(4) Comparison by toxin-antitoxin tests.

Comparison and classification of the botulinus types by means of toxin antitoxin tests is probably the best means now available for this purpose. In the case of non-toxic types, or those with very weak toxins, other means of classification must be attempted. In the earlier work on the botulinus bacteria, it was not realized that more than one type existed. Even now it is not known whether the original type of von Ermengem (1912) for instance, was an A or a B one, and Bergtson (1922) suggests that it was probably more closely related to the C type isolated by her than to either of the other two. Since the work of Burke (1917), the existence and wide distribution of the B type has been recognized. The C type of Bergtson (1922) was established by the failure of its toxin to be neutralized by A and B type antitoxins, though there were in addition, certain cultural characters in which the organisms differed, such as the colony form and some biochemical features. Seddon (1922) established the existence of a C type of the Cl. botulinum in Australia, in association with a paralysis of cattle (Midland disease of Tasmania) and later (1925) demonstrated the toxin of the organism in mixed cultures from carrion in other parts of Australia. Seddon gave his organism the name of Bacillus parabotulinus, a name which has not so far been altered by other workers, but it is obvious that the name cannot be allowed to stand indefinitely in view of the fact that the related types are all classified as Cl. botulinum of some type. There does not seem to be any real justification for changing the name Cl. botulinum C to Cl. luciliae as has been done in Bergey's Determinative Bacteriology.

Weinberg (1927) in a criticism of the nomenclature adopted by Theiler and Robinson (1927) in relation to Cl.parabotulinum bovis, suggested that the name Cl. botulinum D should be substituted for the one given. Meyer and

Gunnison (1929) in a brief descriptive article on the organism called Cl. parabotulinum bovis by Theiler and Robinson, suggest that it be called Cl. botulinum type D. (Theiler and Robinson). The reason given is that the toxin is not neutralised by the antitoxins of eitherthe A, B or C types and therefore, the organism should constitute a fourth or D type. The writer is inclined to agree with this classification, and the organism will therefore be referred to as Cl. botulinum type D.

The organism described by Theiler and the writer (1928) in association with certain cases of botulism in horses in South Africa, and given the name Cl. parabotulinum equi, may eventually prove not to be a new type. In a personal communication to the Director of Veterinary Services Dr. R. Graham of the Illinois Agricultural Experiment Station, U.S.A. stated that his type C antitoxin neutralised the toxin of the Cl. parabotulinum equi. This observation has recently been confirmed by the writer who has in addition been able to show that the C toxin was neutralised by Cl. parabotulinum equi antitoxin. It is difficult, however, to explain the difference in virulence of the South African and American types for animals of the same species as will be noted in the section of this paper dealing with comparative virulence.

The writer's ideas and suggestions in relation to the classification and nomenclature of the botulinus types will be further discussed in the final consideration of our knowledge in relation to them.

In the comparative work on toxins and antitoxins of this group, the following types have been available:

(1) Cl. botulinum A (A 223 Lister Institute)
(2) " " B (B 95 " ")
(3) " " C (Graham U.S.A.)
(4) B. parabotulinus (Seddon, Australia)
(5) Cl. botulinum D (Cl. parabotulinum bovis)
(6) Cl. parabotulinum equi (Theiler and Robinson 1927)
(7) Cl. botulinum 333 (not yet typed;isolated from Lamsiekte carrion).

The abovementioned toxins were all from pure cultures. The following toxins were available as well but were from mixed cultures from toxic material. They produced typical symptoms of botulism in guinea pigs, but the cultures from which they were obtained might have contained more than one type of botulinus organism. From the results in the toxin antitoxin tests, however, this does not seem to have been the case.

Toxins (mixed bacterial cultures).

(8) Rat 3) From carcases of rats allowed to decompose
(9) " 5) in closed fruit jars
(10) " 6)

(11) 334) From carcases of cattle dead of lamsiekte at
(12) 335) Armoedsvlakte, Bechuanaland.
(13) 335a)

(14) 628 From an outbreak of botulism in horses on a farm between Johannesburg and Pretoria.

(15) 11 From an outbreak of botulism in donkeys in Johannesburg. The culture was from the carcas of a dead cat.

