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THE TOXIC ANTIGENIC FACTORS PRODUCED BY *CLOSTRIDIUM BOTULINUM* TYPES C AND D

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ABSTRACT

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The toxic factors produced by *Clostridium botulinum* (Van Ermengem, 1896), types C and D were determined. Monospecific antisera were prepared against the different factors. It was shown that type C_{α} produces factors C_1, C_2 and D; type C_{β} produces factor C_2 and type D produces factors C_1 and D. It was also shown that the International Standard Type C Antitoxin contains antibodies against factors C_1, C_2 and D and the International Standard Type D Antitoxin contains antibodies against factors C_1 and D.

INTRODUCTION

The presently recognized *Clostridium botulinum* types A, B, C, D, E and F are a heterogeneous group of organisms which vary in their cultural and biochemical characteristics. The only reliable method of identification is based on the immunologically distinct toxic factors they produce, but the classification on this basis is not entirely elucidated for all the types. Serologically the toxins of types A and B appear to be homogeneous. The toxins of types E and F may be serologically related because large amounts of type E antitoxin will neutralize small amounts of type F toxin. The position with types C and D is somewhat controversial.

Pfenninger (1924) found that serum prepared against the type now known as $C\alpha$ neutralizes the toxins of types $C\alpha$ and $C\beta$, while the serum against type $C\beta$ neutralizes only the toxin of the homologous strain. Mason & Robinson (1935) presented results indicating that their type C strains produced three toxic components, C_1, C_2 and a small amount of D. Their type D cultures contained chiefly D toxin, but also small quantities of the C fraction. However, they did not indicate which C fraction was present. Bulatova, Matveev & Samsonova (1966) state that Pfenninger's findings have "insufficient basis" and quote the results of Prévot & Brygoo (1953) and Guillaumie, Kréguer & Geoffroy (1955) in support of their own conclusions, i.e. that $C\alpha$ and $C\beta$ antisera cross-neutralize with $C\alpha$ and $C\beta$ toxins. Bulatova *et al.* also demonstrated that type C produces a small amount of type D toxin and that type D produces both type $C\alpha$ and type $C\beta$, in addition to the type D toxin.

In the Republic of South Africa, where botulism due to types C and D is an important disease of cattle in certain regions, approximately five million doses of vaccine are used annually. It is important to know exactly which toxic factors are produced by the organisms responsible for the disease, in order to ensure the inclusion of the correct antigens in the vaccine and monitoring of the response of vaccinated animals to the different antigenic components. Up to the present the immunity of experimental and farm animals has been interpreted as the degree of resistance to the composite toxic culture filtrates of the two types of organism, as determined by serum-neutralization tests in mice.

The main purpose of the study reported here was to identify the toxic antigens produced by *C. botulinum* types C and D. Whereas previous investigators attempted to unravel this problem by using crude toxic culture filtrates and sera produced by injecting formol-toxoids into experimental animals, the problem was approached by preparing antisera against the individual toxic factors. With the monospecific antisera thus obtained some of the toxic factors in culture filtrates could be neutralized. By neutralizing all but one toxic factor, monospecific toxins could be prepared and through the addition of different combinations of monospecific antisera to a crude toxic culture filtrate all the different toxic factors could be singled out in turn for further neutralization tests. By testing these single toxic factors against antisera prepared against the culture filtrates of *C. botulinum* types C and D, it was possible to determine which toxic antigenic factors are produced by these organisms.

The intention was not to investigate as many strains as could be obtained, but rather to establish methods whereby the various factors could be identified with relative ease. The main factors produced by type C were designated C_1 and C_2 and the main factor produced by type D was named D in accordance with the practice adopted by Mason & Robinson (1935).

MATERIALS AND METHODS

Preparation of toxins

The *C. botulinum* types $C\alpha$ and D used for this study were the highly toxigenic vaccine strains prepared by this Institute. The type $C\beta$ 11772 ATCC strain was of low toxigenicity, but since other strains which were tested gave no better results, it was decided that sufficient toxin was produced for the purpose of this study.

Toxic cultures were obtained in three different ways:

(a) By the method (S.W.) of Sterne & Wentzel (1950). This method consistently gave highly toxic cultures with strains $C\alpha$ and D and quite usable toxins with type $C\beta$.

(b) By growing the organisms in Robertson's meat broth at 37°C for 7 days, highly toxic cultures were obtained with types $C\alpha$ and D but not with type $C\beta$.

