Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 5, Number 1, July, 1935.

The Antigenic Components of the Toxins of *Cl. botulinum* Types C and D.

By J. H. MASON, F.R.C.V.S., F.R.S.E., Empire Marketing Board Research Fellow;

E. M. ROBINSON, F.R.C.V.S., D.V.Sc., Dr.Vet.Med., Sub-Director of Veterinary Services, Onderstepoort.

INTRODUCTION.

Intoxications caused by the toxins of the C and D types of *Cl. botulinum* have been reported from different parts of the world (America, Australia, Tasmania, and South Africa), the diseases produced usually being associated with the consumption of carrion or decomposed material. Although the fact that the C and D types do cause intoxications in the domestic animals is well established, the relationship of the toxins of the C type to another and their relationship to the D type is not in the same position. Bengtson (1922, 1923) reported that toxic filtrates of the C type organism isolated from fly larvae were not neutralized by A or B type antitoxin and that C antitoxin did not neutralize A or B toxins. Seddon (1922), discussing his parabotulinus bacillus, noted that there was no cross protection between it and the A or B types. Pfenniger (1924) stated that 0·2 c.c. to 0·5 c.c. of C antitoxin neutralized two fatal doses (for guinea-pig) of C toxin, but that this amount of toxin was not neutralized by A, B, or Seddon antitoxins. Further, Seddon toxin was neutralized by his C antitoxin, but not by A or B.

Doyle (1923) noted that the toxin associated with limberneck in chickens, investigated by him, was not neutralized by A or B type antitoxins. Graham and Boughton (1924) were able, with antitoxins produced with type C toxin, to neutralize Seddon toxin, and, further, antitoxin made with the toxin produced by limberneck (type C) germs neutralized homologous, heterologous, and fly larvae toxins. As already reported (Robinson, 1930), one of us (E.M.R.) showed that the South African C type toxin (equine origin), a C type toxin (Graham, U.S.A.), and the Seddon toxin cross-neutralized; however, D antitoxin did not neutralize any of these toxins. Further, in a personal communication, Graham stated that his C antitoxin neutralized *Cl. parabotulinum* toxin (i.e. C type toxin of South African equine origin). Meyer and Gunnison (1929), examining cultures, received from this institute, stated that the D type toxin was not neutralized by A, B, or C antitoxins. Gunnison and Meyer (1932), working with a disease in ducks associated with *Cl. botulinum* type C, showed that antitoxins prepared from the duck strain and

from strain 526 Ca neutralized the duck, 526 Ca, Seddon, and 550 Cb toxins, whereas Seddon (Cb) antitoxin neutralized only the homologous and the 550 Cb toxins. Bennetts (1933) showed that the toxin prepared from an impure culture of a type C strain was neutralized by the Australian parabotulinum antitoxin and by C antitoxin. Theiler and Robinson (1928) stated that the D type antitoxin (5·0 c.c.) did not neutralize two lethal doses of the South African C, the Seddon, American C, or the A type toxins.

From the foregoing it is clear that unanimity of opinion does not exist as regards the relationship of the C toxins to one another. The possibility exists that a cause of this may be that the antitoxins used were of very low titre. This opinion is supported by the fact that large amounts of antitoxin (up to 5·0 c.c.) were required to neutralize only a few fatal doses of toxin. However, the literature would indicate that the D type (South African bovine, associated with lamsiekte) toxin is monospecific.

In view of the lack of uniformity of results and as work on another group of anaerobes, the *Cl. welchii-Cl. ovitoxicus* group, showed that the toxins produced by individual members may share the same toxic fractions [Wilsdon (1931), Glenny *et alia* (1933), Montgomerie and Rowlands (1934), Dalling (1934), Gill (1933)], work was commenced on six C type, one D type, and one A and one B type toxins. In the authors' opinion, the results obtained show that in the C and D groups there is, as in the *Cl. welchii* group, a sharing of toxic antigenic fractions.

METHODS.