(16) Zoo strain. (From intestinal contents of wild ducks in Pretoria Zoo).

(17) Bekker strain (From carcase material from cases of botulism in sheep at Bredasdorp, Cape Province

(18) Carlisle strain (From intestinal contents of turkeys dead of botulism on a farm near Pretoria.

All the toxins both from pure and impure cultures were filtered through a small Seitz filter before use in tests.

Antitoxins.

(1) Combined Cl. botulinum A and B (Parke Davis & Co.)
(2) Cl. botulinum B (from an immunized goat)
(3) " " C (from Graham. Univ. of Illinois U,S.A
(4) " " D, or
" parabotulinum bovis (lamsiekte carrion).
(5) " " equi (botulism in horses, Theiler Robinson (1927).
(6) Rat 3 strain (from the carcase of a decomposed rat)
(7) 335 strain (from lamsiekte carcase material).

Numbers 6 and 7 are the strains referred to in the toxin list as 8 and 12 respectively.

With the exception of antitoxins (1) and (3) the sera were obtained by hyperimmunising goats. Rabbits were used at first, but provided such small quantities of serum and were so liable to die of intercurrent diseases during the immunisation process, that they were later given up in favour of goats.

In an article by the writer (1929) now in the press, the process used in the immunisation of goats is described, and may be briefly given here, as the article may not appear in print until after the present article has been presented.

In the case of each toxin where a hyper-immune serum was produced against it, an anatoxin was first made from it by the addition of .4% formalin and incubation at 37°C until the toxin lost its toxicity. The period of incubation varied with the virulence of the toxins, the more virulent the toxin the longer the incubation period necessary.

In immunisation of a goat, the writer commenced first with anatoxin, using as an initial dose 5 c.c. This was followed after 10 days by a dose of 10 c.c. Ten days later a dose of 20 c.c. was given. A week after this 10 M.L.D's of pure toxin were given and subsequently the dose was doubled every week until 2000 M.L.D's were given. The serum of the goat would then usually neutralize up to 500 M.L.D's for the guinea pig in a dose of 1 c.c. Further hyper-immunisation was not attempted as the serum was then sufficiently potent for cross toxin antitoxin tests. During the hyperimmunisation process, none of the goats showed any symptoms of botulism. Five different hyperimmune sera were produced and all were very satisfactory.

Graham and Thorp (1929) carried out a number of experiments on the detoxification of toxins of Cl.botulinum A, B and C types. They showed that the time necessary for detoxification by formalin was very variable and expressed the opinion that immunisation by means of anatoxins might prove a very valuable method.

As regards the interrelation of the A, B and C types of the Clostridium botulinum, the first attempt at a classification of them by toxin antitoxin tests was made by Pfenninger (1924). He was able to establish the identity of various C types and to show that Seddon's B.parabotulinus

toxin was neutralised by a C type antitoxin. An interesting observation made by Pfenninger was that the antitoxin of the B. parabotulinus type neutralised the homologous toxin only and not other C types. As will be seen in the tests to be described, Cl. parabotulinum equi toxin is neutralised by C type antitoxin and conversely the C type toxin is neutralised by Cl. parabotulinum equi antitoxin. Although therefore, as judged by toxin antitoxin tests B. parabotulinus and Cl. botulinum C are not apparently identical, Cl. parabotulinum equi and the C type are.

In the course of a large series of toxin antitoxin tests carried out by the writer, the observations of other workers have been supported and extended. In all the tests the approximate M.L.D.'s of the toxins were determined just previously.

Table I. 26/3/26.

Toxin		Antitoxin		Result
Cl.botulinum D (Cl. parabot bovis)	10 M.L.D's	Cl.botulinum D,	5 c.c.	Survived
"	10 M.L.D's	"	1 c.c.	"
"	10 "	none		Died in 48 hours
Cl.parabot. equi	2 "	Cl.botulinum D,	5 c.c.	Died in 72 hours
"	2 "	none		-do-
B.parabot. (Seddon)	2 "	Cl.botulinum D,	5 c.c.	-do-
"	2 "	none		Died in 48 hours
Cl.botulinum C,	2 "	Cl.botulinum D,	5 c.c.	Died in 96 hours
"	2 "	none		-do-
Cl.botulinum A,	10 "	Cl.botulinum D,	5 c.c.	Died in 72 hours
"	2 "	"	5 c.c.	-do-
"	2 "	none		Died in 96 hours

From this table the difference of the toxin of the lamsiekte organism (Cl. botulinum D) from the other toxins used is clearly established. At the time, other antitoxins were

not available but were obtained later, when further tests were carried out. A polyvalent antitoxin against the A and B types of the Cl. botulinum was obtained from the Burroughs, Welcome Co., and with it a series of tests was carried out. These results are given in table 2.

