as determined by the turbidimetric method, had decreased from 47·7 to 43·5 E.U./ml. All the values in the table refer to the same filtrate.

It will be seen that the haemolytic activity of a mixture of α and θ toxins (α+active θ, or α+reactivated θ) is no greater than that of α alone (or α+inactivated θ). When two series of dilutions, both containing the same concentration of α toxin, but one containing θ toxin as well, are incubated with red cells, the series with the θ toxin will show haemolysis in some of the dilutions and the series without θ toxin will not; but if both series are cooled the extent of haemolysis will be found to be the same in both. At first it was thought that this

Table 2

	Preparation	Toxins involved	H.U./ml.
1	Fresh filtrate, in phosphate buffer	Active θ	471
2	Fresh filtrate after reduction, in phosphate buffer	Active θ	480
3	10-day-old filtrate in phosphate buffer	Inactivated θ	23
4	10-day-old filtrate, after reduction, in phosphate buffer	Reactivated θ	473
5	Fresh filtrate, after adsorption with R.B.C., in phosphate buffer, with or without reduction	None	0
6	Fresh filtrate, in calcium saline, by 'hot-cold' method	α + active θ	1145
7	10-day-old filtrate, in calcium saline, by 'hot-cold' method	α + inactivated θ	1100
8	10-day-old filtrate, after reduction, in calcium saline, by 'hot-cold' method	α + reactivated θ	1000
9	θ-free filtrate, in calcium saline, by 'hot-cold' method, corrected for partial loss of α	α alone	990

phenomenon was due to the inhibition of α by θ, by competition for the red blood cells. The fact that θ toxin is adsorbed by the red cells before α toxin seemed to support this view. It was thought that every α unit inhibited by θ was counterbalanced by a θ unit, i.e. 1000 α units + 500 θ units would amount to 500 active α units + 500 inhibited α units + 500 active θ units = 1000 active haemolytic units. If this were the case, then reversibly inactivated θ toxin should not be adsorbed by the red blood cells; otherwise the values of the preparation 7, α + inactivated θ, should be lower than that of α alone, or of α + active θ. In fact, as was mentioned above, inactivated θ is adsorbed to the extent of 70 %. The fact that the combined haemolytic activities of α and θ toxins are not additive therefore remains to be explained.

With the exception of a filtrate which is discussed below, the values in Table 2 are typical of the filtrates so far examined. The ratio H.U./E.U. in untreated filtrates, and in filtrates from which the θ has been adsorbed, has always been from 20 to 23, and the ratio of M.L.D./E.U. in the same preparations, has, within the limits of the errors of the assay for toxicity, always been approximately 2.

Recently a filtrate, apparently prepared in the same manner as the previous one, was obtained which gave the results shown in Table 3. The filtrate contained 43·5 E.U./ml. and its activity was completely inhibited by *Cl. welchii*, type A antitoxin. It differs from the previous filtrates in the following respects:

(1) The θ activity is apparently doubled (preps. 1–4).

(2) After adsorption with red blood cells there is still some activity (prep. 5). This activity does not require the presence of free calcium ions, and is unstable since it disappears after 4 days (prep. 6); nor can it be recovered by reduction with thiolacetic acid (prep. 7). This apparently unstable activity is again shown in the difference between 1686 H.U./ml. for prep. 8, and 1430 H.U./ml. for preps. 9 and 10.

(3) In preps. 9, 10 and 11 the ratio H.U./E.U. is about 31, as compared with the ratio about 21 previously found.

These results can be explained by assuming that two more haemolysins have appeared. For convenience in discussion these hypothetical haemolysins will be called X and Y.