(c) By using specially prepared sausages. Horse meat was minced and tightly packed into cellophane sausage casings of 8 cm diameter. A growing culture of *C. botulinum* was injected into the sausage which was incubated at 37°C for 21 days. The toxin was extracted from the contents of the sausage with isotonic saline solution. Fairly potent toxic solutions were obtained with all three types, although not as regularly with type $C\beta$ as with the others.

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All toxic cultures were centrifuged and the supernatant fluids were clarified by Seitz filtration.

For the toxoiding of toxic fluids their amino-nitrogen content was determined by the copper method of Pope & Stevens (1939) and formalin was added according to the formula of Holt (1950) for diphtheria toxin. The pH was maintained at 7,0 and incubation continued at 37°C until 0,2 ml injected intravenously failed to kill white mice. On an average toxoiding took 11 days.

Alum-precipitated toxoid was prepared by adding alum to give a final concentration of 2 per cent at a pH 6,0.

For the preparation of dried toxin from types Cα and D, cultures were grown in Robertson's meat broth. Potent toxins were produced by this method and in the gel-diffusion test, fewer precipitation lines were formed with these toxins than with those produced by the S.W. method.

The meat broth toxic culture filtrates (pH 7,0) were subjected to serial fractionation with ammonium sulphate using increments of 2 g per 100 ml. Although toxin was precipitated over a wide range of concentrations, most was obtained in the 19 g to 21 g per 100 ml fraction. This fraction was collected from 10 l of filtrate, washed three times with an aqueous solution of ammonium sulphate (21 g ammonium sulphate per 100 ml of isotonic saline solution), dialyzed against distilled water at 4°C for approximately 7 days and subsequently freeze-dried. The Cα dry toxin contained 200 MLD per mg and the D, 10 000 MLD per mg.

Dried toxin was prepared from type Cβ by adding 40 g of ammonium sulphate per 100 ml of clarified toxic culture produced by the S.W. method. The pH was kept at 7,0. The precipitate was collected by centrifugation, washed three times with an aqueous solution of ammonium sulphate of equal concentration to the precipitating mixture, dialyzed at 4°C and subsequently freeze-dried. The dried toxin contained 1 MLD per mg.

The mean weights of mice, rabbits and guinea-pigs used were 25 g, 4,5 kg and 0,7 kg respectively.

Toxin-antitoxin mixtures were injected intravenously nto white mice and the results recorded for 3 days.

Initial production of antisera in horses against toxic cultures of types Cα ,Cβ and D

Toxic cultures of types Cα, Cβ and D grown by the S.W. method were used for this purpose. Horses were given two injections of alum-precipitated toxoid at an interval of 40 days. They were then rested for 2 months and given another course of two injections of toxoid followed by two injections of toxin at fortnightly intervals. They were bled a week after the last injection and the serum was preserved with 0,02 per cent Thiomersal without prior refinement.

The production of monospecific C_2 antiserum

The toxic filtrate of *C. botulinum* type Cβ grown by the S.W. method was clarified and treated with varying concentrations of ammonium sulphate at pH 7,0. Three distinct precipitates formed at 20 g, 27 g and 39 g per 100 ml. The first and third were only slightly toxic after dialysis. The second precipitate was toxic and half was toxoided, alum-precipitated and used for immunizing a rabbit according to the schedule in Table 1.

One week after the last injection the rabbit was exsanguinated and its serum preserved with 0,02 per cent Thiomersal. This antiserum neutralized type Cβ toxin, but 0,4 ml had no neutralizing effect on 5 MLD type Cα or type D toxin. (This does not necessarily imply that types Cα and D do not produce the same factor as type Cβ in low concentration, because this factor could have been diluted out when using a test dose of 5 MLD.)

TABLE 1 Schedule of immunizing a rabbit with type Cβ antigen

Day	ml of APT* subcut	ml of toxin subcut
1	2,0	—
40	2,0	—
54	3,0	—
68	3,0	—
82	—	4,0
96	—	4,0

*APT = Alum precipitated toxoid

From the results reported later it will become clear that this serum is monospecific for the C_2 factor.