Table 2. 1/9/26.

Toxin		Antitoxin	Result
Cl.botulinum A,	2 M.L.D's	1 c.c. Cl.botulinum A and B	Survived
"	"	none	Died
Cl.parabot.equi	2 "	1 c.c. Cl.botulinum A and B	"
"	"	none	"
B.parabotulinus (Seddon)	2 M.L.D's	1 c.c. Cl.botulinum A and B	"
"	"	none	"
Cl.botulinum C,	2 "	1 c.c. Cl.botulinum A and B	"
"	"	none	"
Cl.botulinum D, (Cl.parabot. bovis)	2 "	1 c.c. Cl.botulinum A and B	"
"		none	"

From this table it is clear that as shown by other workers, the C and Seddon types differ from the A and B, and in addition, Cl. botulinum D and Cl. parabotulinum equi (C) differ from these types.

Until recently the writer had been unable to make an equi (C) serum in any animal species, but after commencing the anatoxin method of immunisation, a good serum was obtained. Most of the work done by the writer on toxin-antitoxin tests, has therefore, been carried out in the last year. The original observations on Cl. botulinum D were repeated and confirmed, and a series of comparative tests was carried out with other types.

Recently an efficient antitoxin against the toxin of Cl. parabotulinum equi (C) has been prepared, and it is of interest to note its effect on Cl. botulinum C toxin.

Table 3.

Toxin		Antitoxin		Result
Cl.parabot equi (C)	5 M.L.D's	2 c.c.	Cl.parabot equi C	Survived
Cl.botulinum C	"	"	"	"
Cl.parabot equi (C)	"	none		Died

The two toxins are, therefore, identical. Graham, in a personal communication to the writer, stated that he had found his C type antitoxin would neutralise the Cl.parabot equi toxin. The results in Table 3 therefore confirm his observation.

Taking the Cl. botulinum A, B, C and D as definite types, an attempt was made to classify the toxins mentioned earlier as having been available for testing. Unfortunately in some cases it was not possible to place them absolutely definitely some showing slight variations from the stock types.

When only the Cl. botulinum D antitoxin was available an attempt was made to classify four strains of the Cl. botulinum from carcase material at Armoedsvlakte, Bechuanaland. These are given in the toxin list as follows:-

Cl. botulinum 333 (pure culture)
" 334 (from impure culture)
" 335 -do-
" 335(A) -do-

The results of the test with these four toxins is given in table 4.

Table 4. (24/2/27)

Toxin		Antitoxin		Result
Cl.botulinum 333,	2 M.L.D's	2 c.c.	Cl.botulinum D	Died
"	"		none	"
" 334,	2 "	2	" Cl.botulinum D	Survived
"	"		none	Died
Cl.botulinum 335,	2 "	2	" Cl.botulinum D	Survived
"	"		none	Died
Cl. " 335	2 "			
(A)	"	2	" Cl.botulinum D	"
"	"		none	"
" D	5 "	2	" Cl.botulinum D	Survived
(Cl.parabot bovis)				
"			none	Died

This result was quite unexpected as the toxins had previously all been thought to be of one type. Two of them, Cl.botulinm 3$\frac{3}{4}$4 and 3$\frac{3}{5}$5, may be considered to be identical with Cl. botulinum D and the other two to belong to another type.

These tests with the four strains in Table $\frac{4}{5}$ were repeated later with identical results. It was, therefore, decided to attempt the classification of the two strains which were apparently not of the bovis or D type.

Table 5.