Origin of Cultures.—The cultures 26C, Seddon, and D were all impure, the contaminants being proteolytic non-pathogenic anaerobes. That the only toxin-producing germs contained therein were of the botulinus type was shown by the fact that one certain lethal dose of toxin (for mouse) was not neutralized by 0·1 c.c. of the antitoxins of *Cl. septique*, *Cl. welchii*, *Cl. oedematiens*, *Cl. histolyticum*, *Cl. tetani*, *Cl. ovitoxicus*, and the "lamb dysentery bacillus", or by a mixture of 0·025 c.c. of each of them. A determined attempt to purify the D strain failed. One of us (E.M.R.), some years ago, was able to reduce the number of concomitant micro-organisms to two, and after further work eventually sealed off the culture as pure. However, on subculturing this sealed-off culture two years later, the presence of at least two non-pathogenic anaerobes was noted.

The other C and D strains, all received from Dr. K. F. Meyer, California, appeared, on microscopical and cultural examination, to be pure cultures.

The A and B strains gave no indication of impurity.

26 *C.*—A South African equine strain isolated at these laboratories from a decomposed rat.

Seddon.—A strain received from Dr. Seddon, Australia.

D.—A " bovis " strain, isolated from a bovine dead from lamsiekte, Armoedsvlakte.

A and B.—Received from the Lister Institute, London.

Strain 165, Type D: from K. F. Meyer . Labelled " South African Bovine ".

Strain 178, Type C: from K. F. Meyer. Labelled " South African 550 ".

Strain 191, Type C: from K. F. Meyer. Labelled " Bengtson 526 ".

Strain 192, Type C: from K. F. Meyer. Labelled " Seddon strain from Australia ".

Strain 205, Type C: from K. F. Meyer Labelled " Bengtson 1187 ".

Strain 165 D proved non-toxic, as did a further culture of the same organism received from Dr. Meyer.

Preparation of Toxins.—Robertson's meat broth (horse muscle extract, 1 per cent. peptone, 0·5 per cent. NaCl; the flask or tube containing boiled meat to about one-third the volume), plus 5 per cent. sterile horse serum was used in all cases to produce the toxin. The medium (pH 7·6) was boiled for two hours, cooled quickly, the serum added, inoculated with a 48-hours' culture of the germ, and incubated for seven days at 37° C. The culture was then rendered sterile by passing it through, first, a paper pulp and then a Berkefeld filter candle.

To render the toxin stable the toxic filtrate was saturated with ammonium sulphate (about 55-60 grams per 100 c.c.) and the resultant precipitate pressed, dried *in vacuo* and powdered. Such powder, dissolved in saline, maintained its original (dry) value throughout the experiment.

Production of Antitoxin.—Goats proved suitable for this purpose, being cheap in price and large enough to give a sufficiency of serum for the number of tests carried out. Formol-toxoid was employed in the initial immunization, being prepared by adding 0·3 per cent. commercial formalin (40 per cent. formaldehyde) to the pulped (not candled) toxin at pH 7·4, and incubating this at 37° C. until 0·1 c.c. did not kill a mouse on subcutaneous inoculation. The time necessary for this to occur varied between 1 and 5 weeks. It was found that it was essential to reach this degree of non-toxicity before commencing immunization; on two occasions, the use of a C (South African) equine toxin, the toxicity of which was such that 0·1 c.c. killed a mouse in three days (0·05 c.c. being non-lethal), killed two goats when a dose of 2·0 c.c. was reached. When a dose of toxoid between 20 c.c. and 30 c.c. had been reached, one could with impunity double the dose weekly or change over to unmodified toxin. The final 6-8 inoculations were of toxin, each dose being between 100 c.c. and 200 c.c. in amount. Unless the condition of the goat did not warrant it, the injections were made twice weekly, the previous dose being increased 50 per cent. and later 75 per cent. or 100 per cent.

The course of immunization lasted between two and three months. Trial weekly titrations of bleedings were made, commencing at about the second month; when no rise in antitoxin over the previous one was detected, the animal was exsanguinated, the serum collected from the clot and preserved with 0·5 per cent. phenol. One antitoxin, D(r), was prepared, some years ago, by one of us (E.M.R.) in a horse by the use of the D strain toxin.

