CLOSTRIDIUM BOTULINUM TYPES A AND B TOXIN-ANTITOXIN REACTIONS

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(With 1 Figure in the Text)

INTRODUCTION

Clostridium botulinum antitoxin was first prepared by Kempner (1897) in goats. He used two strains of organism, one of which came from the original 'Ellezelles' outbreak described by van Ermengen. Kempner stated that *C. botulinum* toxin and antitoxin combined according to the law of multiple proportions. Leuchs (1910) prepared antitoxin in horses against two antigenically different strains, and Burke (1919) confirmed the existence of two separate toxigenic types and named them types A and B.

Bengtson (1924) produced a standard type A antitoxin and determined the amount of antitoxin which, if mixed with 100 M.L.D. of toxin, would delay the death of a 250 g. guinea-pig for 96 hr. This amount of antitoxin was regarded as containing 0·1 unit.

Glotowa & Dankerowitz (1935) titrated *C. botulinum* types A and B toxin and antitoxin by injecting the mixtures intravenously, intraperitoneally and subcutaneously in mice. They decided that the intravenous route gave the best results.

Recent studies of purified preparations of *C. botulinum* types A and B toxins by Lamanna & Glassman (1947) showed that these two toxins were markedly different, the type A toxin behaved like a globulin, whereas type B behaved like a more simple protein with an estimated molecular weight of 60,000. The molecular weight of the type A toxin has been estimated as 900,000 (Putnam, Lamanna & Sharp, 1946), and 1,200,000 (Kegeles, 1946).

Wagman & Bateman (1953) reported that in certain circumstances type A toxin dissociates on dilution to form a compound of much smaller molecular weight which however retains its toxic properties.

A number of factors have been shown to influence the combination of toxin and antitoxin in other systems which have been more thoroughly investigated. The speed and firmness of union of toxin and antitoxin were seen to vary according to some quality of the serum, and this quality was first termed avidity by Kraus (1903). Kraus & Baecher (1913) suggested that avidity affected the rate of neutralization and was not fully measured by the official method of standardization.

With certain diphtheria toxin-antitoxin mixtures, the degree of dilution of the components affected the neutral point (Glenny, Pope & Waddington, 1925). Madsen & Schmidt (1926, 1930) demonstrated that more diphtheria toxin could be neutralized by a given quantity of certain antitoxins when more time was allowed

for combination to take place. They also showed that the toxicity of such mixtures was affected by the degree of dilution *in vivo*, so that mixtures which were harmless to a guinea-pig could be lethal to a rabbit. Glenny & Barr (1932*a*, *b*) showed that a strong mixture of diphtheria toxin with a certain non-avid antitoxin, was harmless to a rabbit in large doses given intravenously, but fatal when smaller doses were given, presumably owing to the greater dilution of small doses in the circulation. Using the guinea-pig intracutaneous test they defined a 'dilution ratio' for antitoxins as 'the amount of antitoxin necessary to neutralize the Lr dose of toxin in a total volume of 2 ml. divided by the amount necessary to neutralise the same dose in a total volume of 200 ml.'. They demonstrated by testing ammonium sulphate-precipitated fractions of diphtheria antitoxin, that fractions of different dilution ratio existed in the same serum and that there was a correlation between a high dilution ratio and good protective effect in rabbits.

Glenny, Pope, Waddington & Wallace (1925) defined the 'serum ratio' of an antitoxin as the $\frac{\textit{in vivo}\text{ value}}{\textit{in vitro}\text{ value}}$ of the serum, and showed that the ratio was fairly constant in several bleedings from the same horse, but that it tended to fall slowly as immunization proceeded. Ramon (1930) considered that the speed of flocculation was a measure of the avidity of the antitoxin under test, but Madsen & Schmidt (1930) found that slowly flocculating sera were not always non-avid by the rabbit intravenous method. Glenny & Llewellyn Jones (1931) thought the serum ratio was better correlated with avidity than was the time of flocculation. Barr (1949) showed a correlation between the dilution ratio and the serum ratio of antitoxin except where the serum ratio was greater than 2·0. She found that the greater the toxicity of the test toxin, i.e. the greater the number of M.R.D. per Lr, the lower the dilution ratio of the antitoxin under test appeared to be. The dilution ratios of most sera, however, referred to that of a chosen standard remained constant whatever toxin was used.

Barr, Glenny & Stevens (1954) have pointed out that the proportion of toxoid in a toxic filtrate tends to increase with age, and they showed that diphtheria and tetanus antitoxins have a greater affinity for toxin than for toxoid, and moreover that combination of antitoxin and toxin or toxoid becomes firmer with passage of time; these differences were more pronounced when non-avid antitoxin was used.

Barr (1951) showed that diphtheria antitoxin produced in the early stages of immunization of horses with no natural immunity was of poor avidity, and that the avidity improved after further spaced injections of antigen had been given. A rest after the first two doses of toxoid in the routine production of tetanus antitoxin in horses improves the average value (Glenny, Pope, Waddington & Wallace, 1925). The length of the rest period affects both the quality and quantity of antitoxin produced (Barr & Glenny, 1945).

