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The Bloedpens Strain of *Cl. welchii*, Type B, Wilsdon (the "Lamb Dysentery Bacillus").

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INTRODUCTION.

WHEN the writer arrived at Onderstepoort in 1931, he discussed with his colleague, Dr. E. M. Robinson, the similarity of a lamb dysentery-like-disease, "bloedpens", prevalent in certain districts of South Africa, with lamb dysentery as known in Great Britain. From descriptions of the germ isolated, from a naturally-infected lamb, by Dr. Robinson, he came to the conclusion that in all probability, the disease and the causative microbe were the same as in Britain. To confirm this opinion, toxin, prepared from the bloedpens (BP) strain was titrated intradermally (i.d.) in guinea pigs against a lamb dysentery (L.D.) antitoxin brought from overseas (Wellcome Laboratories). It should be emphasised that the only point at issue was whether or not L.D. antitoxin was capable of neutralizing the toxin; no attempt at accurate work was contemplated. Actually 0·01 c.c. of anti-serum neutralized a test dose of toxin containing between 30 and 50 minimum reacting doses (M.R.D.). On this result, it was decided that the toxins of L.D. and BP were serologically very similar and probably identical. The i.d. method of testing was used because, at that time, no mice were available to carry out intravenous (i.v.) titrations. Later, this lack was made good, and a few rough tests were made by the i.v. route, merely to confirm the i.d. results. The apparently discrepant results obtained intravenously initiated this research. It was found that although L.D. antitoxin in a dose of 0·01 c.c. fully neutralized a certain test dose of BP toxin when the i.d. route was used, as much as 0·1 c.c. was unable to neutralize the same test dose by the i.v. route. The probable reason for this is given in the text.

Until the isolation of the "L.D. bacillus" by Gaiger and Dalling in 1922, and its description by Dalling in 1928, only one serological type of *Cl. welchii* was known, the organism originally described by Welch and Nuttall (1892). Bull and Prichett (1917) showed that the toxins of 27 different strains were neutralized by one antitoxin. One strain, *B. Egens*, isolated by Stoddard (1919) was said to be cultural variant; however, Weinberg and Prévot (1927) and Dalling and the writer (unpublished work) showed that serologically it behaved as *Cl. welchii*. Henry (1922) postulated the

presence of 2 toxic fractions which he named a "haemo"- and "myo"-toxin. The writer (unpublished work) was unable to confirm this (by absorption with red cells and muscle) and the results of Dalling *et al.* (1928) and Mason and Glenny (1928) showed that antitoxins of widely different origin all neutralized the toxin of one *Cl. welchii* strain, the titrations being carried out intramuscularly (i.m.) or intravenously (i.v.) in mice or haemolytically against rabbit red cells. Further, the writer has, over a period of 7-8 years, titrated antitoxins of different origin against the toxins of about 6-8 different welch strains; neutralization took place in every instance. Simonds (1915) classified *Cl. welchii* according to its fermentative action on various sugars. Dalling and the writer (unpublished work) received from another laboratory 4 strains classified according to his method; toxins prepared from them were neutralized by an antitoxin prepared against *Cl. welchii*.

The foregoing brief review indicates strongly that the toxins produced by classical *Cl. welchii* are serologically identical. No indication has yet been given that this germ produces more than one toxin, and further, the methods of testing applied (i.v. mouse, i.m. mouse, i.d. guinea pig, and haemolytic) would point to its various actions being due to the same toxic principle. However, in view of the intensive work that is being carried out in various laboratories in different parts of the world on the L.D.—*B. paludis*—*B. ovitoxicus* group of welch-like germs and of the interest that this will stimulate in further investigations into the true welch group, it would occasion no surprise if definite serological variants of *Cl. welchii*, more akin to welch than to L.D., were reported upon in the future.

Apart from some minor cultural variations, the main difference between the L.D. bacillus and *Cl. welchii* was shown to be in their toxins (Dalling, 1928); the L.D. toxic molecule contained some welch toxin as evidenced by the ability of its antitoxin to neutralize welch toxin. Welch antitoxin, on the other hand, had no effect upon L.D. toxin, showing that its toxin contained none of the L.D. fraction. McEwen (1930) described another welch-like germ, *B. paludis*. The toxin of one strain (CT 40) was found by Mason, Ross and Dalling (1931) and by Mason (1933) to be serologically identical with that of L.D.

Bennetts (1932) isolated from the bowel of sheep infected with "Entero-toxaemia", a welch-like germ, *B. ovitoxicus*, and Gill (1933) recovered the same or a very similar organism from the gut of lambs infected with "Pulpy kidney disease". Bennetts stated that *B. ovitoxicus* toxin was neutralized by L.D. and ovitoxicus but not by paludis antitoxins, that welch toxin was neutralized by these three antisera, that L.D. toxin was neutralized by L.D., paludis, but not by ovitoxicus antitoxins and that paludis toxin was neutralized by L.D. and paludis but not by ovitoxicus antitoxin. Gill was able to neutralize the toxin of his strain with one batch of L.D. antitoxin but not with a second batch; the toxins and antitoxins of ovitoxicus and of his germ cross neutralized, whereas paludis antitoxin failed to neutralize either ovitoxicus toxin or that of his bacillus. It should be noted that both the L.D. antitoxins used were prepared under Dalling's supervision at the Wellcome Laboratories. (The reason for

one batch protecting and the other failing to do so will become clear from what follows.) Wilsdon (1931, 1933), examined a considerable number of welch-like germs and was able to group them on the type of toxin they produced. This classification was briefly as follows:—

Type A contains one type of toxin (factor W)—classical *Cl. welchii.*

Type B contains three types of toxins (factors W, X and Z)—L.D. bacillus.