Titrations of Toxin and Antitoxin.—Mice of about 15-18 grams were employed as test animals, the route of injection being the subcutaneous. Whilst it is realized that the guinea-pig has been the test animal of choice for the titration of botulinus toxin and antitoxin, the mouse proved to be quite as accurate an indicator, and further was cheaper, more easily housed in large numbers, and obtainable in uniform body weight.

Dry toxin was dissolved in saline in the proportion of 100 mgm. to 5.0 c.c. and from this solution dilutions again made in saline. The approximate minimal fatal dose was then worked out, and whilst no attempt was made to ascertain this very accurately, sufficient mice were injected around the probable $L.D._{50}$ (Trevan) point to be reasonably certain that a certain fatal dose could be predicted. The end point was sufficiently sharp, with all toxins at about the M.L.D. level to allow a 30 per cent. drop in toxin amount to result in a " live " or " paralysed " instead of a " death ".

For antitoxin titrations, a test dose of toxin was chosen after a few preliminary trials. Beyond ensuring that this dose contained at least 10 sure fatal doses no attempt was made to have the same number of lethal doses in the test dose of each toxin. This dose, mixed with varying amounts of antitoxin, was left for one hour at room temperature (20°-26° C.) and injected into mice. Where preliminary titrations showed that a serum was poor or deficient in antitoxin, one certain fatal dose of toxin was mixed with from 0·1 c.c. to 0·5 c.c. of antitoxin and injected.

In all cases, observation was carried on for seven days, results being recorded as " lived ", " paralysed ", or " dead ".

EXPERIMENTAL.

In the first series of titrations, the neturalizing power of the antitoxins of 26 C, Seddon, and D [including D(r)] was tested against toxins made from these strains. An example of such a test is given in Table 1.

TABLE 1.

Toxin: 26C. *MLD*: 0·0003 c.c. *T.D.*: 0·01 c.c.
Antitoxin: Seddon.

Dose (c.c.)	D.	P.	L.
0·0007	4	0	0
0·0008	3	1	0
0·001	0	1	4
0·0012	0	0	3

(M.L.D. = minimum lethal dose. T.D. = test dose. D = died. L = lived. P = paralysed, i.e. showed paralysis during the seven days' observation but survived seven days. In this titration 0·001 c.c. of antitoxin is taken as a neutralizing dose.)

Similar titrations were carried out with all the toxins and antitoxins, at least three and usually six to eight mice being injected at those levels just around the neutral point. Table 2 summarises the titrations of the toxins of 26 C, Seddon, and D against the antitoxins of these strains. The central unenclosed figure is the neutralizing dose of antitoxin.

TABLE 2.

Toxin.	MLD.	T.D.	Antitoxins.			
			26 C.	S.	D.	D(r).
26 C	0·0004	0·01	(3–3½) 0·003– 0·0035 [1]	(1) 0·001 [1]	(50) 0·05 [1]	(200–240) 0·1– 0·12 [1]
S	0·0001	0·01	(4) 0·005– 0·006 [1½–2]	(1) 0·0015 [1½]	(66) 0·1 [2]	(5⅓) 0·08 [4]
D	0·000002	0·01	(1¾) 0·02– 0·025 [600]	(1) 0·03– 0·035 [1200]	(1/600) 0·02 [2/5]	(1/440) 0·025 [1/8]

T.D. of D toxin v 26 C and S antitoxins = 0·00001 and 0·000025 c.c. respectively.
T.D. of 26 C toxin v D(r) antitoxin = 0·005 c.c.
T.D. of S toxin v D(r) antitoxin = 0·001 c.c.
All unenclosed figures = c.cs.
Figures in [ ] = ratio of one toxin to another.
Figures in ( ) = ratio of one antitoxin to another.
Central unenclosed figure = neutralizing dose of antitoxin.

The conclusions to be drawn from Table 2 are (1) the toxins 26 C and Seddon are antigenically the same and (2) that D toxin contains some C antigen and the C and Seddon toxins some D antigen, but that the amount of heterologous antigen contained in each toxin varies greatly, as does the amount of heterologous antitoxin contained in each serum.