Jerne (1951) pointed out the difficulty of assigning an antitoxic value to non-avid sera which did not combine proportionately with toxin at different levels of test and for which the curve of neutralization showed a different slope from that of the standard antitoxin in use.

Bowmer (personal communication) has shown that far more antitoxin is required to neutralize fractions of the test dose of *C. botulinum* type D toxin than would be expected.

The work reported here was undertaken to examine the behaviour of *C. botulinum* types A and B toxin-antitoxin reactions in the light of investigations previously carried out on other systems.

MATERIALS AND METHODS

All toxin-antitoxin titrations were made by intraperitoneal injection of albino mice weighing 20–25 g. The mice were observed daily, and the end-point of a titration was assessed on the basis of death or survival on the fourth day. When death occurred at one level on the 1st, 2nd or 3rd day and the next mouse in the series survived, the end-point was arrived at by interpolation. Each test consisted of a series of toxin-antitoxin mixtures in which the volume of toxin used, the test dose, was kept constant, and the volumes of diluted antitoxin differed from each other by 20 %. A series of four or five test mixtures were prepared and injected into single mice. This procedure was repeated on subsequent days until enough tests had been performed, the volumes of diluted antitoxin in each test series differing from those of the previous one by 10 %. For example, if the volumes of serum added in the first series were 0·33, 0·4, 0·5 and 0·60 ml. then the volumes of serum added in the next series might be 0·36, 0·44 0·55 and 0·66 ml., the decision to start the series above or below the previous one depending on the result of the previous test. In this way the 20 % differences between antitoxin values in mixtures were bridged in alternate tests. The arithmetic mean of the tests on one serum was taken as the antitoxic value of that serum.

The toxins were preserved in 66 %, v/v, neutral glycerine and diluted daily as required in horse-muscle digest broth at pH 7·0. The antitoxins were diluted in weak (M/200) phosphate buffer at pH 6·5. All antitoxins, except where otherwise stated, were prepared by hyperimmunization of horses by routine methods.

The test dose of toxin was pipetted into small bottles and the calculated amount of antitoxin dilution added. The bottles were capped and mixed by inversion. The mixtures were allowed to stand for 1–1½ hr. after which time the whole volume of each mixture was removed and injected into the test animal.

Provisional standard sera of *C. botulinum* types A and B and a glycerinated toxin of each type were supplied by the Microbiological Research Department of the Ministry of Supply.

DEFINITIONS

Combining power of an antitoxic serum is the toxin neutralizing power of the serum. It may be expressed in antitoxin units/ml. when the serum is tested against a fixed dose of toxin in parallel with a standard antitoxin under constant conditions. The term may be used, however, to express the different neutralizing capacity of a serum under different conditions against the same test dose of toxin, in which case the difference cannot be expressed in antitoxin units because these have a constant value in relation to the standard, regardless of the conditions of the test. The term

combining power may also be applied to the capacity of toxin or toxoid to fix antitoxin.

Level of test is an expression used to indicate the concentration of toxin and antitoxin in a series of test mixtures and the relationship of this concentration to the antitoxin standard. A quantity of toxin which when mixed with 0·1 unit of standard antitoxin will just kill the test animal on the 4th day after injection contains by definition one L+/10 dose of toxin. The L+/10 level of test was used in this work in the comparison of potency of different samples of antitoxin. If toxin-antitoxin mixtures are made up in which the same volume of toxin is used previously diluted 1/10 or 1/100 then the tests are being carried out at the 0·1 L+/10 or the 0·01 L+/10 level of test, similarly, if the toxin is 10 or 100 times more concentrated the corresponding levels of test will be 10 L+/10 or 100 L+/10.

Serum ratio is the ratio

$$\frac{\textit{in vivo} \text{ value of the serum (antitoxin units/ml.)}}{\textit{in vitro} \text{ value of the serum (antitoxin units/ml.)}} \text{ at an accepted level of test.}$$

Dilution ratio is the ratio of the volumes of antitoxin required for neutralization at the 100 L+/10 and at the 0·01 L+/10 levels of test, the figures being corrected for the 1/10,000 dilution of the serum between these two levels of test.

In order to estimate the dilution ratio of *C. botulinum* sera according to the original definition for diphtheria antitoxin (Glenny & Barr, 1932*a*), it would be necessary to estimate the volume of antitoxin needed to neutralize the 100 L+/10 dose of toxin in a total volume of 1·0 ml. and that needed to neutralize the same dose of toxin in a total volume of 10 l. It was more convenient in practice to dilute both toxin and antitoxin an appropriate amount previously and inject the mixtures of these dilutions. Thus in fact the tests with dilute toxin were done at the 0·01 L+/10 level of test, and the volumes of mixtures injected at both levels of test were the same. In some cases dilution ratios were calculated from tests carried out at the 10 L+/10 and 0·01 L+/10 levels of tests (Tables 7 and 8).