Type C contains 2 types of toxin (factors W and Z)—*B. paludis.*

Type D contains two types of toxin (factors W and X)—*B. ovitoxicus.*

Further, he showed that this D type and the *B. ovitoxicus* produced maximum toxin after 3-5 days' incubation of the culture at 37° C. Montgomerie and Rowlands (1933) isolated welch-like germs from the intestine of lambs infected with lamb dysentery. The toxins produced therefrom behaved in the following fashion: paludis antitoxin neutralized the toxins only when they were produced from a young (18 hours) culture and when the test dose of toxin contained only a few (5) M.L.D.; toxins of 6-30 days' cultures were not affected by it. Ovitoxicus antitoxin (Wilsdon Type D) neutralized only when the toxin had origin from an old (6-30 days) culture. One L.D. antiserum (A) neutralized the toxin produced in 18 hours', 6 days' and 30 days' cultures; another L.D. antitoxin (B) behaved in the same way as did the paludis serum. (Both these antitoxins were prepared at the Wellcome Laboratories.) A combination of ovitoxicus and paludis antitoxins neutralized toxins of all ages. The authors conclude that the toxin of the L.D. bacillus contains 2 toxic fractions: one, present in high concentration during the early period of incubation and neutralizable by paludis antitoxin and the other, present in low concentration during the early states of incubation, but increasing with incubation and neutralizable by *B. ovitoxicus* antitoxin. Dalling (1934), working in collaboration with Montgomerie and Rowlands, examined the L.D. cultures in use in 1932 (these being subcultures of the original sealed-off 1922 culture) and found that these were unable to form the ovitoxicus type of toxin, whereas a direct subculture from the 1922 tube produced a filtrate containing both the L.D. and the ovitoxicus type of toxin. Further, he states that he examined the antitoxins used by Montgomerie and Rowlands; that (A above) which neutralized their toxins, no matter their age, was of earlier production and that (B above) which neutralized only "young" toxins was of more recent preparation. He concludes that, at some time between 1922 and 1932, the L.D. bacillus lost the power to produce the ovitoxicus type of toxin, at least, in amounts detectable by the methods employed.

What has immediately preceded explains the apparently discrepant results obtained by Bennetts and Gill; Bennetts obviously received the earlier and Gill the later type of L.D. antitoxin.

[It may be stated, at this point, that Dalling (1932) and the writer [addendum (1) to this paper] have brought forward evidence to show that *B. ovitoxicus* may produce the L.D. type of toxin.]

The final article that need be mentioned is that of Glenny *et al.* (1933). They examined the toxins and antitoxins of the L.D. bacillus, *B. paludis* and *Cl. welchii* and were able to detect 4 toxic fractions in the toxins. These they designated "*alpha*"—the toxin of classical *Cl. welchii* present also in *B. paludis* and the L.D. bacillus (agni) filtrates; "*beta*"—a necrosis-producing, non-haemolytic toxin, not present in welch but the predominant constituent of L.D. and paludis filtrates; "*gamma*"—a constituent of L.D. (agni) filtrates, lethal to mice and not neutralized by alpha or beta antitoxins. Its presence could only be inferred because sera were encountered with a high protective power against L.D. (agni) filtrates as judged by the intradermic injection of guinea pigs and low protective power in mice (i.v. inoculation). This toxin is probably contained to a slight extent by paludis filtrates; "*delta*"—a haemolysin produced by *B. paludis* and probably to a much less extent by L.D. (agni); and "*epsilon*"—a necrosis-producing toxin associated with Wilsdon's Type D (*B. ovitoxicus*) bacillus. This toxin is neutralized by L.D. sera prepared prior to 1930, but not by recent batches.

A brief summary of the results of research on the toxins of the Welch-L.D.-paludis-ovitoxicus group is as follows. There are three main toxins produced (following Glenny's nomenclature):—"*alpha*" produced by all members, "*beta*" produced by L.D. paludis, and according to Dalling and the writer's earlier results to a slight extent by ovitoxicus but not by classical *Cl. welchii* and "*epsilon*", produced by the original L.D. strain and by *B. ovitoxicus* but not by *B. paludis* or *Cl. welchii*. "*Gamma*" is a lethal but non-necrotic fraction produced by L.D. and probably to a slight extent by paludis and "*delta*" is a haemolysin formed by paludis and to a slight extent by L.D.

METHODS.

Toxin Production.

Robertson's meat broth was used in all instances, being thoroughly boiled and rapidly cooled just prior to inoculation. After 18 hours' or 5 days' incubation at 37° C., the culture was passed through paper pulp and then through a Berkefeld candle, and if the toxin was to be used in the liquid state was preserved by adding enough phenol-ether (equal parts) to make a 1·0 per cent. concentration. Dry stable toxin was obtained by saturating the toxic filtrate with ammon. sulph., pressing the precipitate, and drying it *in vacuo* over H_2SO_4. For use, 100 mgm. of dry toxin was dissolved in 5·0 c.c. of saline.

Toxoid Production.

Enough formalin (40 per cent. formaldehyde) was added, either to the whole culture (freed from meat particles) or to the toxic filtrate, brought to pH 7·4, to make a 0·4 per cent. concentration and the formalised material incubated at 37° C. until more than 0·25 c.c. (injected i.v.) was required to kill a mouse. This usually took from 3 to 7 days.

Antitoxin Production.

Goats were used throughout, immunization commencing with the subcutaneous injection of formol-toxoid and finishing with unmodified toxin.

Neutralization Tests.