In table 3 the results of titrating fresh brews (precipated with ammonium sulphate and dried as before) of 26 C and Seddon toxins against antitoxins of 192 C, 178 C, 191 C, and 205 C are summarized. The data given therein confirms the results shown in Table 2, viz., that the toxins, 26 C, and Seddon, are antigenically the same.

TABLE 3.

Toxins.	MLD.	T.D.	Antitoxins.			
			192	178	191	205
26C	0·001	0·01	(12) 0·006 [1]	(7) 0·0035 [1]	(12) 0·006 [1]	(1) 0·0005 [1]
S	0·00025	0·01	(8–10) 0·02– 0·025 [4]	(4–6) 0·01– 0·015 [4]	(8–10) 0·02– 0·025 [4]	(1) 0·0025 [5]

Central unenclosed figure = neutralizing dose of antitoxin (c.c.).
All unenclosed figures = c.c.
Figures in [ ] = ratio of one toxin to another.
Figures in ( ) = ratio of one antitoxin to another.

In Table 4 is summarized the titration of the toxins of 192 C, 178 C, 191 C, and 205 C against the six C antitoxins.

TABLE 4.

Toxins.	MLD.	T.D.	Antitoxins.					
			26 C.	Seddon.	192 C.	178 C.	191 C.	205 C.
192 C.	0·0005	T.D. =	0·1 (3–4) 0·01 [1/3]	0·1 (1) 0·0025– 0·003 [1/4–1/3]	0·025 (27) 0·017 [1/3]	0·025(13-16) 0·01– 0·012 [1/3]	0·1 (10–12) 0·02– 0·03 [1/4]	0·1 ($1\frac{1}{3}$–$1\frac{5}{8}$) 0·004 [1/3]
178 C.	0·0001	T.D. =	0·1 ($3\frac{1}{2}$) 0·035 [1]	0·1 (1) 0·01 [1]	0·01 (25) 0·025 [1]	0·01 (10–12) 0·01– 0·012 [1]	0·01 (17) 0·017 [$1\frac{5}{12}$]	0·1 ($1\frac{1}{2}$) 0·015 [$1\frac{1}{4}$]
191 C.	0·0004	T.D. =	0·01 (4) 0·035– 0·045 [1–$1\frac{1}{2}$]	0·1 (1) 0·01 [1]	0·002 (5) 0·01 [2]	0·01 (10) 0·1 [5/6]	0·01 ($\frac{1}{2}$) 0·004– 0·005 [1/30]	0·01 ($\frac{1}{4}$) 0·0025 [1/5–1/4]
205 C.	0·00015	T.D. =	0·1 (3) 0·03– 0·035 [1]	0·1 (1) 0·01– 0·012 [1]	0·01 (20–25) 0·025 [1]	0·01 (10–12) 0·012 [1]	0·01 (10–12) 0·012 [1]	0·01 (1–$1\frac{1}{5}$) 0·012 [1]

All unenclosed figures = c.c.
Top unenclosed figures opposite T.D. = test doses used.
Figures in [ ] = ratio of one toxin to another.
Figures in ( ) = ratio of one antitoxin to another.
Central unenclosed figures = neutralizing dose of antitoxin.

From Tables 3 and 4 the following information may be gathered: the toxins 26 C, Seddon, 192 C, and 178 C are antigenically indistinguishable. As, however, 192 C and 178 C are merely purified cultures of 26 C and Seddon respectively, one can say that, by the methods employed, no difference in the antigenic constitution of their toxins can be elucidated. Further, reference to Table 2 indicates that the amount of the D toxin fraction of their make-up does not differ much, if at all. These two toxins and the two Bengtson strains, 191 C, and 205 C, share two antigens, one of which may be termed the "main" and the other the "subsidiary" fraction. In the elimination of the 191 C toxin titration from Table 4, the subsidiary fraction would remain unrevealed, and the other three toxins would be considered identical. It is obvious from the table that the expected ratios are not obtained with the use of 191 C toxin; three and possibly four figures are "out", two of them beyond the limits of experimental error and of the method of testing. If the toxin molecule be considered as containing three fractions C_1, C_2, and D, then the apparent discrepancy obtained with the use of 191 C toxin may be explained. The particular brew employed contained an excess of the C_2 fraction and, as follows, the antitoxin produced by it contained an excess of C_2 antitoxin.