Index of efficiency of sera is defined as the quantity of serum that should be required to neutralize fractions or multiples of the L+/10 dose of toxin (if toxin and antitoxin combined proportionately at all levels of test), divided by the quantity of serum actually found necessary by experiment.

RESULTS

The combining power of antitoxin at different levels of test

The provisional standard *C. botulinum* type A antitoxin, with an accepted value of 270 units/ml., was use to estimate the test dose of a type A toxin (ABA 31) equivalent to 0·1 unit. This volume of test toxin, i.e. the L+/10 dose was then used to standardize a number of sera. Attempts were made to estimate the test dose of toxin equivalent to 0·01 and 0·001 unit of the provisional standard. The results obtained were so different from what would be expected from the law of multiple proportions that it was decided to test a number of sera against fractions and multiples of the test dose of toxin to see if a serum could be found with a more normal behaviour. By using the same volume of different dilutions of the test

toxin, the sera were therefore tested against the 100 L+/10, 10 L+/10, 0·1 L+/10 and 0·01 L+/10 doses of toxin. Neutralization did not follow the law of multiple proportions: as the dilution of toxin increased, considerably more antitoxin was required to neutralize it than would have been expected from that law. The dilution ratios of the sera differed considerably (Table 1, fig. 1).

Two *C. botulinum* type B antitoxins were tested at different levels of test, and the dilution ratios calculated from the results obtained at the 10 L+ and 0·001 L+ levels of test were 0·087 and 0·110 respectively. The best type A antitoxin tested over an equivalent range had a dilution ratio of 0·063 and the majority of type A antitoxins tested had dilution ratios between 0·025 and 0·05. There was a progressive decline in the index of efficiency of the type A antitoxins at all levels of test

Table 1. *The index of efficiency of* C. botulinum *type A sera titrated against multiples and fractions of the L+/10 dose of toxin contained in a fixed volume*

Test dose of toxin	Provisional standard	Index of efficiency of sera			
		Ex 1551	8469 B	8480	P 183 A
100 L+/10	2·50	4·13	2·10	2·07	2·00
10 L+/10	2·39	2·69	1·80	1·83	1·54
L+/10	1·00	1·00	1·00	1·00	1·00
0·1 L+/10	0·29	0·22	0·27	0·29	0·35
0·01 L+/10	0·064	0·060	0·080	0·083	0·096
Antitoxin value units/ml. L+/10 level of test	280	65	5000	4100	6500
Dilution ratios:					
Arithmetic means	0·0256	0·0145	0·0381	0·0401	0·0480
Geometric means	0·0243	0·0141	0·0383	0·0397	0·0475

The index of efficiency of a serum is defined as that quantity of serum that should be required to neutralize a fraction or multiple of the L+/10 dose of toxin (if toxin and antitoxin combined proportionately at all levels of test), divided by the quantity of serum actually found necessary by experiment.

Analysis of variance on the logarithms of observed dilution ratios shows that highly significant differences occur between sera. The common-error variance corresponds to a standard deviation of about ± 15 % for a *single estimate*.

from 100 L+/10 to 0·01 L+/10; with the two type B antitoxins, however, the index of efficiency at the 10 L+ and the L+ levels of test was the same and the decline in the index of efficiency commenced at the 0·1 L+ level and was progressive on further dilution to the 0·001 L+ level.

Time of combination and the combining power of mixtures of dilutions of toxin and antitoxin

A number of *C. botulinum* type A sera, which had been shown in previous tests to have dilution ratios between 0·05 and 0·014, were retested, but the mixtures were left to combine for 24 hr. instead of 1–1½ hr. before injection into mice. The quantity of serum required to neutralize the 100 L+/10 dose of toxin was unchanged within the limits of error by this manoeuvre, but the quantities of serum

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required to neutralize the L+/10 and 0·01 L+/10 doses of toxin were found to be much less than those required by the normal method of test (Table 2). The bulk dilutions of toxin required to make the mixtures in the above tests were kept for 24 hr. and retested the following day by the routine method to ensure that there had been no change in combining power as a result of standing overnight in dilute solution.