Toxin and antitoxin were mixed, allowed to stand for one hour at room temperature (18°-26° C.) and injected i.v. into mice. Results were recorded, after 5 days, as " live ", " dead " or " symptoms, lived ", i.e. showed nervous phenomena but survived the 5-day period. In the intradermic titrations in guinea pigs, the total volume injected was 0·2 c.c. and the results were recorded after 48 hours as +++++N, ++N, +N, ±N, the " N " referring to the inflammatory necrotic lesion produced. In the haemolytic tests, haemolysin (toxin) and antihaemolysin (antitoxin) were mixed, enough saline added to bring the volume to 1·5 c.c. and after one hour's interval, 1·0 c.c. of a 2·5 per cent. suspension of well-washed rabbit red cells was added. After 2 hours at 37° C. and 2 hours at room temperature, results were recorded as " complete " (C), " partial " (P), " large trace " (L.T.), " trace " (tr), or negative (N) haemolysis.

EXPERIMENTAL.*

Origin of the cultures used:—

The bloedpens culture	Isolated from a naturally-infected lamb by Dr. E. M. Robinson.
Cl. welchii, Type B ...	(*a*) The " 1930 " variety, brought from the Wellcome Laboratories by the writer. This is a subculture (many generations old) of
	(*b*) The " original " strain, isolated by Dalling and Gaiger in 1922.
Cl. welchii, Type C ...	McEwen's C.T. 40 strain.
Cl. welchii, Type D ...	Bennetts' *B. ovitoxicus* (R2), as received from Dr. Bennetts.

Morphology and Cultural Characters of the Bloedpens Strain.

The bloedpens strain showed no morphological or cultural characteristic that distinguished it from classical *Cl welchii*. Its fermentative action on the various " sugars " (1·0 per cent. in 1·0 per cent. peptone water) after 14 days' incubation at 37° C. in a MacIntosh and Fildes' jar was as follows: acid and gas formation in saccharose, galactose, glucose, lactose, maltose, dextrine, raffinose, glycerine and inosite; no action on salicine, adonite, mannite and dulcite. Gelatin was liquefied, alkaline egg medium clotted and milk fragmented (" stormy fermentation "). Loeffler's serum medium was partially liquefied, but inspissated horse serum was not attacked.

* *Cl. welchii* will be referred to as " Type A ", the L.D. bacillus as " Type B ", *B. paludis* as " Type C ", and *B. ovitoxicus* as " Type D ". Glenny's nomenclature will be used to designate the toxic fractions.

Pathogenicity.—The intramuscular injection of 0·1 c.c. of an 18 hours' meat broth culture of BP into guinea pigs caused death in from 15 to 18 hours. The post mortem appearance was very similar to that produced by *Cl. welchii*, Type A, to such a degree that the writer could not differentiate, with certainty, those animals killed with the BP organism from those killed with *Cl. welchii*. On a few occasions, the post mortem picture recalled that produced by *Cl. oedematiens*. The germ could be recovered from the heart blood and liver of the animal shortly after death.

Cross Immunity Tests in Guinea Pigs.

(Bloedpens and Type B.)

Formol-toxoids were prepared from the BP and Type B strains. The latter germ was of the 1930 variety and thus incapable of forming the epsilon toxic fraction.

BP Toxoid Immunized Guinea Pigs.—These received two subcutaneous injections of 2·0 c.c. of toxoid at three weeks' interval, and were tested for immunity thirteen days after the second inoculation by the cumulative M.L.D. method (see Mason, Ross and Dalling, 1931). Table 1 records the results.

Table 1.

Amount of Toxin tolerated by G.Ps. Immunized with BP Toxoid.

Test Toxin.
(M.L.D. withstood.)

G.P.	BP 1.	Type B, 1930, 1.
1.....	1–4	
2.....	1–4	
3.....	16	
4.....	32–64	
5.....		1–4
6.....		8–16
7.....		>64

[The "1" of BP 1 and Type B 1930, 1 means that the toxin was obtained from a culture incubated for one day (18 hours). The toxoid used to produce immunity was made from a toxin of a one-day culture.]

Type B, 1930 *Toxoid Immunized Guinea Pigs.*—The toxoid was prepared from a one-day toxin. The injections, the test toxins and the method of test were the same as noted for "BP" toxoid immunized guinea pigs. Table 2 gives the results.

A further test was carried out on two guinea pigs which had received two injections of 2·0 c.c. each of Type B, 1930 toxoid and had survived the intravenous administration of 12 M.L.D. of toxin. One week later, they survived the i.v. injection of 16 but not 32 lethal doses of BP 1 toxin.

TABLE 2.

Amount of Toxin tolerated by G.Ps. Immunized with Type B, 1930, 1 *Toxoid.*

Test Toxin.
(M.L.D. withstood.)

G.P.	BP I.	Type B, 1930, 1.
1.....	<1	
2.....	<1	
3.....	1–4	
4.....	4–8	
5.....	8–16	
6.....	16–32	
7.....		1–4
8.....		16–32
9.....		>32
10.....		>32
11.....		>32

The results presented in tables 1 and 2 show that the two toxins have at least one important antigen in common. The anti-toxin binding values of the two test toxins used to estimate the degree of immunity produced cannot be compared, since, as will be shown later, they differ qualitatively.

THE TOXIN PRODUCED BY THE BLOEDPENS STRAIN.

The "One-day" Toxin (BP 1).

A dry toxin was prepared from an 18 hours' culture; the M.L.D. for mice by i.v. injection was 0·001 c.c. From the results obtained in the immunization of guinea pigs (tables 1 and 2), and from a few rough preliminary titrations in mice, it was anticipated that Types B and C antitoxins would neutralize this toxin in multiple proportions. Such was not the case. In titrating Type B toxin (1930 variety) and antitoxin, the result can usually be forecasted with some certainty 2 to 4 hours after the injection of the toxin-antitoxin mixtures. Using a test dose of BP 1 toxin of 0·1 c.c. (100 M.L.D.) the following results were obtained in the titration (mouse i.v.) of two Type B and one Type C antitoxins (table 3).

