139. THE BIOCHEMISTRY OF THE GAS GANGRENE TOXINS

I. ESTIMATION OF THE α TOXIN OF *CL. WELCHII*, TYPE A

BY WILLIAM EDWARD VAN HEYNINGEN

From the Biochemical Laboratory, Cambridge

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UNTIL recently the lethal factor, α toxin, occurring in the toxic filtrates of *Cl. welchii*, type A, cultures has been estimated mainly by two methods, namely, the determination of toxicity by intravenous injections into mice, and the determination of haemolytic activity *in vitro*. In addition the necrotic activity has been determined by intradermal injections into guinea-pigs. These three methods have been described by Ipsen *et al.* [1939]. As means of specifically estimating α toxin these methods are open to various objections. (For a distinction of the known toxins of *Cl. welchii*, type A, see R. G. Macfarlane *et al.* [1941], Dalling & Stephenson [1941] and the next paper in this series.) Trevan [1927] has given a detailed account of the errors unavoidably associated with the determination of toxicity by the mouse intravenous method. Apart from its inaccuracy this method also suffers from the fact that large numbers of mice are required, which are expensive and difficult to obtain. The estimation takes 2 or 3 days, and, moreover, is not specific in that it is a measure of the combined lethal activity of α toxin and such other lethal toxins as might occur with it.

The *in vitro* haemolytic assay of the α toxin is more rapid and less expensive than the mouse intravenous method, but has, until recently, been obscured by at least one other haemolysin, the θ toxin, in the toxic filtrates.

In work on the purification and separation of the toxins of *Cl. welchii* filtrates it is necessary to have an accurate, specific and convenient method for the determination of α toxin. The basis for such a method has recently been found by Nagler [1939] who observed a specific reaction between α toxin, but not toxoid, and normal human serum. This reaction is shown by the development of an opalescence soon after the reactants have been mixed, and he has used it in the determination of α toxin and of α antitoxin, and also of α toxoid [Nagler, 1941]. His method consists in determining the least amount of toxin (in toxin, toxin-antitoxin, or toxin-toxoid-antitoxin mixtures) which will give an observable degree of opalescence with the serum after 16 hr. incubation. For toxin determinations a series of dilutions of the unknown toxin is compared with a series of dilutions of a standard toxin, and, similarly, appropriate controls are used in the toxoid and antitoxin estimations. R. G. Macfarlane *et al.* [1941] have since shown that a similar reaction takes place between α toxin and the crude lecitho-vitellin solution obtained by filtering a suspension of egg yolk in isotonic saline. This solution is more sensitive to the toxin than normal human serum, and the reaction requires the presence of free calcium ions. These workers also estimate α toxin by observing the least amount of toxin which will bring about an opalescence, but not a flocculation, with the egg yolk solution after incubation overnight. The present writer has confirmed that toxoid formed by treating *Cl. welchii* toxin with formalin does not give this reaction.

In the method described below a departure is made from the procedure of observing the lowest amount of toxin which will bring about a noticeable reaction; instead, the degree of turbidity developed in a given time is correlated with the amount of toxin.

Materials and apparatus

The egg-yolk solution is made by beating the yolk of a day-old egg in 500 ml. of isotonic (0.9 %) saline which is 0.005 *M* with respect to calcium acetate. After about 20 g. kaolin have been stirred in, the suspension is filtered by gravity through ordinary filter paper, and the filtrate sterilized by passing through a Seitz E.K. or Ford G.S. filter pad. The sterile solution, which is slightly opalescent, and which varies in colour from blue-yellow to fairly deep yellow, is then stored in the refrigerator in 25 ml. amounts in sterile cotton wool-plugged test-tubes.

The standard toxin is prepared by dialysing fresh toxic filtrate against pure glycerol for 48 hr. at 0°, as described by M. G. Macfarlane & Knight [1941]. This glycerinated toxin is sterile and retains its activity undiminished for at least 9 months.

When turbidities were compared with an absolute standard a neutral grey filter of density 1.5, made by the Ilford Research Laboratory, was used in conjunction with a Leitz colorimeter. This colorimeter is fitted with a green filter in the eyepiece, and its plungers are coupled and therefore move into the blank and unknown solutions to the same depth. The neutral grey filter is placed in the path of the beam of light which has passed through a blank consisting of egg-yolk solution which has been incubated with isotonic saline. The other light beam passes through the unknown turbidity.