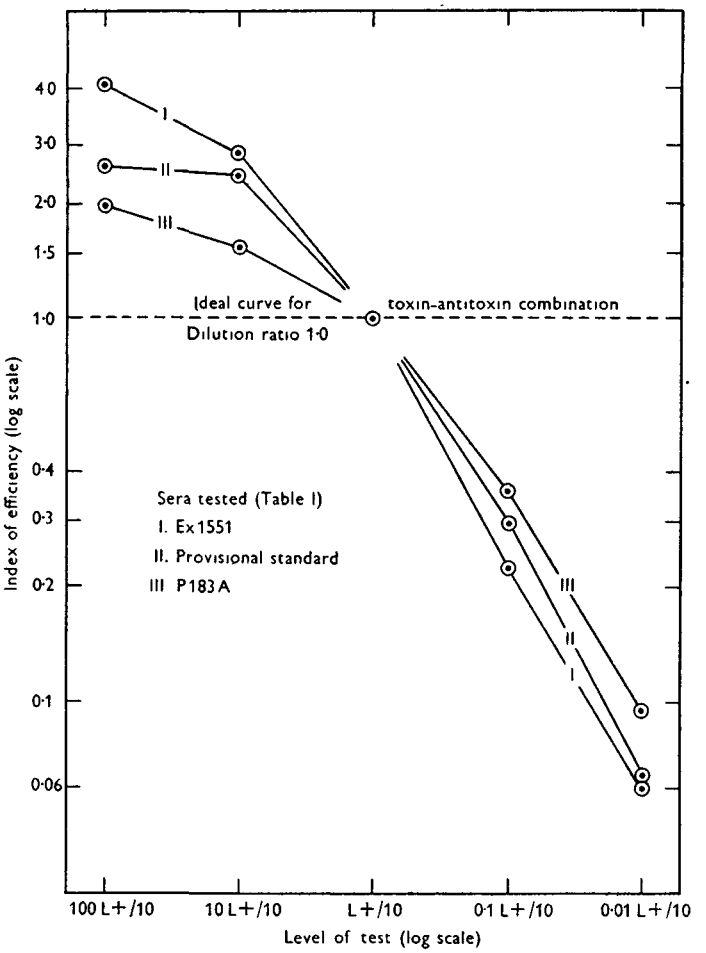

Fig. 1. The index of efficiency of three *C. botulinum* type A antitoxic sera estimated at different levels of test showing the rate of reduction of the combining power of the sera from high to low levels of test.

Comparison between serial dilutions of strong mixtures and mixtures of serial dilutions of toxin and antitoxin

Eight mixtures containing the 100 L+/10 dose of type A toxin and a suitable range of volumes of the provisional standard serum were made up to a constant volume and allowed to stand. After 1½ hr. serial 1/10 dilutions of each mixture were made so that a series of tests were prepared at the equivalent of the 10 L+/10, L+/10, 0·1 L+/10 and 0·01 L+/10 levels of test, and sufficient material was made to allow injection of the diluted mixtures 1½ and 24 hr. after diluting.

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It was observed (Tables 2 and 3) that when mixtures of dilutions were made the index of efficiency of the sera steadily declined to a very low figure at the 0·01 L+/10 level of test; with dilutions of strong mixtures the index of efficiency of the serum declined much less, and at the 0·01 L+/10 level of test there was in fact an increase in the index of efficiency. This increase was associated with a broadening of the

Table 2. *The index of efficiency of* C. botulinum *type A sera titrated against fractions and multiples of the L+/10 dose of toxin by injection of the test mixtures after standing (A) 1½ hr. and (B) 24 hr. at room temperature.*

	Index of efficiency of sera		
Test dose of toxin	Provisional standard	Ex 1551	8480
A 100 L+/10	2·50	4·13	2·07
L+/10	1·00	1·00	1·00
0·01 L+/10	0·064	0·060	0·083
Dilution ratios	0·0256	0·0145	0·0401
B 100 L+/10	2·50	4·28	1·95
L+/10	1·46	1·59	1·49
0·01 L+/10	0·18	0·10	0·22
Dilution ratios	0·0720	0·0233	0·122
Index of improvement of dilution ratios	2·81	1·61	3·04

Table 3. *The index of efficiency of the* C. botulinum *type A provisional standard serum titrated against fractions and multiples of the L+/10 dose of toxin by mixing dilutions and by diluting strong mixtures of the components, and injecting after different times*

	Index of efficiency by various methods			
	*Mixture of dilutions of toxin and antitoxin. Mixtures allowed to combine for		Dilution of strong mixtures of toxin and antitoxin. Mixtures diluted and allowed to combine for	
Test dose of toxin	1½ hr.	24 hr.	1½ hr.	24 hr.
100 L+/10	2·50	2·50	2·22	2·14
10 L+/10	2·39	—	1·96	1·96
L+/10	1·00	1·46	1·43	1·64
0·1 L+/10	0·29	—	1·12	1·07
0·01 L+/10	0·064	0·18	1·96	1·79
Dilution ratios	0·0256	0·0720	0·883	0·836

* Extracted from Table 2.

end-point of the titration which suggested that the number of available lethal doses was no longer adequate for accurate testing (Table 3). When the dilutions of strong mixtures were kept 24 hr. before injection there was no appreciable change in the index of efficiency of the sera at any level of test. This suggests, that once combined in strong solution, botulinum type A toxin-antitoxin complexes do not tend to dissociate to any great extent on dilution, and no appreciable progressive dissociation occurs with time.