Table 3. *Properties of filtrate possibly containing additional toxins*

	Preparation	Hypothetical toxins involved	H.U./ml.
1	Fresh filtrate, in phosphate buffer	Active θ	958
2	Fresh filtrate, after reduction, in phosphate buffer	Active θ	1018
3	4-day-old filtrate, in phosphate buffer	Partially inactivated θ	455
4	4-day-old filtrate, after reduction, in phosphate buffer	Reactivated θ	1000
5	Fresh filtrate, after adsorption with R.B.C., in phosphate buffer	X	200–300
6	Same as 5, after 4 days, in phosphate buffer	Inactivated X	0
7	Same as 6, after reduction, in phosphate buffer	Inactivated X	0
8	Fresh filtrate, in calcium saline, by 'hot-cold' method	Active $\theta + X + \alpha + Y$	1686
9	4-day-old filtrate, in calcium saline, by 'hot-cold' method	Partially inactivated $\theta + \alpha + Y$	1430
10	Same as 9, after reduction, in calcium saline, by 'hot-cold' method	Reactivated $\theta + \alpha + Y$	1430
11	Preparation 6, in calcium saline, by 'hot-cold' method, corrected for partial loss of α	$\alpha + Y$	1360
12	α, calculated from H.U./E.U. = 21 and E.U./ml. = 43·5	α	915

X is an unstable haemolysin, which is not adsorbed by red blood cells; it is unlike α in that it does not require the presence of free calcium ions, and its loss of activity, unlike that of θ, cannot be restored by reduction. It contributes 200–300 H.U./ml.

Y is a more stable haemolysin, which is also not adsorbed by red blood cells; it is apparently associated with α, and seems to require the presence of free calcium ions. In the 4-day-old preparations it increases the H.U./E.U. ratio from 20 to about 30. It contributes about 500 H.U./ml. (the difference between the values in sections 11 and 12).

If these assumptions are correct, then the haemolytic activities of $\alpha + X + Y$, unlike those of $\alpha + \theta$, are additive.

The lethal activity of the filtrate was not determined. These findings with the latest filtrate must be treated with reserve, as they have been observed with only one filtrate. They are mentioned here in case other workers should not be able to confirm the results set out in Table 2. The discussion below is not concerned with this latest filtrate.

Discussion

It is interesting to examine some of the earlier work on the toxins of *Cl. welchii* in the light of the results reported above. Numerous workers in the past [e.g. Glenny *et al.* 1933] have demonstrated a close parallelism of the lethal and haemolytic properties of the toxic filtrates. As it has since been demonstrated that there are at least two haemolysins, one of which (θ) is only slightly lethal, and easily inactivated, it might be considered surprising that it has been possible to demonstrate such parallelism. The results here reported, however, show that, provided that the haemolytic activity is estimated by a 'hot-cold' method, and not in phosphate buffer, the value obtained is always that of the α toxin. (This might not hold if there were more θ units/ml. than α units.) It appears to be

independent of the amount of θ toxin, and of its state of activation. Glenny *et al.* did their haemolytic estimations in isotonic borate buffer, and they took their readings after 2 hr. incubation and overnight standing at room temperature. Standing overnight at room temperature is probably sufficient to complete 'hot-cold' haemolysis.

Other workers have adsorbed the toxins on red blood cells. Thus Wuth [1923] treated 3 ml. of *Cl. welchii* filtrate with 3 ml. undiluted sheep's red blood cells for 2 hr. at 0°, and found that all the haemolytic activity had been removed. His method of estimating haemolysis was such that it probably included haemolysis by α toxin. Since he used a very high concentration of red cells, and since he left the toxin-red cell mixture to stand for 2 hr. it is probable that he adsorbed both θ and α toxins. He was also able to adsorb all the haemolysin on powdered fibrin. When he treated an unstated amount of toxin with 0·02 ml. of 1 % methyl alcohol solution of lecithin, 90 % of the haemolytic activity was lost. This was conceivably due to competitive inhibition of the α toxin by lecithin, which M. G. Macfarlane & Knight [1941] have shown to be a substrate for an enzyme which is probably α toxin.