Toxin		Antitoxin		Result
Cl.botulinum	333, 2 M.L.D's	2 c.c.	Cl.parabot equi (C)	Survived
"	334	"	"	Died
"	335	"	"	"
"	335 (A)	"	"	Survived
Cl.parabot equi (C)		"	"	"
"		"	no antitoxin	Died

The results in this table are the exact opposite of those in Table 4. The toxins 333 and 335(A) therefore appear to be of the C type.

Using an antitoxin made against Cl. botulinum (335 A) one gets the results in Table 6:

Table 6.

Toxin		Antitoxin		Result
Cl.botulinum	33$\frac{4}{5}$ 2 M.L.D's	Cl. botulinum 335 (A)	3 c.c.	Died
" "	33$\frac{3}{4}$ "	"	"	Survived

This result affords further evidence of the relationship of strains 334 and 335)A), amd that 333 and 334 differ. Using an antitoxin made against the toxin of rat 6 which will later be shown to be a D type, apparently identical with that of Cl.parabotulinum bovis (Cl. bot. D), it can be shown that it neutralises the toxin of Cl. botulinum 334 but not that of 333.

Table 7.

Toxin		Antitoxin		Result
Cl.botulinum 334	2 M.L.D's	Cl.botulinum (Rat 6)	3 c.c.	Survived
" 333	"	"	"	Died

According to the results obtained from the various tests described, it would appear definite that one is dealing with at least two types of the Cl. botulinum on the farm Armoedsvlakte, Bechuanaland, Cape Province. The farm in question was originally purchased by the Government as being the most suitable place for undertaking research work on "lamsiekte" in cattle, as the mortality from the disease was very heavy before preventive measures against it were introduced.

Five strains of the Cl. botulinum from the above farm have, therefore, been classified by toxin antitoxin tests. One was the original Cl. parabotulinum bovis strain (D), and the other four were the strains Cl.botulinum 333, 334 335 and 335(A). It may be of interest to mention that strain 333 was from a guinea pig whose carcase became toxic when exposed in a wire cage at Armoedsvlakte, the wire having such a fine mesh that only dust could pass it.

These strains must therefore be classified as follows

(1) Cl. botulinum D or Cl. parabotulinum bovis
(2) Cl. botulinum 334 is a Cl. botulinum D type
(3) " 335 "
(4) " 333 is a Cl. botulinum C type
(5) " 335(A) "

The three toxins from carcases of rats which had been allowed to decompose in fruit jars were then tested. Against one of these, the toxin from rat 6, a powerful antiserum had been made by hyperimmunizing goats. As a preliminary step, therefore, it was thought advisable to try whether all three of the toxins were identical or not by the use of antitoxin for rat 6 toxin.

Table 8.

Toxin				Antitoxin		Result
Cl.botulinum	(rat 6)	5	M.L.D	Cl.botulinum	(rat 6) 3 c.c.	Survived
"	"	3	5 "	"	"	"
"	"	3	1 "	"	"	"
"	"	5	5 "	"	"	Died
"	"	5	1 "	"	"	Survived
"	"	6	5 "	no antitoxin		Died

The toxins of rat 6 and 3 were, therefore, apparently identical but that of rat 5 was different from the other two. The possibility of the toxin of rat 5 being a mixture of more than one toxin cannot be overlooked and as will be seen from some of the other toxin antitoxin tests the result with impure toxins and antitoxins made from them have been in some cases rather irregular.

In the next table the results of tests using antitoxin from rat 6 toxin and various toxins are given. It will be seen that a guinea pig which received toxin of rat 3 was not protected, but it probably did not die of botulism. In table 8 it has definitely been shown that the toxins of rats 3 and 6 are the same and the tests have been repeated and confirmed this result.

Table 9.

Toxin			Antitoxin		Result
B.parabotulinus Seddon	5	M.L.D	3 c.c.	Cl.botulinum (rat 6)	Died
Cl.parabot. equi (C)	5	"		-do-	"
" botulinum A	5	"		-do-	"
" " B	5	"		-do-	"
" parabot bovis (D)	2	"		-do-	Survived
" botulinum (rat 6)	5	"		-do-	"
" (" 3)	5	"		-do-	Died
" (" 5)	5	"		-do-	"
" (" 6)	5	"	no antitoxin		"

From this table it would appear that the toxin of rat 6 is of the bovis or D type.