As there was the possibility that 191 C toxin readily dissociated, *in vivo*, from a toxin-antitoxin mixture, a number of tests were put up to check the point. On three different occasions, mixtures were made, and their effect on mice ascertained after they had stood for one hour at room temperature and at 5° C. for 24 hours. The results did not differ materially from those recorded in Table 4.

In table 5 are recorded the results of the titration of four C type toxins against the D and D(r) antitoxins.

TABLE 5.

Antitoxins.	Toxins.				
	T.D.	192 C.	178 C.	191 C.	205 C.
D	T.D. =	0·01 [1] 0·05 (1)	0·001 [21/2] 0·02 (1)	0·0025 [1/2] 0·12 (1)	0·005 [1/2] 0·1 (1)
D(r)	T.D. =	0·005 [1] 0·1 (1/4)	0·001 [3/4] 0·15 (1/7)	0·0005 [=] >0·3 (=)	0·001 [1] 0·1 (1/5)

T.D. = Test dose of toxin used. (c.c.)
Central unenclosed figure = neutralising dose of antitoxin (c.c.).
Figures in [ ] = ratio of one toxin to another.
Figures in ( ) = ratio of one antitoxin to another.

From the data given in Table 5 it is apparent that the amount of C antitoxin contained in the two D sera is not the same, in each instance D(r) being much the poorer; it will be seen that 0·3 c.c.

of D(r) antitoxin was unable to neutralize one M.L.D. of 191 C toxin. The possibility exists that this discrepant result may be explained by the amounts of the C_1 and C_2 fractions contained in the toxins and antitoxins. As Table 4 shows, 191 C toxin contains an excess of the C_2 fraction; the lack of the anti-C_2 fraction in D(r) antitoxin would explain its inability to neutralize.

Table 2 showed that cross-neutralization existed between the toxins and antitoxins of 26 C, Seddon, and D. Using a fresh brew of D toxin, a series of tests were put up to ascertain the neutralizing power of all the C antitoxins against it. Table 6 summarizes the results.

TABLE 6.

(M.L.D. of new D toxin = 0·000002 c.c.)

Antitoxins (c.c.).

	26 C.	S.	205 C.	192 C.	178 C.	191 C.
Toxin D (c.c.)	0·1 neutralized 0·000007–0·00001	0·1 neutralized 0·00001–0·000015	0·1 neutralized 0·00001–0·000015	0·5	0·5	0·5
				Did not neutralize one M.L.D.		

The antitoxins 26 C, Seddon, and 205 C in doses of 0·1 c.c. were able to neutralize a few M.L.D. of D toxin, but the three other C sera, even in doses of 0·5 c.c. were incapable of neutralizing one fatal dose. The antitoxic fraction of 191 C serum was salted out by half saturation with ammonium sulphate; the precipitate was dialysed against distilled water for three days and the inner fluid fanned down to one-quarter of the original volume. Although the anti-C (191) value of this concentrate was three times that of the original, 0·5 c.c. was incapable of neutralizing one M.L.D. of D toxin. The possibility existed that the goats used to produce 192, 178, and 191 antitoxins did not receive enough of the D fraction (presumed to be present in small quantity in the C toxic filtrates) to give rise to the production of D antitoxin in demonstrable amount. Further, the C fraction of the toxin so preponderated over the D that unless the animal body had exhausted its power of producing C antitoxin, there would be little chance of D antitoxin being produced in appreciable quantity. Immunization was therefore undertaken in three new goats, and carried on for three months, finishing up the process by injecting 100-200 c.c. toxin ten times within three weeks. The 192 goat was then bled out, but the other two were rested for a fortnight and then " shocked " by injections of 100-200 c.c. of toxin given every second day for ten days. By this method antisera were obtained containing demonstrable amounts of D antitoxin in the case of 191 and 178 antitoxins. Table 7 gives the results.