When unknown turbidities are compared with the turbidity produced by the standard toxin an ordinary Bausch and Lomb colorimeter is used. The eyepiece is fitted with the blue filter provided by the makers as well as with a Wratten B tricolour green filter. While readings are being taken extraneous light must be prevented from falling on the walls of the colorimeter cups by using the light guard provided with the colorimeter. Any good colorimeter is suitable, but it is recommended that one fitted with a built-in and adjustable light source be used.

The toxin-egg-yolk solution reaction

When amounts of toxin corresponding to 1–6 M.L.D. are incubated for 15 min. at 38° with 2 ml. of the egg-yolk solution fairly dense turbidities are produced, which fall outside the range of a nephelometer, but which can be conveniently measured in an ordinary colorimeter. Thus if 6 ml. of a toxin solution containing 1 M.L.D./ml. are incubated with 2 ml. egg-yolk solution for 15 min. at 38°, and the resultant turbid suspension is diluted, the degree of turbidity, as compared with the neutral grey filter, density 1.5, is directly proportional to the dilution. This is shown in Fig. 1. The abscissa shows the degree of dilution of turbidity and the ordinate the reciprocal ($1/d$) of the depth in mm. of turbidity corresponding to density 1.5.

The degree of turbidity produced by a given amount of toxin varies with different eggs, and with the age of the egg solution. In Fig. 2 the values of $1/d$ for density 1.5 are plotted against the amount of toxin used. Curves I and II were obtained with the same egg-yolk solution, but in II the egg-yolk solution was 24 hr. older than in I. In curve III a different egg-yolk solution was used. In all three curves the same toxin (ammonium sulphate-precipitated) was used.

In order to exclude variations due to different eggs and to the age of the egg-yolk solutions, the turbidities produced by different amounts of toxin were compared with the turbidity produced by an arbitrary unit of the standard toxin. An arbitrary egg unit (E.U.) was chosen as a fixed amount of the standard

Fig. 1. Effect of dilution on turbidity.

Fig. 2. Turbidity produced with different egg-yolk solutions compared with an absolute standard.

toxin corresponding to approximately 2 M.L.D. The activity curve shown in Fig. 3 was prepared as follows. The standard toxin was diluted with isotonic saline to give a solution containing 3 E.U./ml. Amounts of this dilution from 0.15 to 1.0 ml. were pipetted into a series of test tubes and in each case the toxin solution was made up to 1 ml. with isotonic saline. To each tube 2 ml. of the egg-yolk solution were then added, and the toxin-egg-yolk solution mixtures were incubated at 38°. After 15 min. the tubes were taken from the bath, and t

each of them 5 ml. of an isotonic saline solution containing 2–3 antitoxin units/ml. were added. The antitoxin solution was added to stop the reaction and to increase the turbid suspension to a convenient volume for use in the colorimeter. The turbidities obtained with the various amounts of toxin were then compared in the colorimeter with the turbidity produced by 1 E.U. of the standard toxin. In Fig. 3 the ratios of the reading for the standard turbidity to the readings for the unknown turbidities (S/U) are plotted against the amounts of toxin. It will be seen that over a threefold range (0.7–2.1 E.U.) the curve is linear and the degree of turbidity is directly proportional to the amount of toxin. During the summer months of this year (1941) all the eggs used gave the same activity curve, which did not change until the egg-yolk solutions were 7–10 days old,

Fig. 3. Activity curve. Effect of toxin concentration on turbidity produced.

provided that the solutions were kept sterile and in the refrigerator. Lately (September, October) it has been noticed that, while all fresh egg-yolk solutions give the curve in Fig. 3, the shape of the curve varies rapidly with the age of the solution, sometimes after 4 or 5 days, often sooner. Occasionally an egg is still found which gives a constant curve for at least a week, and all egg-yolk solutions do not vary in the same way. It seems that while the blue-yellow egg-yolk solutions give less absolute turbidity, they are not more variable than the more yellow solutions. The variability in the recent eggs is either seasonal, or due to the war-time diet of the hens.

The 15 min. incubation period used in the construction of the activity curve was chosen because, as can be seen from Fig. 4, the reaction is still proceeding at constant velocity after this time. In Fig. 5 it will be seen that 2 ml. of the egg-yolk solution are sufficient to 'saturate' the toxin under the conditions employed.

If equal amounts of toxin and egg-yolk solution are incubated together in different dilutions, and the resultant turbid suspensions are then all diluted to

Fig. 4. Course of the reaction between 1 E.U. of toxin and 2 ml. of egg-yolk solution.