Table 10 is practically a repetition of table 8.

Table 10.

Toxin			Antitoxin			Result
Cl.botulinum	rat	6	10 M.L.D.	3 c.c.	Cl.botulinum (rat 6)	Survived
"	"	"	1 "	"	"	"
"	"	"	5 10 "	"	"	Died
"	"	"	3 10 "	"	"	Survived
"	"	"	6 10 "	no antitoxin		Died
"	"	"	6 1 "	"	"	"

The results in this table confirm those in Table 8. Two further tables numbers 11 and 12 are of interest in the typing of the strain from rat 5.

Table 11.

Toxin		Antitoxin		Result
Cl.botulinum	(rat 3) 2 M.L.D.	Cl.botulinum (335A)		Died
"	(" 5) 2 "	"		Survived
"	(" 6) 2 "	"		Died
"	(335A) 2 "	no antitoxin		Died

Cl. botulinum (335A) is of the C type of toxin, therefore, one must place the toxin of rat 5 under this type.

Table 12.

Toxin		Antitoxin		Result
Cl.botulinum	(rat 3) 2 M.L.D.	Cl.botulinum (rat 6) 3 c.c.		Survived
"	(rat 5) 2 "	"		Died
"	(rat 6) 2 "	"		Survived
"	(rat 6) 2 "	no antitoxin		Died

The results in Table 12 confirm those in Table 10, and show that even in the amount of 2 M.L.D's the toxin of rat 5 is not neutralised by Cl. botulinum (rat 6) antitoxin. Rat 6 toxin appears definitely to be a D type. The toxins of the three rats may, therefore, be classified as follows:

Cl. botulinum (rat 6) is a Cl.parabot. bovis or Cl. botulinum D type.
Cl. botulinum (rat 3) is a Cl. parabot. bovis or Cl. Botulinum D type.
Cl. botulinum (rat 5) is a Cl. parabot. equi type, that is Cl. botulinum C.

Although one might expect the toxins of all three rats to belong to one type, there seems to be no reason why they should not differ, as they were caught in different parts of the laboratory grounds. Details of the experimental work carried out with carcases of rats appear in an article by the writer (1929), shortly to appear but at present in the press. They were carried out with the idea of throwing some light on the origin of the outbreak of botulism in mules which occurred at this Laboratory in 1924, and from which the strain of Cl. botulinum known as Cl. parabotulinum equi was isolated, later found to be identical with Cl. botulinum C of Bergtson (1922) and Graham and Boughton (1923). On testing the two toxins from outbreaks of botulism in horses and donkeys in Johannesburg district, and given in the toxin list as 11 and 628, interesting results were obtained.

Table 13.

Toxin	Antitoxin	Result
Cl.botulinum (11) 2 M.L.D's	Cl.botulinum D 3 c.c.	Survived
"	Cl.parabot equi (C) 3 c.c.	Died
"	Cl.botulinum (rat 6) 3 c.c.	"
"	" (550) 3 c.c.	"
"	" A 1 c.c.	"
"	" A & B 1 c.c.	"
"	no antitoxin	"

On repeating the test similar results were again obtained with one exception, in that the antitoxin of rat 6 protected against toxin 11 which may, therefore, be classified as a D type.

Table 14.(Contd. next page).

Table 14.

Toxin			Antitoxin			Result
2 M.L.D.	Cl.botulinum	(628)	3 c.c.	Cl. botulinum	D	Survived
"	"	"	3 "	"	C (equi)	"
"	"	"	3 "	"	554 (D type)	Died
"	"	"	3 "	"	550 (C type)	Survived
"	"	"	1 "	"	A	Died
"	"	"	1 "	"	A & B	"
"	"	"	no antitoxin			"

The results in this table are not uniform in that one guinea pig given Cl. bot D antitoxin survived as well as two with C type antitoxins. A further test was, therefore, carried out with similar results. On carrying out a further test the results were again similar with the exception that the D antitoxin in this case failed to protect, but the control survived as well.

In order to get a better idea of how this toxin should be classified a larger dose of it was used, the results being given in table 15:

Table 15.