TABLE 7.

(M.L.D. of D toxin = 0·000002 c.c.)

Antitoxins.

	191 C.	178 C.	192 C.
Toxin D	0·5 c.c. neutralized 0·000004 c.c.–0·000005 c.c.	0·1 c.c. neutralized 0·000015 c.c.	0·5 c.c. did not neutralize one M.L.D.

As a control to the large dose of serum (0·5 c.c.) used, as shown in Table 7, experiments were carried out using 0·5 c.c. of normal goat serum and 0·5 c.c. of antitoxin prepared in goats against *Cl. welchii*, Type B, *Cl. chauvoei*, *Cl. ovitoxicus*, and *Cl. septique*. In no instance did this amount neutralize one sure lethal dose of D toxin. The results given in Table 7 show that the toxins of 191 C and 178 C contain a small amount of D toxin; the failure of 192 antitoxin may mean either that its toxin contains none of the D type toxin or that immunization was not carried far enough to allow of the production of demonstrable amounts of antitoxin.

The A and B type toxins appeared to be monospecific. A test dose (about 20 M.L.D.) of A toxin was neutralized by 0·015 c.c. of its own antitoxin, whereas 0·2 c.c. of B, C (26 C, S, 191 C, 192 C, 178 C, and 205 C), and D antitoxins failed to neutralize one fatal dose. Further, from 0·1 c.c. to 0·2 c.c. of A antitoxin did not neutralize one lethal dose of the toxins of the B, D, and the various C toxins. In the same way, B toxin was neutralized by its own antitoxin, one test dose, about 100 lethal doses, being rendered non-toxic by 0·01 c.c. of antitoxin; however, its toxin (one M.L.D.) was unaffected by the other antitoxins and its antitoxin had no neutralizing power on the other toxins.

DISCUSSION.

The results just recorded show that the toxins of the C and D botulinus group contain more than one toxic component. That there are three antigens concerned is fairly obvious—the 2 C fractions, C_1 and C_2, and the D. However, no proof has been presented that these are the only antigens present, this investigation merely showing that, by ordinary methods and reasonably careful technique, some of the fractions making up the toxic molecule may be demonstrated. It is realized that a great deal more work would have to be done in order to arrive at anything approaching finality on the question. To explain an apparent discrepancy, the hypothesis has been formulated that the C toxin is composed of a C_1 and a C_2 fraction. Assuming this to be correct, there is the definite possibility, arguing from the analogy of the *Cl. welchii-ovitoxicus* group, that the proportion of the C_1 and C_2 fractions produced in culture would vary, depending upon the age of the culture. As an example it may be stated that *Cl. welchii*,

Type B, produces, in the early stages (12-24 hours) of growth only the *Cl. paludis* type of toxin, whereas if growth is allowed to continue, this diminishes greatly, to be replaced in large measure by the *Cl. ovitoxicus* type of toxin.

Further, as has been shown in the *Cl. welchii* group, the animal to be immunized also enters into the question; depending upon its " natural " immunity and no doubt also on factors unknown, the antitoxin produced will differ qualitatively as well as quantitatively. Thus, it is obvious, arguing from such a standpoint, that this investigation serves merely as a pointer to further work.

A second and obvious explanation for the sharing of antigens is that the cultures were mixtures of more than one C and the D types. This is a possible but extremely improbable explanation. Against it are the following facts: (1) the cultures originated in Australia, South Africa, and America; it is unreasonable to suggest that in each case the C (two varieties) and the D types were picked up; (2) there is some regularity in the anti-C content of D sera and the anti-D content of C sera (Tables 2, 5, 6, and 7). It will be seen that 0·02 c.c. to 0·1 c.c. of D antitoxin neutralizes 6 to 20 fatal doses of C toxin, whereas from 0·1 c.c. to 0·5 c.c. of the C antitoxins was necessary to neutralize two to seven lethal doses of D toxin. In toxic filtrates made from a mixture, one would expect greater variation of the C and D fractions, as judged by the anti-C and anti-D content of the sera. As, in the production of the antitoxins, several (2 to 5) different brews of toxin were used to produce each antitoxin and as the anti-C content of the D sera and the anti-D content of the C sera was always very low, it would appear unlikely that a mixed culture is the explanation of the sharing of antigens. Again with regard to the C toxins—if the irregularity obtained with 191 C toxin and antitoxin is to be explained by assuming contamination, then all the C cultures contained this contaminant. As before stated, such an occurrence is extremely unlikely.