The production of monospecific C_1 antiserum

C. botulinum type Cα was grown in Robertson's meat broth. The toxin was precipitated from the clarified culture filtrate with 19 g to 21 g ammonium sulphate per 100 ml, and then washed and dialysed as described above. The toxic liquid fraction was toxoided and alum-precipitated and used for immunizing a horse according to the schedule in Table 2.

TABLE 2 Schedule of immunizing a horse with an alum-precipitated, formalinized toxic fraction from a type Cα culture

Day	ml injected subcut
1	2
43	5
112	5
125	10
133	20
136	40
141	80

The horse was bled one week after the last injection and its serum refined according to the pepsin digestion method described by Pope (1939). The serum was tested against crude type Cα toxin prepared by the S.W. method, using the Ramon flocculation test as modified by Glenny & Okell (1924) for titrating diphtheria toxin and antitoxin. The toxin was diluted 1:10; final volumes of 1,0 ml toxin and antitoxin were used in 8 × 1 cm tubes which were shaken mechanically in a waterbath at 38°C. After 5 minutes flocculation occurred in the tube containing toxin and antitoxin in a proportion corresponding to the neutral point as determined *in vivo* using a test dose of 5 MLD.

Subsequently, 100 ml of toxin was mixed with antitoxin in the same proportion as in the indicating tube and allowed to flocculate. The floccules were collected and washed four times in isotonic saline and resuspended in saline to a density corresponding to Brown's No. 6 tube. Formalin to a final concentration of 0,2 per cent was added and the mixture incubated at 38°C for 4 days. This was necessary since it was found that mice were killed by the intraperitoneal injection of 0,5 ml of the untreated suspension. Alum to a final concentration of 0,5 per cent was added to the suspension and the pH adjusted to 6,0. This was used for immunizing rabbits according to the schedule in Table 3.

The rabbits were bled 1 week after the last injection, their serum pooled and preserved with 0,02 per cent Thiomersal.

TABLE 3 Schedule of subcutaneous injection of floccule suspension into rabbits

Day	ml suspension
1	0,5
21	0,5
30	1,0
37	2,0
42	3,0

It was found that, using a test dose of 5 MLD of dried toxin, 0,4 ml of this serum protected only against Cα toxin and not against D. Neither did the same quantity have any neutralizing effect on 2 MLD of Cβ toxin. As will be seen later, this serum is monospecific for the C_1 toxic fraction, while antiserum produced against crude Cα toxin also has a neutralizing effect on 5 MLD of type D and 2 MLD of Cβ toxin.

However, when these rabbits had been rested for 5 months and then given injections of 2 ml, 4 ml and 5 ml of the same suspension at 14 day intervals and bled 1 week after the last injection, 0,4 ml of this serum protected against 5 MLD of dried toxin of types Cα and D, but not against 2 MLD of type Cβ toxin. It was concluded that some type D toxin, evidently produced by type Cα during growth by the S.W. method, was adsorbed onto the floccules, but was insufficient to produce an antibody response during the initial course of immunization.

The production of monospecific D antiserum

It is well known that cross-neutralization takes place between types Cα and D toxins and their antisera. Therefore, it was clear that in attempting to produce a monospecific serum against factor D, the Cα toxic factor would have to be removed from the toxic type D culture to be used as antigen. The factor produced by Cβ was not under consideration, because antiserum produced by hyperimmunizing a horse with crude type D toxin derived from a S.W. culture had no neutralizing effect on 2 MLD of Cβ toxin.

In the previous section, use was made of the specific flocculating ability of horse antiserum against factor C_1 when mixed with a solution containing the corresponding factor. Monospecific D factor antiserum was therefore produced as follows: Type D toxic culture fluid prepared by the S.W. method was flocculated with factor C_1 antiserum and the floccules removed. The flocculation was first done with 1 ml amounts of toxin to determine the correct proportion of serum to toxin and then repeated with 100 ml quantities. After removal of the floccules by centrifugation, the supernatant was filtered through a Seitz clarifying pad and then formalinized and alum-precipitated.

Three rabbits were each given a subcutaneous injection of 3 ml of the horse antiserum against factor C_1 in order to produce a passive immunity. After an hour they were given a subcutaneous injection of the material described in the previous paragraph, i.e. type D antigen from which the C_1 factor had been removed. It was assumed that the passive immunity would suppress the immunogenic stimulus resulting from any C_1 antigen that might still be present in the material injected.

After 6 weeks the three rabbits were treated as follows:

(1) One was immunized passively against factor C_1 as before and subsequently given an injection of 3 ml of the same material used for the primary stimulus.