Toxin		Antitoxin			Result
5 M.L.D.	Cl. bot 628	3 c.c.	Cl. bot	D (bovis)	Died
"	"	3 "	"	C (equi)	Survived
"	"	3 "	"	C (550)	"
"		none			Died

A further series of tests was carried out, the results being given in Table 16.

Table 16.

Toxin			Antitoxin		Result
2 M.L.D.	Cl.botul.	628	3 c.c.	(Cl. botul. 554 D)	Died
"	"		3 "	(" 550)	Survived
"	"		3 "	(" C)	"
"	"		5 "	(" D Bovis)	Died
"	"		no antitoxin		"

From this and previous tables, therefore, it would seem that Cl. botulinum 628 toxin must be classified as a C type. A small quantity of Seddon antitoxin having been made, it was decided to try the effect of it on the 628 toxin. The results, as will be seen from table 17, were rather unexpected. The antitoxin was made by the use of anatoxin alone and on testing it, 5 c.c. was found to protect against about 20 M.L.D's of B. parabotulinus toxin.

The rabbits used for the test were given doses of 3, 5, 7 and 10 c.c. of anatoxin intraperitoneally, at weekly intervals, being bled a week after the last injection.

Table 17.

Toxin		Antitoxin		Result
2 M.L.D's	Cl.botul.(628)	5 c.c.	B.parabot.(Seddon)	Survived
2 "	" D (bovis)	"	"	Died
2 "	" C	"	"	"
2 "	B.parabot. (Seddon)	"	"	Survived
2 "	"	no antitoxin		Died
2 "	Cl.botul. C U.S.A.	"	"	"

From this table it appears that Cl. botulinum 628 is of the B. parabotulinus type, that is to say, it is a variant of the C type. This observation is of considerable interest, as it tends to show that there are probably intermediate types of the Cl. botulinum not corresponding exactly to either the A, B, C or D types. Pfenninger's (1924) observation as to the antitoxin against B. parabotulinus only neutralising its own particular toxin, is confirmed.

The toxin of Cl. botulinum 628 may, therefore, be considered as a variant of the C type corresponding very closely to the Cl. botulinum of Seddon (B. parabotulinus).

The last three toxins to be typed were as follows:

(1) Zoo strain (from intestinal contents of wild ducks in Pretoria Zoo.

(2) Bekker strain (from carcase material from cases of botulism (lamsiekte)in sheep at Bredasdorp, Cape Province,

(3) Carlisle strain (from intestinal contents of turkeys in an outbreak of botulism in these birds on a farm near Pretoria).

The first strain was obtained from the coeca of a species of ornamental duck, one of eighteen, which died at Pretoria Zoo after a heavy rainfall. These ducks were all in one small pond, and all showed typical symptoms of botulism. The outbreak is described in an article by the writer (1929), but unfortunately the source of the infection could not be traced. Strains of an organism of the botulinus type were obtained from the coeca of three of these ducks.

The second one referred to as Bekker strain, was of great interest, as it was obtained from an outbreak of lamsiekte (botulism) in sheep. The disease in the Bredasdorp area of the Cape Province is associated with a definite phosphorus deficiency in the pasture, causing the sheep to show a marked tendency to develop osteophagia. There is an unusual brittleness of bone shown by the sheep in this area and fractures of ribs or long bones are very common. Lamsiekte in sheep is uncommon in the Union of South Africa though occasionally seen in goats. Recently, Sigwart (1929) has published a paper on "Lamsiekte in Sheep" in South West Africa. The symptoms described leave no doubt as to the disease being true lamsiekte as seen in cattle. It occurs chiefly in Afrikander sheep, but may also be seen in the merino. Carcase material from the farms where the disease occurs has not yet been tested for botulinus toxins, but material will shortly be available for this purpose.

The third strain (Carlisle) was obtained from the coecal contents of a turkey, one of fifteen young adult birds which died on a farm near Pretoria. They were all affected at the same time and showed definite symptoms of botulism. The source of the infection could not be traced, but the turkeys were running free and the owner could not be certain what they had been eating.

In the first table the three strains were tested with a combined Cl. botulinum A and B antitoxin.

Table 18.