Fig. 5. Rate of the toxin-egg-yolk solution reaction as a function of 'substrate' concentration. Curve I with 1 E.U. of toxin. Curve II with 2 E.U. of toxin.

the same volume, it will be found that the turbidity produced decreases with the increasing dilution during incubation. This is shown in Fig. 6.

Fig. 6. Effect of dilution on development of turbidity by 1 E.U. of toxin with 2 ml. egg-yolk solution.

Estimation of α toxin

The method now employed for the estimation of α toxin is the following.

Three or more 1 ml. volumes of different dilutions of the toxin are incubated in test tubes with 2 ml. of the egg-yolk solution for 15 min. at 38°. Then 5 ml. of the isotonic saline solution containing 2–3 antitoxin units/ml. are added to each tube and the resultant turbid suspensions compared in the colorimeter with the turbidity produced by 1 ml. of a solution of the standard toxin containing 1 E.U. With a good colorimeter the readings can be easily matched with an error of not more than 2%. From the ratio S/U thus determined the corresponding number of E.U. for each amount of toxin can be read off from the activity curve. In this manner a triplicate determination of the activity of the unknown toxin in E.U./ml. can be obtained. If the activity of the toxin is entirely unknown it might, of course, be necessary to carry out a preliminary orientation with a series of twofold dilutions. If it is found that the three values for E.U./ml. do not agree, but decrease with decreasing amounts of toxin used, then the activity curve is no longer being obeyed. During the summer months the activity curve was obeyed for at least a week, and if a fresh egg-yolk solution was made up every week this difficulty was very rarely encountered.

In Table 1 some typical results obtained by this method are shown. The maximum difference in a set of triplicate readings is seldom > 8%, and generally < 5%. The last set of three values shown in this table is typical of those obtained with an egg-yolk solution which is no longer obeying the activity curve in Fig. 3. When such results were obtained during the summer months the egg-yolk solution was discarded, but with the recent eggs this has not been feasible, as the solutions often vary from day to day in the activity curve obtained. This difficulty can easily be surmounted by plotting a new activity curve on each day when it is desired to carry out estimations. The time required to obtain a daily 10-point activity curve does not exceed 45 min. Alternatively, when only a few estimations of activity are required, the activity curve need not be used, and the value of E.U./ml. can be determined by extrapolation. That is, 4 or 5 dilutions of the unknown toxin, some containing less and some more than 1 E.U. are incubated with the egg-yolk solution and the turbidities compared with the standard turbidity. The results are then plotted, and by extrapolation the amount of toxin which gives the same turbidity as 1 E.U. can be inferred.

Table 1. *Triplicate values obtained in determinations of a toxin*

Average E.U./ml.	Toxin used ml.	<i>S/U</i>	E.U.	E.U./ml.	Max. difference %
36.5	0.0500	1.79	1.82	36.4	3.3
	0.0375	1.35	1.35	36.0	
	0.0250	0.93	0.93	37.2	
38.3	0.0500	1.86	1.90	38.0	4.2
	0.0375	1.41	1.41	37.6	
	0.0250	0.98	0.98	39.2	
171	0.0067	1.20	1.20	179	8.2
	0.0050	0.84	0.84	168	
	0.0033	0.50	0.55	165	
192	0.0067	1.32	1.32	193	1.5
	0.0050	0.95	0.95	190	
	0.0033	0.64	0.64	192	
203	0.00500	1.00	1.00	200	3.0
	0.00375	0.78	0.78	206	
	0.00250	0.45	0.55	204	
235	0.0100	2.28	2.40	240	4.3
	0.0075	1.70	1.72	230	
	0.0050	1.18	1.18	236	
239	0.0067	1.57	1.57	234	4.2
	0.0050	1.22	1.22	244	
	0.0033	0.80	0.80	240	
1013	0.0033	3.00	3.40	1020	6.0
	0.0025	2.44	2.60	1040	
	0.0017	1.64	1.64	980	
—	0.0500	2.72	3.00	60	—
	0.0375	2.03	2.10	56	
	0.0250	1.19	1.19	48	

Determination of combining power (test dose)