Conclusions.

Toxic broth filtrates of one A, one B, six C, and one D types *Cl. botulinum*, and the antitoxins produced by injecting these filtrates into goats have been tested, from the standpoint of working out the antigenic " make up " of the toxins. The results obtained indicate:—

(1) The A and the B types are monospecific.

(2) The C types contain three components (*a*) C_1, (*b*) C_2, and D. The D fraction is contained in only very slight amount.

(3) The D type contains chiefly the D, but also a small quantity of the C fraction.

REFERENCES.

BENGTSON, I. A. (1922). Preliminary Note on a Toxin-producing Anaerobe isolated from the larvae of *Lucilia Caesar*. *Publ. Health Report*, Vol. 37, Part I, p. 164.

BENGTSON, I. A. (1923). A Toxin-producing Anaerobe isolated from Fly Larvae. *Publ. Health Report*, Vol. 38, Part 1, p. 340.

BENNETTS, H. W. (1933). Botulism in Sheep in Western Australia and its Association with Sarcophagia. *Jnl. of Counc. Sci. and Indust. Res.*, Vol. 6, No. 3, p. 137.

DALLING, T. (1934). A Note following Montgomerie's and Rowlands' Article. *Vet. Jnl.*, Vol. 90, No. 8, p. 337.

DOYLE, L. P. (1923). Limberneck in Chickens. *J. Amer. Vet. Med. Ass.*, Vol. 16, p. 754.

GILL, D. A. (1933). Pulpy Kidney Disease of Lambs (an Acute Enterotoxaemia of Bacterial Origin). *Research Report, New Zealand Dept. of Agric., Wellington.*

GLENNY, A. T.; BARR, M.; JONES, M. LL.; DALLING, T.; AND ROSS, H. E. (1933). Multiple Toxins Produced by Some Organisms of the *Cl. welchii* group. *J. Path. and Bact.*, Vol. 37, p. 35.

GRAHAM, R., AND BOUGHTON, I. D. (1924). *Cl. botulinum* type C associated with a Limberneck-like Disease in Chickens and Ducks. *J. Amer. Vet. Med. Ass. N.S.*, Vol. 17, p. 723.

GUNNISON, J. B., AND COLEMAN, G. E. (1932). *Cl. botulinum* type C associated with Western Duck Disease. *J. Inf. Dis.*, Vol. 57, No. 3, p. 542.

MEYER, K. F., AND GUNNISON, J. B. (1929). South African Strains of *Cl. botulinum* and *parabotulinum*, XXXVII. With a description of *Cl. botulinum* type D n. sp. *J. Inf. Dis.*, Vol. 45, p. 106.

MONTGOMERIE, R. F., AND ROWLANDS, W. T. (1934). Lamb Dysentery in North Wales. *Vet. Jnl.*, Vol. 90, No. 8, p. 323.

PFENNIGER, W. (1924). Toxico-immunologic and Serologic Relationship of *B. botulinus*, type C and *Parabotulinus* " Seddon ", XXII. *J. Inf. Dis.*, Vol. 35, p. 347.

ROBINSON, E. M. (1930). The bacteria of the *Cl. botulinum* C and D types. *16th Rpt. Dir. Vet. Serv. Union of S.A.*

SEDDON, H. R. (1922). The Specific Identity of *Bacillus parabotulinus*. *J. Comp. Path. and Therap.*, Vol. 35, p. 275.

WILSDON, A. J. (1931). Observations on the Classification of *Bacillus welchii*. *Sec. Rept. Direct. Instit. Anim. Path. Univ. Cambridge*, p. 53.