(2) One was given a secondary stimulus by injecting 3 ml of the same material used for the primary stimulus.

(3) The third rabbit received 5 ml subcutaneously of material prepared as follows: guinea-pigs were dosed *per os* with 1 ml of toxic type D culture prepared by the S.W. method. After 3,5 hours they were exsanguinated and the serum was separated from the pooled blood. This serum contained about 20 MLD of toxin as determined by intravenous injection into mice. It was toxoided by the addition of a final concentration of 0,1 per cent formalin and incubated at 38°C for 8 days. A final concentration of 1 per cent alum was then added at pH 6,0.

This procedure was applied on the assumption that in the process of digestion all secondary toxic factors would be eliminated and only some of the major toxic fraction of type D would be absorbed into the blood.

The three rabbits were bled 1 week after the secondary stimulus. A quantity of 0,4 ml of the three sera failed to neutralize 5 MLD of Cα and 2 MLD of Cβ dried toxins. It was subsequently shown that they are monospecific for factor D.

RESULTS

Analysis of the toxic factors produced by C. botulinum *types C and D.*

Initially, antisera produced in horses against toxic cultures of types Cα, Cβ, and D grown by the S.W. method were tested against the dried toxins described under Materials and Methods. For Cα and D toxin the test dose was 50 MLD and for Cβ toxin 5 MLD. It was found that the Cα antiserum neutralized Cα, Cβ and D toxins; type D antiserum had the same effect on Cα and D toxins, but failed to neutralize Cβ toxins; type Cβ antiserum neutralized Cβ toxin but not the Cα and D toxins. These results are summarized in Table 4.

TABLE 4 Toxin-neutralization results obtained with dry toxin and sera produced against the crude toxic cultures

Serum	Toxin		
	Cα	Cβ	D
Cα	+	+	+
D	+	—	+
Cβ	—	+	—

The negative results indicating a lack of cross neutralization between Cβ and D toxins and antisera are significant and it is fair to conclude that the two produce distinct antigenic toxins. Cα and D toxins and antitoxins show cross-neutralization and therefore contain a common factor or factors. Cβ serum does not neutralize Cα toxin but Cα serum neutralizes Cβ toxin which indicates that they must have a toxic factor in common. The explanation is that the factor common to Cα and Cβ is produced by Cα in small quantities and this lesser factor is diluted out when the test dose of Cα toxin is reduced to 50 MLD.

Legitimate objections to the technique as applied are that the individual factors cannot be identified and that subsidiary toxic factors are lost when a small number of MLD are used as test dose with weaker antisera. Consequently, an alternative method was followed. The organisms were cultured and their toxic

culture filtrates were formalinized and injected into rabbits. After four injections of toxoid at fortnightly intervals the rabbits were given four injections of unchanged toxin at fortnightly intervals and bled 1 week after the last injection. It was believed that through the extended course of immunization antibodies would be formed against all toxic factors including those produced in minimal amounts. Sera were obtained against types Cα, Cβ and D toxins produced by the S.W. method and by the sausage culture method. For comparative purposes the International Standard Antisera against types C and D (obtained from The State Serum Laboratories, Copenhagen) were included in the test.

As toxins for the toxin neutralization test the following were used in the manner described:

(1) *Type Cα*: The dried 19 to 21 per cent ammonium sulphate fraction was dissolved in saline to yield a concentration of 4 mg/ml (equivalent to 200 MLD/ml). On the assumption that C_2 and D factors were still present, 0,5 ml of each of the C_2 and D monospecific sera were added to every ml of toxin solution. After 30 min at room temperature the mixture, now containing 200 MLD/2 ml was diluted and dispensed into tubes in 2 ml amounts to give 200, 100, 50, 20 and 10 MLD per 2 ml in consecutive tubes. Control tubes containing only toxin and C_1, C_2 and D monospecific serum in lieu of test serum were included. The contents of the tubes were injected intravenously into mice in 0,4 ml quantities, using two mice per tube. The final test in mice therefore involved 0,2 ml quantities of the sera tested against 20, 10, 5, 2 and 1 MLD of toxin. The schedule and results are set out in Table 5.