Henry [1923] found that when fresh toxic filtrates of *Cl. welchii* were treated with thick suspensions of red blood cells for 24 hr. at 0°, there was a decrease in lethal activity. When stale filtrates were treated similarly there was no decrease in lethal activity. It was shown above that red cells will adsorb all of the θ, and some of the lethal α toxins from fresh filtrates, but only about 70 % of the θ, and none of the α, from stale filtrates. Henry also showed that a 'myotoxin', presumably the α toxin, could be adsorbed on fresh chopped guinea-pig muscle.

It is obvious that there is still a great deal to be done in connexion with the toxins of *Cl. welchii*, and the work reported in this paper can only be regarded as preliminary. In view of the close relationship of streptolysin O and θ toxin, it would be interesting to determine whether the combined haemolytic activities of streptolysin O and α toxin are non-additive. Smith & Price [1938] found that the addition of broth or glycerol to dilutions of staphylococcus β haemolysin abolished the 'hot-cold' haemolytic effect; with glycerol the same end point was obtained at 37° as in 'hot-cold' lysis, and with broth the end point was slightly displaced. It is necessary to see whether the same effect can be observed with the α toxin of *Cl. welchii*.

While it is possible to obtain θ-free preparations of α toxin by adsorption with red blood cells, it would be convenient to find another adsorbent, preferably inorganic. Red blood cells are not very suitable for the adsorption of θ toxin on a large scale. Moreover, it is not possible to recover the θ toxin from the red blood cells. A few inorganic adsorbents, namely kaolin, kieselguhr, alumina C_γ and dialysed iron have already been tried, but without success.

Formal proof that the factors which are responsible for the 'hot-cold' haemolysis, the lethal action, the necrotic action, the reaction with egg-yolk solution and the lecithinase activity are identical is probably impossible to provide. There is, however, much evidence that these varied activities are, in fact, due to one toxin, the α toxin. M. G. Macfarlane & Knight [1941] have demonstrated the parallelism of toxicity, development of turbidity with egg-yolk solution and lecithinase activity. They found this parallelism not only in various samples, but also in samples which had been partially inactivated by bubbling with nitrogen. The present writer has similarly demonstrated parallelism of toxicity, turbidity production and 'hot-cold' haemolysis. Parallelism has also been demonstrated for necrotic and lethal activities (see, for example, Glenny *et al.* [1933]). The demonstration of parallelism of necrotic and lethal activities

with other activities cannot be very close because of the inaccuracies associated with their assay.

The objection to the type of evidence just discussed is that two or more separate toxins having similar physical properties and showing different activities could also exhibit such parallelism. But if several different antisera were assayed against the same toxin preparation by measurement of the above-mentioned five types of activity, and if these five methods all gave concordant values for each of the antisera, then that would be convincing evidence that the five types of activity are due to a single toxin. If these activities were due to different toxins it would be extremely unlikely that their respective antitoxins would occur in corresponding proportions in an antiserum, and even more unlikely that these proportions would hold for a number of antisera. Moreover, the percentage error in the mouse intravenous method, or in the necrotic assay, is much lower in antitoxin or test dose determinations than in straight determinations of activity, so that lethal or necrotic activity could be correlated more exactly with other activities. Parallelism of the various properties by antitoxin assay has already been established by a number of workers [see, for example, Glenny *et al.* 1933; Nagler, 1939; 1941; R. G. Macfarlane *et al.*, 1941; and M. G. Macfarlane & Knight, 1941]. It is desirable, however, that work on these lines with θ-free toxin be extended.

Finally it must be emphasized that all the work reported in this paper was done with a single strain; obviously it is necessary that other strains should be studied. Crook [1941] has demonstrated parallelism of toxicity and reactivity with egg-yolk solution and normal human serum in 40 batches of filtrates from 14 type A strains.

SUMMARY

1. The toxins of *Cl. welchii*, type A, have been partially purified.
2. θ toxin has been separated from α toxin.
3. α toxin has been shown to be a 'hot-cold' haemolysin.
4. The combined haemolytic activities of α and θ toxins are not additive.

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