Toxin			Antitoxin			Result
2	M.L.D's	Cl.botulinum (Carlisle)	1	c.c.	botulinum A + B	Died
2	"	Cl.botulinum (Bekker)	1	"	"	"
2	"	Cl. botulinum (Zoo)	1	"	"	"
5	"	Cl.botulinum A	1	"	"	Survived
5	"	" B	1	"	"	"
5	"	" A	no antitoxin			Died
5	"	" B	"		"	"

The three strains were, therefore, not Cl. botulinum A or B types.

Further tests were then carried out using three different antitoxins, Cl. botulinum D, Cl. botulinum C (equi type) and Cl. botulinum 550, also an equi type. The results were uniform, as will be seen from the following three tables numbers

Table 19.

Toxin			Antitoxin			Result
5	M.L.D.	Cl.botulinum Carlisle)	2	c.c.	Cl.botulinum C (equi)	Survived
5	"	Cl.botulinum (Bekker)	"		"	Died
5	"	Cl.botulinum (Zoo)	"		"	Survived
5	"	Cl.botulinum (equi)	"		"	"
2	"	Cl. "	no antitoxin			Died

The turkey and zoo strains therefore corresponded to the Cl. botulinum C type.

Table 20.

Toxin			Antitoxin			Result
5	M.L.D.	Cl.botulinum (Carlisle)	3	c.c.	Cl.botulinum 550 (C type)	Survived
5	"	" (Bekker)	"		"	Died
5	"	" (Zoo)	"		"	Survived
5	"	" 550 (C)	"		"	"
2	"	" 334 D	2		"	Died

The results in this table confirm those in the previous one, the antitoxin being of the same type.

Table 21.

Toxin			Antitoxin		Result
5 M.L.D.	C.botulinum	(Carlisle)	3 c.c.	Cl.botulinum D	Died
5 "	"	(Bekker)	"	"	Survived
5 "	"	(Zoo)	"	"	Died
5 "	"	(334) D	"	"	Survived
2 "	"	"	no antitoxin		Died

The strain from material from cases of lamsiekte in sheep, therefore, belongs to the Cl. botulinum D type. This is a very interesting fact in view of the distribution of the disease in cattle and sheep.

The three strains just described may, therefore, be classified as follows:

(1) Cl. botulinum (Zoo strain, from birds) is a C type
(2) " " (Bekker " , from sheep) " " D "
(3) " " (Carlisle" , " turkey) " " C "

It may be of interest to give a composite table showing the results obtained with toxin antitoxin tests on all the strains used:

TABLE SUMMARISING TOXIN ANTITOXIN TESTS.

Cl.botulinum D (Cl.parabot.bovis)	Cl.botulinum C (Cl.parabot.equi)	Cl.botulinum C (Graham, U.S.A.)	B.parabotulinus (Seddon)	Cl.botulinum (Rat 3)	Cl.botulinum (Rat 5)	Cl. botulinum 554 (Rat 6)	Cl. botulinum 333
D (bovis) = +	C (equi) = +	D (bovis) = -	D (bovis) = -	bovis (Rat 6) = +	C (335A) = +	D bovis +	C (equi) +
D (bovis) = +	C = +	C = +	A + B = -		C (equi) = -	D (Rat 6) = +	D (bovis) -
A = -	D (bovis) = -	C (equi) = +	C = +		D (bovis) = -	D (Rat 6) = +	Rat 6 (bovis) -
B = -	A + B = -	A + B = -	B.parabot. = +		A = -	C (335 A) = -	
A + B = -		B.parabot. = -			B = -		
C (equi) = -					D (Rat 6) = -		
↑ D type Cl.botulinum 334 ↓	↑ C type Cl.botulinum 335 ↓	↑ C type Cl.botulinum 335(A) ↓	↑ C type Cl.botulinum 628 (Horses in Johannesburg) ↓	↑ D type Cl.botulinum 11 (Donkeys, Johannesburg) ↓	↑ Probably C type Cl.botulinum (Turkey strain) ↓	↑ D type Cl.botulinum (Bekker, sheep lamsiekte) ↓	↑ C type Cl.botulinum (Zoo bird strain) ↓
C (equi) = -	C (equi) = -	C (equi) = ∓	D (bovis) = -	D (bovis) = +	C (equi) = +	335(A) = ∓	C (equi) = +
D (Bovis) = +	D (bovis) = +	C (equi) = +	D (bovis) = -	C (equi) = -	C (equi) = +	C (equi) = -	C (equi) = +
D (Rat 6) = +	C (equi) = -	D (bovis) = -	C (equi) = +	D (Rat 6) = +	C (equi) = +	C (equi) = +	C (equi) = +
C (335A) = -	D (bovis) = +	D (bovis) = -	C (335A) = +	C (335A) = -	D (bovis) = -	C (equi) = -	D (bovis) = -
			A + B = -	A = -	D (bovis) = -	D (bovis) = +	D (bovis) = ∓
			B.parabot. = + (Seddon)	A + B = -	D (bovis) = -	-do- = +	A + B = -
					A + B = -	-do- = ∓	
						A + B = -	
D type	D type	C type	C type	D type	C type	D type	C type