TABLE 5 Neutralization tests with different sera against toxin rendered monospecific for factor C_1

SERUM	MLD of C_1 toxin per dose				
	20	10	5	2	1
Normal rabbit	++	++	++	++	++
D monospecific	++	++	++	++	++
C_2 ,, ,,	++	++	++	++	++
C_1 ,, ,,	LL	LL	LL	LL	LL
International Type C	LL	LL	LL	LL	LL
Type Cα	LL	LL	LL	LL	LL
Type Cβ	++	++	++	++	++
International Type D	LL	LL	LL	LL	LL
Type D	LL	LL	LL	LL	LL

\+ = death within 3 days
L = alive after 3 days

The results show that type Cβ antiserum had no influence on factor C_1 toxin and neither did C_2 nor D monospecific serum in excess of the quantities originally added neutralize factor C_1 toxin. On the other hand, types Cα and D and the International Standard types C and D antisera neutralized factor C_1 toxin.

(2) *Type D:* This was tested in the same way as type Cα. The dried 19 to 21 per cent ammonium sulphate precipitated fraction was made up in saline to a concentration of 10 000 MLD/ml. On the assumption that this still contained some C_1 toxin, 1 ml of C_1 monospecific serum was added for every ml of toxin solution. The rest of the procedure as described for type Cα was followed. Consequently, in the final test in mice 0,2 ml of the sera were tested against 1 000, 200, 100, 20, 10, 2 and 1 MLD of toxin. The schedule and results are set out in Table 6.

Type Cβ antiserum did not neutralize factor D toxin and neither did C_1 monospecific serum in excess of the amount originally added have any influence on factor D toxin. Types Cα, D and the International Standard Antisera for types C and D neutralized factor D toxin (Table 6).

TABLE 6 Neutralization tests of different sera against toxin rendered monospecific for factor D

SERUM	MLD of D toxin per dose						
	1000	200	100	20	10	2	1
Normal rabbit	++	++	++	++	++	++	++
C_1 monospecific	++	++	++	++	++	++	++
D monospecific	LL	LL	LL	LL	LL	LL	LL
Type Cα	LL	LL	LL	LL	LL	LL	LL
Type Cβ	++	++	++	++	++	++	++
International Type C	LL	LL	LL	LL	LL	LL	LL
Type D	LL	LL	LL	LL	LL	LL	LL
International Type D	LL	LL	LL	LL	LL	LL	LL

\+ = death within 3 days
L = alive after 3 days

(3) *Type Cβ:* Since the dried ammonium sulphate precipitated Cβ toxin was weak, the tests as applied to types Cα and D could be done only at the 5, 2 and 1 MLD levels. Because no cross-neutralization between type Cβ antiserum and C_1 and D toxic factors could be detected in previous tests, antisera against these factors were not included with the type Cβ toxin used for the test. The sera tested against type Cβ toxin and the results are given in Table 7.

TABLE 7 Neutralization tests with different sera against type Cβ toxin

SERUM	MLD of Cβ toxin per dose		
	5	2	1
Normal rabbit	++	++	++
Type Cα	LL	LL	LL
Type Cβ	LL	LL	LL
International type C	LL	LL	LL
Type D	++	++	++
International type D	++	++	++

\+ = death within 3 days
L = alive after 3 days

From the results it can be seen that type Cβ toxin is neutralized by its homologous antiserum, type Cα and the International Standard Type C antisera.

The same series of tests were done with sera obtained from rabbits after immunizing them against toxin obtained from the growth of organisms in sausages. The same immunization procedure as used for the S.W. culture toxins was used. The results are recorded in the following three sections of Table 8.

The results in Table 8 show that the C_1 toxic factor is neutralized by serum produced against Cα toxin and to a lesser extent by serum produced against D toxin. The D toxic factor is neutralized by serum against type D toxin and to a lesser extent by serum against Cα toxin. In contrast to the previous findings, type Cα

toxin did not stimulate the production of antibodies against factor C_2.