The change in dilution ratio during immunization of horses

Two normal horses were each given three small doses of type A toxoid, and after 3 months' rest were started on a routine course of hyperimmunization with type A toxoid. Samples of blood were taken at intervals during immunization. The dilution ratios of the sera obtained from these test bleedings were measured in the usual way. The ratios were found to improve steadily during immunization, but the greatest improvement occurred during the 3 months' rest period, i.e. between 91 and 170 days: thereafter the ratios tended to become constant (Table 4).

Table 4. *The influence of the stage of immunization on the dilution ratios of* C. botulinum *type A sera. The index of efficiency of sera titrated at different levels of test from two horses during immunization*

	Index of efficiency of sera							
	H 8916 Days from beginning of immunization				H 8917 Days from beginning of immunization			
Test dose of toxin	91	170	185	198	91	170	185	198
100 L+/10	3·33	2·21	1·92	1·88	3·17	1·79	1·64	1·76
L+/10	1·00	1·00	1·00	1·00	1·00	1·00	1·00	1·00
0·01 L+/10	0·055	0·058	0·070	0·075	0·060	0·072	0·073	0·082
Dilution ratios	0·0166	0·0262	0·0365	0·0398	0·0189	0·0402	0·0445	0·0466
Antitoxin value u./ml. L+/10 level of test	165	95	3000	4250	30	42	1100	1900

Table 5. *Dilution ratios of seven fractions of* C. botulinum *type A serum* (8480) *obtained by ammonium sulphate precipitation*

Fraction	Precipitation limits ammonium sulphate (g./l.)	Index of efficiency at levels of test			Dilution ratios
		100 L+/10	L+/10	0·01 L+/10	
1	0–140	1·95	1·00	0·090	0·0462
2	140–160	1·73	1·00	0·063	0·0364
3	160–180	2·09	1·00	0·069	0·0330
4	180–200	2·04	1·00	0·080	0·0392
5	200–220	2·00	1·00	0·072	0·0360
6	220–250	2·14	1·00	0·086	0·0402
7	250–300	1·77	1·00	0·094	0·0531

The dilution ratios of different fractions of horse antitoxin

A quantity of *C. botulinum* type A serum from one horse was fractionated with ammonium sulphate into seven separate successive fractions commencing with material obtained by precipitation with 140 g./l. of the salt. The precipitates produced at different salt concentration were dialysed against running tap water and rendered isotonic by the addition of sodium chloride and the antitoxin content estimated at the 100 L+/10, L+/10 and 0·01 L+/10 levels of test. The dilution ratios of the fractions varied somewhat, but the first and last fractions had the best ratios which were significantly higher than the ratios of the majority of the other fractions (Table 5).

The dilution ratios of guinea-pig sera after two doses toxoid

Serum was obtained from three groups of 15 guinea-pigs bled 10 days after the second of two doses of type A toxoid given at 28 days interval. The three groups of animals had received unprecipitated formol toxoid or the same toxoid adsorbed on aluminium phosphate or precipitated by potash alum. The sera from each group were pooled and tested at three levels of test (100 L+/10, L+/10 and 0·01 L+/10). The dilution ratios calculated from these results were much lower than those of any hyperimmune horse sera tested (Table 6).

Table 6. *The index of efficiency at different levels of test and the dilution ratios of three pools of guinea-pig sera from animals bled after two doses of* C. botulinum *type A toxoid*

	Index of efficiency of sera at test levels			
Serum pool	100 L+/10	L+/10	0·01 L+/10	Dilution ratios
DX 4450	3·75	1·00	0·038	0·0102
DX 4452	4·91	1·00	0·044	0·0090
DX 4453	5·33	1·00	0·060	0·0113

Table 7. *The dilution ratios of a group of sera titrated with two different preparations of* C. botulinum *type A toxin*

	Dilution ratio of				
Test toxins	LD 50 per L+/10	Provisional standard	Ex 1551	8469	8480
PTA	1000	0·063	0·057	0·129	0·137
ABA 31	3000	0·027	0·022	0·044	0·046
*D.R. against PTA / D.R. against ABA 31		2·33	2·59	2·93	2·98

* D.R. = Dilution ratio.

In this table the dilution ratios were calculated from titrations carried out at the 10 L+/10 and 0·01 L+/10 levels of tests, since the toxin PTA was not sufficiently potent to work at the 100 L+/10 level.

The influence of different preparations of type A toxin on dilution ratios

The *C. botulinum* type A sera previously tested at various levels with the toxin ABA 31 were retested using another preparation (PTA) of type A toxin. This preparation could however only be used at a maximum level of test of 10 L+/10 because the potency was not great enough to permit testing at the 100 L+/10 level of test. The dilution ratios obtained were compared with those obtained with the original test toxin ABA 31 over an equivalent range and all were found to be much higher (Table 7).