The toxin is given above the column and the antitoxins in the column. Where an antitoxin appears more than once in a column it means the test was repeated. Result appears under the column.

\+ - neutralised

\- - not neutralised

∓ - doubtful result.

There is no doubt that classification of types of Cl. botulinum toxin by cross toxin antitoxin tests forms the best means for the purpose at present available. With impure cultures one may sometimes get toxins which are difficult to classify absolutely definitely, but even with them one can generally accomplish this satisfactorily.

The bearing of this work on the question of immunisation against botulism in animals, more particularly cattle, sheep and goats, is of great interest.. It shows that any such immunisation, if it were ever considered advisable, would have to be done with polyvalent antitoxins in the case of passive and anatoxins in that of active immunisation.

GENERAL CONCLUSIONS.

It is clear from the work carried out by many investigators, including the writer, that apart from the A and B types of the Clostridium botulinum ,one has to dealwith at least two other types in nature, namely the C and D types.

All the types dealt with by the writer can be classified either under the C or the D one, as judged by comparative toxin-antitoxin tests. The position of the B.parabotulinus of Seddon is still somewhat uncertain though it is definitely related to the C type.

Meyer (K.F), in a private communication to the writer mentions that he obtained an organism of the Cl.botulinum A type from a culture sent to him by the writer. This is the only record of the occurrence of this particular type in South Africa.

It would appear that the organism described by Theiler and the writer as "Clostridium parabotulinum equi" must definitely be regarded as identical with the C type of Bengtson and, that of Graham and Boughton as judged by toxin antitoxin tests. There is no doubt, however, that it is very much more toxic for horses than the American types.

The organism described as "Clostridium parabotulinum bovis" must now definitely be classified as "Cl. botulinum D" (Theiler and Robinson) as suggested by Meyer and Gunnison (1929). It must, however, be emphasised that organisms which belong to the C type exist on "lamsiekte" farms and therefore, "lamsiekte" can no longer be considered as due to one particular toxin.

In the foregoing pages the writer has attempted to summarise our knowledge of the organisms of the Cl.botulinum C and D types at the present date, and give a survey of the known facts in connection with them.

The comparison by morphological and cultural characteristics does not point to any special features which would be of value in distinguishing the bacteria classified under these two types, in fact, one is struck by their close resemblance in almost every particular.

Comparative toxicity tests cannot be regarded as of much value in differentiating the types dealt with. There are marked differences in toxicity for certain animal species shown by different types, but it is quite possible, judging from our knowledge of exo-toxins in general, that the same type of the Clostridium botulinum isolated from different sources might have a very variable toxicity.

Comparative agglutination tests have given very interesting results, and are of undoubted value in indicating the relationships of various Clostridium botulinum types. The group relationships which exist are very well brought out by these tests.

Finally, comparisons by toxin antitoxin tests were carried out and proved by far the most valuable means of classifying these organisms. One particular advantage of them is that one can attempt the classification of toxins from impure cultures with a fair degree of success.

The writer is well aware of the defects which still exist in our knowledge of the bacteria of the Clostridium C and D types, but recognises the value of from time to time reviewing our knowledge in connection with a particular subject or part of a subject, and it was with this idea that the present article was written.

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