TABLE 3.

Titration of a large test dose (0·1 c.c. = 100 *M.L.D.*) *of B.P.*1 *toxin against antitoxins.*

Antitoxins (cc).

Type B (1789).	Type B (SC).	Type C (pal).
0·004 ÷ ½ hr.	0·02	0·004 ÷ ½ hr.
0·005 ÷ ½ hr.	0·025 All showing symptoms	0·005 ÷ ½ hr.
0·007 ÷ 3 hr.	0·03 5 hours.	0·007 ÷ 4 hr.
0·01 ÷ o/n	0·04 All dead o/n	0·01 ÷ 5 hr.
0·015 ÷ o/n	0·1	0·1 ÷ o/n
0·1 ÷ o/n	0·25	0·25 ÷ o/n
0·25 ÷ o/n	0·5	0·5 ÷ o/n
0·5 ÷ o/n		

[÷ = died; o/n = overnight; hr. = hour(s). Type B (1789) and (SC) were prepared from a "1930" strain.]

The results in table 3 show that such a relatively enormous dose as 0·5 c.c. of Types B and C antitoxins was unable to neutralize the test dose of BP 1 toxin although the appearance of the mice after two or three hours indicated that protection would be afforded. That the antitoxins used were of high value is shown by the fact that relatively small amounts (0·004 c.c.—0·015 c.c.) neutralized 20 to 100 M.L.D. of Type B, 1930 and Type C toxins. However, by reducing the test dose of BP 1 toxin to 0·01 c.c. (10 M.L.D.) full protection was obtained, but 0·05 c.c. was not neutralized by 0·1 c.c. of antitoxin and 0·025 c.c. sometimes was and at other times was not neutralized. Table 4 summarises the results obtained, using a test dose of 0·01 c.c. of BP 1 toxin.

TABLE 4.

Titration of a Small Test Dose (0·01 c.c. = 10 *M.L.D.*) of *BP* 1 *Toxin against Antitoxins.*

Antitoxins (cc).

Type B (1789).	Type B (SC).	Type C (pal).
0·0007 ÷ o/n ÷ 2d.	0·0012 ÷ 3h. ÷ 5h.	0·0006 ÷ o/n + 2d. + 3d.
0·0008 ÷ o/n ÷ 3d.	0·0015 //// 4d.	0·0007 ÷ o/n + 2d.
0·001 /// 4d.	0·002 / 4d.	0·0008 + 2d. / 4d.
0·0012 // 4d.		0·0009 // 4d.

(÷ = died; / 4d. = mouse alive and well on the 4th day; h. = hours.)

Before considering one point of importance in Table 4, the result of determining the M.L.D. of a mixture of 0·1 c.c. of BP 1 toxin and 0·015 c.c. of Type B (SC) antitoxin (theoretically a neutral mixture from the results given in table 4) will be presented. This mixture was set up in a multiple of 5; 0·25 c.c., 0·2 c.c., 0·15 c.c. and 0·1 c.c. killed mice overnight; the mouse receiving 0·075 c.c. was ill after 18 hours, showed " jumpy " symptoms in 3 days but was alive after 6 days. In the titrations recorded in table 4 and in this one, a number of mice did not die until the 2nd or 3rd day, and one mouse showed nervous phenomena not previously seen in titrating Type B toxin and antitoxin. In a further series of tests put up to determine the rise in antitoxin content of the blood of goats immunized with Types B and C toxoids, these nervous phenomena and late deaths were again encountered. It was at this stage that the writer consulted (by letter) Major T. Dalling, who was engaged upon the toxins produced by the original Type B culture and the " 1930 " subculture of it. He (T.D.) suggested that the BP strain was the same as the original Type B culture and that the inability of the Type B antitoxin to neutralize a large test dose was due to the fact that it contained no epsilon antibody and the toxin did contain a small amount of the epsilon fraction. This point and the relationship of the toxins of BP, Types B, C and D produced from young (18 hours) and old (5 days) cultures was next investigated.

As noted, 0·1 c.c. (100 M.L.D.) of BP 1 toxin was not neutralized by as much as 0·5 c.c. of two " 1930 " Type B antitoxins and one Type C antitoxin, but 0·01 c.c. was neutralized by quite small amounts. This indicated definitely that, in the BP 1 toxin, there were at least two toxic fractions, one neutralizable by Type B, 1930 antitoxin and another, quantitatively in less amount, not neutralizable by this antitoxin. When a test dose of 0·01 c.c. was employed, this fraction was diluted below one fatal dose and thus was unable to indicate its presence.

The experiments, summarised in table 5, show that, although Type B, 1930 antitoxin was incapable of neutralizing 0·1 c.c. of BP 1 toxin and 0·1 of BP 5 and Type D 1 and 5 antitoxins did not neutralize 1½ M.L.D. of this toxin, a mixture of Type B, 1930 antitoxin and one of the three mentioned antitoxins did neutralize 0·1 c.c. of BP 1 toxin. (BP 5 and Type D 1 and 5 means that the antitoxins were produced by means of filtrates of 1 or 5 day cultures.)

The results presented in table 5 confirm the view earlier expressed, that BP produces an epsilon and a beta toxic fraction. Further, it is shown that the antitoxins produced with the 5 day BP toxin and the 1 and the 5 day Type D toxins contained so little of the anti-Type B (beta) fraction that 0·1 c.c. was unable to neutralize 1 M.L.D. of Type B, 1930 toxin. However, that Type D can produce, under suitable conditions, some beta toxin will be discussed in an addendum. Further, results, to be recorded later, show that BP 1 antitoxin neutralizes Type D toxin (not neutralizable by Type B, 1930 antitoxin).