In preliminary work on the determination of the combining power of toxins by means of the turbidimetric method described above it was found that when low concentrations of toxin and antitoxin were used the results were anomalous. Fig. 7 shows the results obtained when a series of 4 ml. volumes, each containing 3 E.U. toxin/ml. and 0.04–0.08 antitoxin unit/ml., were left to stand together at room temperature for 1 hr. After this period 1 ml. aliquots from each 4 ml. volume were incubated with 2 ml. egg-yolk solution, and the residual activity was determined by the method described above. The values thus obtained were subtracted from 3 to give the number of E.U. of toxin neutralized by the various amounts of antitoxin. The three curves shown are the results of three apparently identical experiments carried out on the same day. While the amount of toxin neutralized by a given amount of antitoxin varies considerably, it will be seen that in each case a given increase in the amount of antitoxin is equivalent to the same increase in the amount of toxin neutralized. Thus the increase of 0.04 antitoxin units, from 0.04 to 0.08, corresponds to an increase of 1.07, 1.13 and 1.12 E.U. of toxin neutralized in curves A, B and C, respectively, the average being 1.11 E.U. From the value 1.11 E.U./0.04 antitoxin unit it can be calculated that 0.2 unit of antitoxin neutralize 5.55 E.U. of toxin. The $L+5$ value, or test dose, of a toxin, as determined by the mouse intravenous method, is defined as that amount of toxin which, in the presence of 0.2 unit of antitoxin will kill some, but not all, of the mice injected. Since 1 E.U. is approximately 2 M.L.D., the test dose of the toxin can be calculated to be approximately

6.05 E.U. (5.55 E.U. + 1 M.L.D., or 0.5 E.U.) or, since the toxin used contained 200 E.U./ml., 0.030 ml. The $L+1/5$ value for the toxin, as determined by the mouse intravenous method, was 0.024 ml.

Fig. 7. Neutralization of toxin by antitoxin in low dilution.

It was found that when the concentrations of toxin and antitoxin in the mixtures were increased the results obtained were more reproducible, and, under the conditions employed, the toxin-antitoxin combination seemed to take place in simple stoichiometric proportions. Triplicate values for test dose of a toxin could be carried out in a little over an hour by setting up a series of tubes, each containing in a 1 ml. volume 0.2 antitoxin unit and increasing amounts of the toxin. After 30 min. at room temperature 2 ml. of the egg-yolk solution were added to each of the toxin-antitoxin mixtures and the residual activity of the toxin was determined in the usual way. A typical experiment is shown in Table 2.

Table 2. *Determination of combining power*

ml. toxin dilution containing 10 E.U./ml.	0.4	0.5	0.6	0.7
ml. antitoxin dilution containing 1 unit/ml.	0.2	0.2	0.2	0.2
ml. isotonic saline	0.4	0.3	0.2	0.1
E.U. toxin added	4.0	5.0	6.0	7.0
E.U. found remaining	0	0.6	1.5	2.6
E.U. neutralized by 0.2 antitoxin unit	—	4.4	4.5	4.4

Average 4.43

Test toxin contains 44 E.U./ml.
Test dose 4.43 E.U. or 0.10 ml.

The test dose is here defined, not as that amount of toxin which, in the presence of 0.2 antitoxin unit, contains a limited amount of free toxin, but as that amount of toxin which is just completely neutralized by 0.2 antitoxin unit. The antitoxin used in these experiments was the Standard Gas Gangrene (Perfringens) Antitoxin issued by the Department of Biological Standards of the National Institute of Medical Research at Hampstead.

Determinations of combining power carried out by the method outlined above have so far proved to be entirely satisfactory, and no anomalous results have been obtained. It must be emphasized, however, that so far only about 10 determinations have been carried out, and this number is not large enough to establish the efficacy of the method.

Moreover, in the toxins so far studied, the toxin-toxoid ratio must either have been high, or the affinities of the toxin and toxoid for antitoxin must have been the same; otherwise the relationship of toxin neutralized to amount of antitoxin used could not have been a simple stoichiometric one. In such cases the combining power cannot be calculated directly by subtracting the values for residual E.U. from E.U. added; but if the values for residual E.U. are plotted against the E.U. added it will presumably be possible to determine by extrapolation the amount of toxin just neutralized by 0.2 antitoxin units.

The antitoxin concentration in these experiments was 0.2 unit/ml. and in the experiments illustrated in Fig. 7 it varied from 0.04 to 0.08 unit/ml.; it is possible that the anomalous results of the latter experiments were due to dissociation of the toxin-antitoxin complex at the lower concentrations.

Estimation of antitoxin

Conditions have not yet been found for reliable and reproducible estimation of antitoxin, but the results so far obtained are worthy of discussion. The fairly typical results shown in Fig. 8 were obtained with a test toxin containing 938 E.U./ml. and having a test dose of 3.75 E.U., and with an α -antitoxin, kindly provided by Mr Glenny of the Wellcome Physiological Research Laboratory, containing 100 anti- α -units/ml. as estimated in his laboratory by an *in vitro* haemolytic method.