The type A toxin ABA 31 contained 3000 LD 50 per L+/10 dose, whereas the toxin PTA contained only 1000 LD 50 per L+/10 dose. Since it appeared likely that this was due to natural toxoiding of the test toxin PTA, a mixture of a type A toxoid was made with the more toxic toxin ABA 31 so that the L+/10 dose of the mixture contained only 1000 LD 50. This mixture was then used to titrate one of these antitoxic sera at various levels of test, and the results compared with those

obtained with the two other toxin preparations. The neutralizing power of the serum at the lower levels of test was of the same order with the more toxoided toxin PTA and with the toxin-toxoid mixture and appeared to be much greater than with the toxic toxin ABA 31. The dilution ratios of the serum were therefore much higher when a test toxin of high toxoid content was used (Table 8).

Table 8. *The index of efficiency of* C. botulinum *type A serum Ex* 1551 *at different levels of test when titrated against three different preparations of type A toxin, one preparation consisting of a mixture of toxin and toxoid*

	Index of efficiency against test toxins		
Test dose of toxin	ABA 31	PTA	Toxin-toxoid mixture
10 L+/10	2·69	2·01	2·23
L+/10	1·00	1·00	1·00
0·1 L+/10	0·22	0·25	0·28
0·01 L+/10	0·060	0·12	0·12
Dilution ratios	0·0223	0·0597	0·0538
LD 50 per L+/10 dose of toxin	3000	1000	1000

In this table the dilution ratios were calculated from titrations carried out at the 10 L+/10 and 0·01 L+/10 levels of test, since the toxin PTA was not sufficiently potent to work at the 100 L+/10 level.

Analysis of results of potency tests on C. botulinum *type A antitoxins*

In each test of those analysed, six sera were compared with the provisional standard preparation at the L+/10 level of test using four different test toxins. Five tests were carried out on each serum, using each of the four toxins. The accuracy of the results obtained were submitted to statistical analysis and I am indebted to Mr P. A. Young for the following statement:

Differences in absolute results obtained with the various toxins were confounded with the day to day error between tests, and in the analysis of variance the mean square 'between toxins' must be tested for significance against the mean square 'between tests within toxins'. This shows that the variance between results on the different toxins does not significantly exceed the variance between tests using any one toxin.

As would be expected, highly significant differences are demonstrated in the titres found for the different sera, but these titres are not dependent for their values on the toxin used. The latter point, which is probably the most important feature of the analysis, is demonstrated by the very low mean square for the serum-toxin interaction, no matter whether the estimate of error used is that between tests or that within tests.

Since the serum-toxin interaction is neglible, the error variance for a *single estimate* of potency may fairly be taken as the residual mean square from the analysis. This is 0·002818 in log units, giving a standard deviation of ±0·0531 or approximately ±12·5%.

The tables give the results of analysis of variance applied to the data (log values being used), and the geometric mean titres obtained. (Tables 9 and 10.)

Flocculation tests with C. botulinum *type A toxoid*

The antitoxin combining power of a type A toxoid was estimated *in vivo* by a total combining power test. A suitable range of volumes of the toxoid was allowed to combine with 0·2 unit of the provisional standard type A antitoxin. These

mixtures were left for 1 hr. to combine, at the end of which time the L+/10 dose of toxin was added and the mixtures allowed to stand for 1½ hr. before injection into mice. In this way the end-point was obtained when the mixture contained 0·00135 ml. of toxoid. It was assumed that this volume had combined with 0·1 unit of the standard type A antitoxin and that 1·0 ml. of toxoid contained 74 combining equivalents.

A group of type A sera with different antitoxin contents, estimated at the L+/10 level of test, were suitably diluted and a range of volumes differing by 10% were mixed with 0·25 ml. of toxoid, and the optimum flocculation point was found. In this way the number of units of antitoxin (estimated at the L+/10 level of test) combining with 1·0 ml. of toxoid was calculated for each serum. It was found that the combining power of the toxoid by the flocculation method varied with the

Table 9. *Antitoxin titres (units/ml.) of six* C. botulinum *type A sera tested at the L+/10 level of test against four type A toxins*

	Geometric mean titres (u./ml.) against test toxins			
Sera	ABA 31	BAX 38	BAX 39	BAX 40
Provisional standard	270	270	270	270
LX 378	89	80	87	79
S 2	50	52	51	47
RX 5788/1	1281	1229	1313	1248
8916	117	115	110	108
8465	551	576	552	481
8457	1276	1163	1177	1113