TABLE 8 Neutralization tests with sera prepared from sausage cultures against different toxic factors rendered monospecific

(a) C_1 toxic factor

SERUM	MLD per dose				
	20	10	5	2	1
Normal rabbit	++	++	++	++	++
Type Cα	LL	LL	LL	LL	LL
Type D	++	++	+L	LL	LL
Type Cβ	++	++	++	++	++

(b) D toxic factor

SERUM			MLD per dose				
	1000	200	100	20	10	2	1
Normal rabbit	++	++	++	++	++	++	++
Type D	LL	LL	LL	LL	LL	LL	LL
Type Cα	++	++	++	++	LL	LL	LL
Type Cβ	++	++	++	++	++	++	++

(c) Cβ toxin

SERUM	MLD per dose		
	5	2	1
Normal rabbit	++	++	++
Type Cα	++	++	++
Type Cβ	LL	LL	LL
Type D	++	++	++

\+ = death within 3 days
L = alive after 3 days

Discussion and Conclusions

An analysis of the results in Table 5 shows that sufficient toxin remained in each tube to kill the mice injected with the appropriate dose. This toxin was not neutralized by the additional C_2 and D serum added during the test but was, however, neutralized by the C_1 monospecific antiserum. This C_1 monospecific antiserum in turn did not neutralize the D toxin rendered monospecific (Table 6) or the C_2 toxic factor as demonstrated under Materials and Methods.

It therefore follows that the C_1 toxic factor and the C_1 antiserum as used in the test were monospecific.

From the results given in Table 6 it is evident that sufficient D toxin remained in each tube to kill the mice injected with the appropriate dose. This toxin was not neutralized by the additional C_1 serum added during the test. It was, however, neutralized by the D monospecific antiserum. This D monospecific antiserum in turn did not neutralize the C_1 toxin rendered monospecific (Table 5). The D monospecific serum also did not neutralize the C_2 toxic factor as demonstrated under Materials and Methods.

It therefore follows that the D toxic factor as used in the test and also the D antiserum were actually monospecific.

The C_2 toxin and antiserum were previously shown to be monospecific.

A synthesis of the information contained in Tables 5, 6 and 7 allows one to conclude that the antigenic toxic factors produced by the strains of *C. botulinum* investigated when grown by the S.W. method are as follows:

Type	Fractions
Cα	C_1, C_2, D
Cβ	C_2
D	C_1, D

According to the data summarized in Table 8 the factors are distributed as follows when the organisms are grown in specially prepared sausages:

Type	Fractions
Cα	C_1, D
Cβ	C_2
D	C_1, D

The essential difference between the results of the two methods of production is that in the sausages no C_2 is produced by type Cα.

The main value of this study is not so much the elucidation of the toxic factors produced by three strains representing different types, but that an easy method of identifying the toxic factors has been established. The finding that the antigenic factors produced can vary according to the growth medium is equally important.

The monospecific sera have opened the way to exact quantitative studies in the field of vaccine production. Consideration can now also be given to whether a vaccine to protect animals against *C. botulinum* types Cα, Cβ and D should not be prepared by using type Cα only, since it can produce all the required factors under suitable conditions. Whether it produces enough of the different factors will determine the success of such a measure.

Results set down in Tables 5, 6 and 7 show that the International Standard Type C Antiserum contains antibodies against factors C_1, C_2 and D, because no doubt, it was prepared from a type Cα strain. The data also show that the International Standard Type D Antiserum contains antibodies against factors C_1 and D. One is justified in questioning the utility of an international standard which is complex and not specific for a single factor. Under the present circumstances it is even possible that the International Standard Antisera might vary in the proportion of the antibodies they contain against the different factors. The question arises whether it would not be better to adopt monospecific sera as international standards.

Summary

(1) Antisera monospecific for the toxic factors C_1, C_2 and D produced by *Clostridium botulinum* types C and D were prepared.

(2) The monospecific antisera were used in different combinations to neutralize some of the toxic factors in crude culture filtrates leaving one toxic factor in each instance. In this manner monospecific toxic factors were prepared. By using them in neutralization tests the antibodies present in sera produced from crude culture filtrates of *C. botulinum* types C and D could be determined. Thus an indirect method of analysing the toxic factors produced by the organisms was established. The

outstanding advantage of this method is that it avoids missing toxic factors produced in relatively low concentrations.

(3) It was shown that, when the cultures are grown by the method of Sterne & Wentzel (1950), type Cα produces factors C_1, C_2 and D; type Cβ produces factor C_2 and type D produces factors C_1 and D.

When the organisms are grown in sausages, type Cα produces factors C_1 and D, type Cβ factor C_2 and type D factors C_1 and D.

(4) It was found that the International Standard Type C Antitoxin contains antibodies against factors C_1, C_2 and D and the International Standard Type D Antitoxin contains antibodies against factors C_1 and D.

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