A series of 1 ml. volumes, each containing 1 test dose of toxin and increasing amounts of antitoxin, were left at room temperature for 30 min.; 2 ml. of the egg-yolk solution were then added to each tube and the residual activities of the toxin-antitoxin mixtures determined in the usual way. From these figures the percentage test dose neutralized by each amount of antitoxin was calculated. The results obtained from three apparently identical experiments are plotted in Fig. 8. If the value of 88% test dose neutralized by 0.001667 ml. antitoxin, which is common to all three curves, is taken, the value of the antitoxin can be calculated as follows:

$$88\% \text{ test dose} = 0.001667 \text{ ml. antitoxin,}$$

$$1 \text{ test dose} = 0.001667 \times \frac{100}{88} = 0.0019 \text{ ml. antitoxin,}$$

$$= 0.2 \text{ antitoxin units,}$$

$$\text{i.e. antitoxin contains 105 units/ml.}$$

By a similar calculation the other two figures common to curves II and III (36 and 38% test dose neutralized by 0.000667 ml. antitoxin) give a value of 109 units/ml. The broken straight line in Fig. 8 represents a simple stoichiometric reaction between antitoxin (105 units/ml.), and toxin (test dose 3.75 E.U.) in which the toxin-toxoid ratio is high, or the affinities of toxin and toxoid for antitoxin equal. The value 105–109 antitoxin units/ml. is in excellent agreement with the value of 100 units/ml. given by Mr Glenny, especially in view of the fact that these values were found by different workers using entirely different methods with different test toxins.

It is difficult to see why the results obtained in the estimation of antitoxin should be so anomalous when the results of the estimations of combining power

were regular and consistent. It is, of course, possible that the regular results of the combining power estimations were fortuitous. Another possible explanation might lie in the fact that anomalous results have, in the author's experience, always been found when the concentration of antitoxin was appreciably below 0.2 unit/ml. This is shown in Figs. 7 and 8. In Fig. 8 it will be seen that the values of toxin neutralized are inconsistent when the antitoxin concentration falls below 0.001667 ml. antitoxin/ml., or approximately 0.175 unit/ml. In the

Fig. 8. Neutralization of 1 test dose by increasing amounts of antitoxin.

estimations of combining power the antitoxin concentration was kept at 0.2 units/ml., and in the estimation of test dose and antitoxin by the mouse intravenous method, as recommended by the Department of Biological Standards, the antitoxin concentration is of the order of 0.4 unit/ml. It is interesting to note that in Fig. 7, comparing curves B and C with curve A, the tendency is for a given amount of antitoxin to neutralize less toxin, whereas in Fig. 8, the antitoxin appears to show a tendency to neutralize more toxin than the amount represented by the 'ideal' curve.

DISCUSSION

The work so far done on the estimation of combining power and of antitoxin must be regarded as preliminary, and is being extended. It is possible that when concentrations of antitoxin of the same order as those used in the mouse intravenous method are employed more consistent results will be obtained.

The method described above for the estimation of toxin has proved to be of great convenience and satisfactory accuracy in the author's experience extending over about 9 months. It has however, been applied to toxins from one strain only, namely, S 107, and its efficacy can only be established when it has been successfully used by a number of different workers using toxins produced by different strains. It has the advantage over the methods described by Nagler [1939] and by R. G. Macfarlane *et al.* [1941] that the activity of several samples can be determined within an hour, with very satisfactory accuracy, and without

resorting to a large number of serial dilutions. It is particularly useful in studying the toxin-antitoxin reaction since only one tube need be used in the determination of the residual activity in a given toxin-antitoxin mixture. Apart from the saving of time, the short period of incubation employed also enjoys the advantages pointed out by Haldane [1930] associated with the measurement of reaction velocity while the velocity is still constant. The fundamental principles of the methods described in this paper and in that of Macfarlane *et al.* are the same, since in both cases the amount of toxin which produces a given degree of turbidity is determined. In the one case this degree of turbidity is defined in terms of a standard toxin, and in the other it is that amount produced under given conditions which is observable but which does not flocculate: therefore difficulties associated with turbidity determination, such as changes in particle shape or size, are common to both methods. In the author's experience such difficulties have apparently not been encountered.

SUMMARY

1. A turbidimetric method for the estimation of α toxin is described which is based on the reaction between egg-yolk solution and toxic filtrates of *Cl. welchii*, type A.
2. The application of this method to the estimation of combining power and of antitoxin is discussed.

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