Table 10. *Analysis of variance of the data given in Table* 9

Source of variation	Mean square	D.F.
Between sera	7·5740	5
Between toxins	0·0093	3
Serum × toxin interaction	0·0014	15
Between tests within toxins	0·0071	16
Residual	0·0028	80

different sera: the apparent values ranged from 23·5 to 200 Lf/ml. The combining power of the toxoid with the provisional standard serum was 50 Lf/ml., with four out of six sera it was from 145 to 200 Lf/ml. and with two of these 175 and 172 Lf/ml. respectively. The lowest value of all was given by Ex 1551. It is well known that flocculation values are unaffected by the concentration of the components, but the combining power of botulinum sera has been shown to be very different according to the conditions in the *in vivo* test. Table 1 shows that the dilution ratios of five sera tested differed from one another considerably, that Ex 1551 had the lowest dilution ratio, the provisional standard type A antitoxin the next highest and that the three other antitoxins had higher dilution ratios and resembled one another, forming a group with flocculation values for the type A toxoid of 145–175 Lf/ml. It was therefore decided to give an arbitrary value of 170 Lf/ml. to the toxoid, that figure being nearest to the values obtained with the

majority of the sera used for testing it, and the toxoid was then used to estimate the flocculation value of a number of type A sera.

The serum ratios of these type A sera were calculated using the *in vivo* value at the L+/10 level of test and were compared with the dilution ratios previously obtained. Sera with serum ratios between 0·11 and 0·64 had dilution ratios which rose steadily from 0·0145 to 0·0632, but when the serum ratio exceeded 0·64 there

Table 11. *The* in vivo *and* in vitro *values of a group of type A sera and their serum ratios and dilution ratios*

Sera	Antitoxin values at L+/10 level of test in u./ml.	Flocculation values u./ml.	Serum ratios	Dilution ratios
Ex 1551	63	550	0·11	0·0145
Provisional standard	270	1125	0·24	0·0256
8465	870	2050	0·42	0·0265
8467	1800	3400	0·53	0·0480
8448	1030	1600	0·64	0·0632
8480	4250	5250	0·81	0·0401
P 183 A	6750	7500	0·90	0·0480
8235	800	850	0·94	0·0458
8469 A	1424	1275	1·12	0·0444
8469 B	5000	3500	1·43	0·0381

Table 12. *The flocculation values and serum ratios of seven fractions of a* C. botulinum *type A serum* (8480) *obtained by progressive precipitation with increasing concentrations of ammonium sulphate, with the dilution ratios for comparison*

Fractions	Precipitation limits of ammonium sulphate in g./l.	Antitoxin values at L+/10 level of test in u./ml.	Flocculation values in u./ml.	Serum ratios	Dilution ratios
1	0–140	1100	1075	1·02	0·0462
2	140–160	5800	5600	1·04	0·0364
3	160–180	5250	6100	0·86	0·0330
4	180–200	2250	3500	0·64	0·0392
5	200–220	1600	2300	0·70	0·0360
6	220–250	700	—	—	0·0402
7	250–300	170	—	—	0·0531
Original serum	—	4250	5250	0·81	0·0401

ceased to be any progressive rise in dilution ratios (Table 11). Flocculation was slow (6–24 hr.) and the time of flocculation was not correlated with either the dilution ratios or serum ratios.

The type A serum 8480 which had previously been separated by ammonium sulphate precipitation into seven fractions was examined by flocculation. Five of the seven fractions flocculated, but very slowly (1–2 days). The serum ratios declined from 1·02 in the first fraction to 0·70 in the fifth; the sixth and seventh fractions would not flocculate. There was no correlation between the serum ratios and dilution ratios of the fractions (Table 12).

DISCUSSION

The essential difficulty which has been demonstrated in the standardization of antitoxins other than botulinum antitoxin has been attributed to differences in firmness and speed of combination with toxin. Several methods of demonstrating these differences have been used, of which diluting the components before mixing them appears to be the most reliable (Glenny & Barr, 1932*a*).

In the present investigation *all* sera examined were progressively less efficient in combining with toxin as the dilution of toxin and antitoxin was increased. This dilution effect was moreover greater than that demonstrated in other toxin-antitoxin systems.

Although the dilution ratios were worked out at five levels of test covering a dilution range of 1/10,000, none of the type A sera tested, if examined at any two levels of test separated by a 1/100 dilution, had a dilution ratio of 1·0. Much of the work on dilution ratios in other systems has been done over a range of 1/100 dilution, and it has been shown that by suitable methods of immunization it is possible to produce avid antitoxins in the majority of horses that have a dilution ratio of about 1·0, over this range.

The *C. botulinum* antitoxins tested showed a dilution effect of the same order as that found in guinea-pig sera after one dose of diphtheria prophylactic P.T.A.P. (Jerne, 1951).

The dilution effect was considerably reduced when a group of sera was tested with two toxins of high toxoid content, an observation in keeping with that of Barr (1949) with diphtheria toxins and antitoxins.