TABLE 5.

Titration of BP1 Toxin against Antitoxins.

Toxin (cc).	Antitoxin (cc).				Result.
BP 1.	Type B. 1930.	BP 5.	Type D. 1.	Type D. 5.	
0·1.....	0·1	—	—	—	÷ 36 h.
0·0015.....	—	—	—	0·1	÷ o/n
0·0015.....	—	—	0·1	—	÷ ½ h.
0·0015.....	—	0·1	—	—	÷ o/n
0·1.....	0·1	0·1	—	—	÷÷ 36 h.
0·1.....	0·1	0·15	—	—	÷ 3d /4d.
0·1.....	0·1	0·17	—	—	/ 4d.
0·1.....	0·1	0·2	—	—	/ 4d.
0·1.....	0·1	—	0·02	—	÷÷ 1d.
0·1.....	0·1	—	0·025	—	// 4d.
0·1.....	0·1	—	—	0·017	÷ o/n ÷ 2d.
0·1.....	0·1	—	—	0·02	// 4d.
Type B. 1930. 0·0005 (1 M.L.D.).....		0·1÷	0·1÷	0·1÷	
Type C. 0·0005 (1 M.L.D.).....		0·1÷	0·1÷	0·1÷	

[÷ = died; / = lived; h. = hour(s); d. = day(s).]

In table 6, the results of titrating a large (0·1 c.c.) and a small (0·01 c.c.) test dose of BP 1 toxin against BP 1 antitoxin are recorded.

TABLE 6.

BP 1 Toxin, Large and Small T.D. versus *BP 1 Antitoxin.*

Toxin (cc).	Antitoxin (cc).	Toxin (cc).	Antitoxin (cc).
0·01.....	0·01 ÷÷ o/n, ÷ 2, t.		
0·01.....	0·012 ÷ o/n, ÷÷ 2,		
0·01.....	0·015 ÷÷÷ o/n, t,	0·1	0·15 ÷÷÷ o/n
0·01.....	0·017 ÷ 2, ÷ 4,	0·1	0·17 ÷ o/n, ÷ 4d.
0·01.....	0·02 /// 4.	0·1	0·2 ///

(÷ 2 ÷ 4 = died 2, 4 days; t. = showed nervous "turning" symptoms; / = lived.)

The results given in table 6 show that the law of multiple proportions is satisfied when BP 1 toxin and antitoxin are titrated together.

In table 7 the amounts of antitoxin necessary to neutralize a test dose of BP 1, Type B, 1930 and Type C toxins are given.

A consideration of the ratios given below table 7 would indicate that the three toxins were detecting the same antitoxins. Some, somewhat minor, discrepancies occur, but the small number of different toxins and antitoxins used does not warrant the statement that they are of real significance.

TABLE 7.

Amounts of Antitoxin necessary to Neutralize various Toxins by the i.v. Route.

Toxin (cc).	*Antitoxin* (cc).			
	Type B, 1930 (SC).	Type B, 1930 (1789).	Type C (pal.).	BP 1.
BP 1, 0·01	0·0017	0·001	0·0008	0·02
Type B, 1930, 0·005	0·003	0·0009	0·001	0·017
Type C, pal, 0·005	0·002	0·0006	0·0006	0·01

Ratios.

BP 1	→ (2⅛)	⅘	→ (1¼)	1⅔	→ (1)	1⅓	→ (25)	2
Type B, 1930	→ (3)	1½	→ (9/10)	1½	→ (1)	1⅔	→ (17)	1 7/10
Type C, pal.	→ (3⅓)	1	→ (1)	1	→ (1)	1	→ (16⅔)	1

Titrations by the Intradermic Method in Guinea Pigs.

The minimum reacting doses of the three toxins were as follows: *BP* 1, 0·001-0·0015 c.c.; *Type B*, 1930, 0·0005-0·001 c.c.; *Type C, pal.*, 0·0005-0·001 c.c. In table 8 are recorded the amounts of antitoxin necessary to neutralize test doses of the toxins. Table 9 compares the amount of antitoxin required to produce neutralization intradermally and intravenously. The i.d. figures are calculated from those in table 8 and the i.v. figures are those of table 7.

TABLE 8.

Amounts of Antitoxins required to Neutralize Toxins by the i.d. Route.

Antitoxin (cc).

Toxin (cc).	Type B, 1930 (SC).	Type B, 1930 (1789).	Type C (pal.).	BP 1.
BP 1, 0·01	0·012	0·0035	0·0025	0·045
Type B, 1930, 0·025	0·01	0·0025	0·002	0·03
Type C, pal., 0·025	0·006	0·0015	0·0012	0·0175

Ratios.

BP 1	→ (5) 2	→ (1⅕) 2⅓	→ (1) 2	→ (18) 2⅘
Type B, 1930	→ (5) 1⅔	→ (1¼) 1⅔	→ (1) 1⅔	→ (15) 1⅝
Type C, pal.	→ (5) 1	→ (1¼) 1	→ (1) 1	→ (14½) 1
	↑	↑	↑	↑

(The figures under antitoxin represent the amounts of antitoxin required to neutralize the test doses of toxin.)

TABLE 9.

Comparison of the Neutral Points, obtained by i.v. and i.d. Titration.

Toxin (cc).

Antitoxin (cc).		BP 1.	Type B, 1930.	Type C, pal.
Type B, 1930 (SC)	i.v.	0·0017	0·003	0·002
	i.d.	0·0012	0·002	0·0012
Type B, 1930 (1789)	i.v.	0·001	0·0009	0·0006
	i.d.	0·00035	0·0005	0·0003
Type C, (pal.)	i.v.	0·0008	0·001	0·0006
	i.d.	0·00025	0·0004	0·0002
BP 1	i.v.	0·02	0·017	0·01
	i.d.	0·0045	0·006	0·0035

The results given in table 8 show that, by the intradermic method, apparent neutralization of a large test dose (0·1 c.c.) of BP 1 toxin is produced by Type B, 1930 and Type C antitoxins. Further, tests proved that the law of multiple proportions was satisfied when the test dose was raised to 0·2 c.c. or reduced to 0·01 c.c. As 0·025 c.c. of BP 1 toxin plus 0·1 c.c. of Type B, 1930 antitoxin was lethal for mice, it is obvious that 0·1 c.c. of the toxin plus the " 1930 " variety of Type B antitoxin would contain 4 M.L.D of free toxin which, from the evidence presented, is epsilon toxin. A comparison was made of the M.L.D. and M.R.D. of three toxins, not neutralizable by Type B, 1930 antitoxin and the antitoxins of which did not neutralize Type B, 1930 toxin, viz., the toxins of BP 5, and Type D, 1 and 5.

These were as follows:—

Toxin.

	BP 5.	Type D, 1.	Type D, 5.
M.L.D..... cc	0·0015—0·002	0·003	0·0003—0·0005
M.R.D..... cc	0·003	0·015—0·02	0·0005—0·0007

It is seen that the M.L.D. and M.R.D. of the two 5-day toxins do not differ greatly, but in the case of the 1-day Type D toxin the difference is between five and seven times. There is the possibility that such a difference holds good for the epsilon fraction of BP 1 toxin. This view is supported by the fact that when a test dose of 0·2 c.c. of BP 1 toxin is titrated, intradermally, against Type B, 1930 antitoxin, " neutral " or " overneutralized " mixtures produce a pale white area on the skin, not seen when the antiserum contains some of the anti-epsilon fraction. These " reactions " simulate those produced by small amounts of Type D toxin, although paler and less marked and would have been recorded as negative unless the skin were closely examined and the point in question were unknown.

Tables 7, 8 and 9 show that it required absolutely less antitoxin to produce " neutralization " i.d. than i.v.; therefore, one may assume the presence in each toxin of a fraction, having a lethal but not a necrotic effect. That it is not epsilon is proved by the fact that neither Type B, 1930, nor Type C toxins contain this fraction. The gamma toxin of Glenny *et al*, answers very well. They found that, with certain sera, as much as ten times more was required to produce neutralization i.v. and i.d. Until evidence to the contrary is produced, it will be assumed that the difference in the i.d. and i.v. titrations is due to the presence of this gamma toxin.

The ratios given under table 8 show that, by the i.d. route, almost complete agreement was obtained in the titration of the toxins and antitoxins.

It need only be mentioned, with regard to the neutralizing power of BP 5, and Type D, 1 and 5 antitoxins against BP 1, Type B 1930 and Type C toxins, that 0·1 c.c. of none was able to neutralize one M.R.D. of any of the three toxins, thus confirming the i.v. results of table 5.

Before proceeding to present the results of the titration of a 5-day BP toxin, those obtained with a 5-day Type B toxin will be given.

The "5-day" Type B Toxin.

A. Type B "1930" Variety and Type C.

The 5-day toxins produced by these two strains differed from those of one day's incubation only in being weaker, i.e. instead of the M.L.D. being in the region of 0·001 c.c., it was between 0·01 and 0·1 c.c. From 1-10 lethal doses were neutralized by Type B 1930, Type C and BP 1 antitoxins and not by 0·1 c.c. of BP 5 or Type D, 1 and 5 antitoxins.

B. Type B, Original Variety.

The culture originated from one of a series of tubes, sealed-off by Dalling about 1923 and had not, as had the "1930" variety, been subjected to a series of subcultures. A dry toxin was prepared from a meat broth culture, incubated for five days.

Tests showed that 0·1 c.c. of neither Type B, 1930 nor Type C antitoxins were able to neutralize one M.L.D. (0·005 c.c.) or one M.R.D. (0·06-0·07 c.c.) of the toxin. However, that the brews of toxin used to produce the Type B, "original" antitoxin did contain some beta toxin is shown from the results in table 10.

TABLE 10.

Amount of Type B, Orig. 5 Antitoxin required for Neutralization.

Antitoxin (c.c.).

Toxin (cc).		Titration i.v.	Titration i.d.
Type B, 1930	0·1	0·025	0·01
Type C, *pal*	0·1	0·015	0·008
BP 1	0·1	>0·1	0·004**
BP 1	0·01	0·0006	

** The reaction produced by 0·1 c.c. of BP 1 toxin plus 0·003 c.c. of Type B, original 5 antitoxin was intense, being that obtained with beta toxin; with 0·004 — 0·006 c.c. of antitoxin, small, white areas were formed, like those previously discussed.

In table 11, the results of titrating various antitoxins against Type B, original 5 toxin are given, and in table 12, the titrating of Type B, original 5 antitoxin against different toxins is recorded.

TABLE 11.

Amounts of Different Antitoxins required for the Neutralization of Type B, orig. 5 Toxin.