The seven ammonium sulphate precipitated fractions of the type A serum tested had dilution ratios of the same order of difference as has been demonstrated previously with a slightly non-avid diphtheria antitoxin (Glenny & Barr, 1932*a*). When the dilution ratios of two type A sera from horses at various stages of immunization were examined, it was found that the greatest improvement in dilution ratio occurred during and shortly after a 3-month rest period. Thereafter, while active hyperimmunization proceeded the improvement was slight. The horse from which the serum Ex 1551 was prepared was not rested at all after the initial small doses of toxoid, but continued its course of hyperimmunization with steadily increasing doses of toxoid. Sera from this horse gave the lowest dilution ratio and their potency never exceeded 70 u./ml. This rather suggests that further improvement in dilution ratio is not likely, unless a still longer rest period is used. Examination of pooled guinea-pig sera demonstrated the poor avidity of sera from animals given only two doses of toxoid, and bled 10 days later.

It has been shown in other toxin-antitoxin systems that poor avidity is associated with both a low serum ratio and a pronounced dilution effect with test mixtures. The *C. botulinum* antitoxic sera for which both dilution ratio and serum ratio were estimated showed that the two ratios were correlated with one another when the serum ratios were low; but when the serum ratio exceeded 0·64 however the improvement in dilution ratio was not maintained, although serum ratios up to 1·43

were recorded. But it has been shown by Barr (1949) that correlation between serum ratios and dilution ratios with diphtheria antitoxin was good except where the serum ratio was greater than 2·0.

The influence of the time allowed for combination of toxin-antitoxin mixtures on the amount of serum required to neutralize multiples and fractions of the L+/10 dose of toxin was demonstrated both with mixtures of dilutions and with dilutions of mixtures of toxin and antitoxin. The improvement in serum combining power at low levels of test when tests were done with mixtures of dilutions was considerable, the more avid sera improving the most. However, when dilutions of mixtures were used there was no reduction in the amount of serum required for neutralization, with time. This suggests that the fraction of the mixtures which had combined at high concentration and had dissociated on dilution, would not combine at all at low concentration even in infinite time; also, that the dissociation on dilution is very quick. The poor combination of toxin-antitoxin mixtures at the lower levels of test might mean that toxin and antitoxin molecules with receptors of slightly differing configuration had great difficulty in finding a suitable molecule with which to combine. Antitoxin which will fully neutralize a molecule of very high molecular weight may well itself consist of molecules of great complexity, and much care in the immunization of an animal might be required before a generally homogeneous antitoxin could be produced, of which the majority of the molecules would be capable of firm union with toxin.

It has been suggested (Wagman & Bateman, 1953) that type A toxin dissociates in dilute solution under certain circumstances to produce a toxic component of molecular weight of about 60,000. This might well have the effect of producing a number of toxin receptors for antitoxin from one molecule of toxin, an effect which if it occurred would appear to reduce the combining power of antitoxin at low levels of test. This might explain the failure to produce a type A antitoxin with a dilution ratio greater than 0·0632. Bowmer (personal communication) has shown that an antitoxin prepared against *C. botulinum* type D has a dilution ratio of a similar order to those recorded for type A, but he did not observe this with types C and E. It has been shown that the dilution ratios of two type B sera were of a much higher order than those found with type A sera. These differences may be due to the complexity of the antigens.

A number of type A antitoxins were tested at the L+/10 level of test against four type A test toxins. It would be expected that if more than one lethal toxin existed in the toxic filtrates, the proportion of such components would vary in different toxin preparations, as would the proportion of the respective antitoxins in different sera. In such circumstances discrepancies should be observed between the antitoxin values obtained for a group of sera with different test toxins. No such discrepancies occurred in this series of tests.

It is evident from a study of the wide differences in dilution ratio of the sera tested and the varying degrees of toxin-antitoxin combination at different levels of test, and with different times of combination, that any comparison of the potency of *C. botulinum* type A antitoxins is difficult. An approximate value can be allotted if all tests are made under constant conditions. Such a value

cannot however be regarded as absolute, because any change in procedure may not affect the titrations of the standard and unknown sera to the same extent.

SUMMARY

C. botulinum types A and B toxins required larger amounts of antitoxin for neutralization when fractions of the test dose of toxin were used than would be expected.

The degree of this dilution effect, i.e. the dilution ratio, varied with different sera. The dilution ratios with type A sera could be improved by allowing a greater time for combination to occur, or by mixing toxin and antitoxin in high concentration and diluting these mixtures.

The dilution ratios of type A sera from horses rose as immunization proceeded, the greatest rise occurring during the 3-month rest period following three small doses of toxoid.

A type A serum could be separated into fractions of different dilution ratio by precipitation with increasing concentrations of ammonium sulphate.

Type A antitoxins prepared by immunization of groups of guinea-pigs with two doses of toxoid, had dilution ratios far lower than those found in hyperimmune horse sera.

The dilution ratios of sera were lower when tested against a toxic toxin than against a more toxoided one.

A number of type A sera were tested at one level of test against a number of test toxins. There was no evidence of heterogeneity of the toxins and antitoxins tested.

The serum ratios were found to be correlated with the dilution ratios except where the serum ratios exceeded 0·64. Flocculation time was slow and varied from 6 to 48 hr. with different sera tested against the same toxoid.

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