		Toxin (cc). Type B, orig. 5.	Antitoxin (cc).
			Type B, orig. 5.
	i.v.	0·01	0·1
T.D.	i.d.	0·1	0·01
			BP I.
	i.v.	0·02	0·06
T.D.	i.d.	0·1	0·04—0·05
			BP 5.
	i.v.	0·025	0·05
T.D.	i.d.	0·1	0·02
			Type D, 1.
	i.v.	0·05	0·02
T.D.	i.d.	0·1	0·025
			Type D. 5.
	i.v.	0·1	0·03
T.D.	i.d.	0·1	0·004
			Type A, 1.
	i.v.	1 M.L.D.	>0·1
T.D.	i.d.	1 M.R.D.	>0·1

Type B, orig. 5 toxin—M.L.D. —0·005 c.c.
M.R.D. —0·06—0·07 c.c.
(T.D.—test dose.)

Tables 10, 11 and 12 bring out several interesting points. Type B, original 5 antitoxin has a relatively high anti-beta and a low anti-epsilon value—0·025 c.c. and 0·015 c.c. neutralize 0·1 c.c. of Type B, 1930 and Type C toxins respectively, whereas 0·1 c.c. is required to neutralize 1 to 5 M.L.D. of Type D toxin. (It should be noted that the 5-day Type B, original antitoxin was produced by injecting several different brews of 5-day toxin into a goat.) On the other hand, the toxin consists chiefly of the epsilon fraction—0·1 c.c. of neither Type B, 1930 nor Type C antitoxins was able to neutralize 1 M.L.D., whereas 0·02-0·03 c.c. of Type D, 1 and 5 antitoxins neutralized 10-20 M.L.D. This is strong evidence that beta toxin was minimal in amount in Type B, original 5 toxin, since 0·1 c.c. of neither Type D, 1 nor 5 antitoxins neutralized 1 M.L.D. of Type B 1930 toxin (table 5). Little comment can be made on the intradermic titration of Type B, original 5 toxin against the various antitoxins because of the small number of reacting doses in the test dose (slightly more than 1 M.R.D.). It will be noticed that the M.R.D. is about 12-14 times greater than the M.L.D. However, with BP 5 and Type D, 5 toxins, the i.d. test doses of which contained 4 and 12 M.R.D. respectively, much less Type B, original 5 antitoxin was required to neutralize i.d. than i.v.

TABLE 12.

Amounts of Type B, Orig. 5 AT, required to Neutralize various Toxins.

Toxin. (cc).			Antitoxin. (cc). Type B, orig. 5.
		BP 1.	
	i.v.	0·01.....	0·006
T.D.	i.d.	0·1.....	0·004
		BP 5.	
	i.v.	0·01.....	0·01 —0·025
T.D.	i.d.	0·01.....	0·003—0·004
		Type D, 1.	
	i.v.	1 M.L.D.....	0·1—0·2
T.D.	i.d.	N.D.....	
		Type D, 5.	
	i.v.	0·0015.....	0·1
T.D.	i.d.	0·005.....	0·04
		Type A, 1.	
	i.v.	0·05.....	0·02
T.D.	i.d.	0·05.....	0·02

(T.D.—test dose; N.D.—not done.)

The M.L.D. and M.R.D. of the above toxins were as follows:—

	M.L.D. cc.	M.R.D. cc.
BP 1.....	0·001	0·001
BP 5.....	0·0015—0·002	0·0025—0·003
Type D, 1.....	0·003	0·015—0·02
Type D, 5.....	0·0003—0·0004	0·0007
Type A, 1.....	0·005—0·01	0·005—0·01

One further point requires explanation—0·01-0·025 c.c. of Type B, original 5 antitoxin was required to neutralize 0·01 c.c. (5-6 M.L.D.) of BP 5 toxin, 0·1 c.c. for 0·003 c.c. (1 M.L.D.) of Type D, 1 toxin and 0·1 c.c. for 0·0015 c.c. (12-15 M.L.D.) of Type D, 5 toxin. It has already been shown (table 5) that the antitoxins of these toxins contain no demonstrable anti-beta fraction and it will later be shown that they cross-immunize amongst themselves; therefore, it may be safely assumed that the bulk of their "toxic make-up" is the epsilon fraction. It appeared strange that from 0·01-0·025 c.c. of Type B, original 5 antitoxin should be capable of neutralizing 5-6 M.L.D. of one toxin (BP 5), whereas 0·1 c.c. just neutralized 1 M.L.D. of another (Type D, 1) and 0·1 c.c. 12-15 M.L.D. of a third (Type D, 5). The reason becomes clear if the titration of these toxins against Type D, 5 antitoxin is given (table 13).

It is obvious from table 13 that Type D, 1 toxin contains a large amount of toxoid, inasmuch as it requires 35 times more antitoxin (Type D, 5) to neutralize 1 M.L.D. of its toxin than that of BP 5. In the same way, much more Type B, original 5 antitoxin should be required to produce neutrality; this is borne out when the calculation is made.

TABLE 13.

Amount of Type D, 5 Antitoxin and Type B, Orig. 5 Antitoxins required to Neutralize various Toxins.

Toxin cc.	ANTITOXIN CC.					
		Type D, 5.			Type B, orig. 5.	
	T.D. (in MLD.)	Per T.D.	Per MLD.	Ratio.	Per MLD.	Ratio.
BP 5.....	50–60	0·009	0·00018	1	0·0016–4	1
Type D, 5.....	10–15	0·02	0·0013–15	5·6	0·006–8	3 (ab)
Type D, 1.....	7	0·045	0·0064	35	0·1–0·2	25–60

[T.D.—test dose; (ab)—about.]

The 5-Day Toxin of BP and the 1- and the 5-Day Toxins of Type D.

The antitoxins of BP 5 and Type D, 1 and 5 did not neutralize 1 M.L.D. or 1 M.R.D. of Type B, 1930 or Type C toxins nor did the antitoxins of the last two neutralize the toxins of the first three. This shows that the beta fraction was absent in the first three and the epsilon in the last two.

TABLE 14.

Intravenous Titration of Various Toxins and Antitoxins.

Toxin cc.	ANTITOXIN CC.					M.L.D. of Toxin.
	BP 1.	BP 5.	TB. orig. 5.	Type D 1.	Type D 5.	
BP1 .....T.D.	0·1 0·2	1 MLD >0·1	0·01 0·006	1 MLD >0·1	1 MLD >0·1	0·001–15
BP 5.....T.D.	0·1 0·04	0·1 0·017	0·01 0·01–25	0·1 0·0045	0·1 0·009	0·0015–2
Type B, orig. 5.T.D.	0·02 0·04–5	0·025 0·05	0·01 0·1	0·05 0·02	0·1 0·035	0·005
Type D, 1.....T.D.	0·005 0·1	0·007 0·1	0·003 0·1–0·2	0·01 0·025	0·025 0·045	0·003
Type D, 5.....T.D.	0·001 0·04–5	0·0025 0·08	0·0015 0·1	0·005 0·02–25	0·005 0·017	0·0003–4

(TB orig. 5 = Type B, original 5.)

(The figure opposite T.D. is the test dose used. The figure immediately below it is the amount of antitoxin required to neutralize it.)

Tables 14 and 15 record the amounts of the various antitoxins required to neutralize test doses of the various toxins, by i.v. and i.d. titration.

Ratios.

The ratios were arrived at by calculating, from table 14, the amount of antitoxin necessary to neutralize the same test dose of toxin for each toxin. BP 1 toxin is not included because, as has previously been shown, it is made up chiefly of beta toxin and therefore not neutralizable by Type B, original 5, or Type D, 1 and 5 antitoxins.

Toxin.	Antitoxin.				
	BP 1.	BP 5.	TB. orig. 5.	Type D 1.	Type D 5.
BP 5	9	3 7/9	22–55	1	2
Type B, orig. 5	5–6	5	25	1	7/8
Type D, 1	8	5 3/5	13–26	1	3/4
Type D, 5	10	8	16	1	1/3

All five antitoxins are placed in the same order of potency whatever toxin is used for their titration, with the exception that Type D, 5 antitoxin is a little more than twice as weak when tested against BP 5 toxin as when tested against the other toxins. Other, somewhat minor, disagreements occur—BP 1 antitoxin is stronger by one half to double when titrated against Type B, original 5 toxin than against the other toxins; BP 5 antitoxin is weaker and Type B, original 5 antitoxin is stronger when Type D, 5 toxin is used than is the case with the other toxins. The writer does not feel justified in attempting to offer explanations for differences that may, in large measure, depend upon the avidity of the antitoxins, upon the error of the method of testing or upon factors at present unknown. The definite possibility exists that further research will reveal the presence, in D type filtrates, of toxic fractions, other than the epsilon.

Intradermic Titrations.—It must be emphasized, in connection with the i.d. titrations, that much greater difficulty was experienced in obtaining the same results on repeat tests than was the case with the i.v. titrations. In fact, the results noted for Type D, 1 and Type B, original 5 toxins are merely approximations. A probable reason for this is the small number of reacting doses per test dose. Even allowing for the inaccuracy of the i.d. test, it is noticeable that, in every instance, less antitoxin was required to produce "neutrality" i.d. than i.v. Along with this it should be noted that the M.L.D. of each toxin was smaller (by as much as 12-15 times in the case of Type B, original 5) than the M.R.D. These facts point to the presence, in these toxins, of a toxic fraction which is lethal on i.v. injection but which does not produce a skin lesion on i.d. inoculation. The relationship of this to Glenny's gamma toxin has not been fully worked out, but it can be said that it takes the same amount of an epsilon antiserum to produce neutralization (i.v. and i.d.) of an epsilon-containing toxin whether Type B, 1930 antitoxin is added to the mixture or not.

TABLE 15.

Intradermic Titration of Various Toxins and Antitoxins.

Toxin cc.	ANTITOXIN CC.					M.R.D. of Toxin.
	BP 1.	BP 5.	TB. orig. 5.	Type D 1.	Type D 5.	
BP 1.....T.D.	0·1 0·045	1 MRD >0·1	0·1 0·004	1 MRD >0·1	1 MRD >0·1	0·001
BP 5.....T.D.	0·1 0·02	0·1 0·01	0·01 0·003	0·1 0·0025	0·1 0·0025	0·0025–3
Type B. orig. 5.T.D.	0·1 0·04	0·1 0·02	0·1 0·01	0·1 0·025	0·1 0·004	0·06–7
Type D, 1.....T.D.	?	0·05 0·01	?	0·1 0·005 ?	0·05 0·001 ?	0·015–2
Type D, 5.....T.D.	0·0025 0·015–2	0·005 0·012	0·005 0·03	0·01 0·017	0·01 0·015–2	0·0007

(The figure opposite T.D. is the test dose used, the figure immediately below it is the amount of antitoxin required to neutralize it.)

Ratios.

BP 5.....	8	4	12	1	1
Type B. orig. 5	$1\frac{3}{5}$	$\frac{4}{5}$	$\frac{2}{5}$	1	$\frac{4}{15}$
Type D, 1.....	?	4	?	1	$\frac{1}{2}$
Type D, 5.....	4	$1\frac{1}{2}$	$3\frac{1}{2}$	1	1

(The ratios were calculated from table 15 in the same way as noted for the ratios under table 14.)

The ratios given under table 15 show no correlation whatever. Disregarding the figures for Type B, original 5 and Type D, 1 toxins, by the use of which consistent results could not be got, and considering only those for BP 5 and Type D, 5 toxins, it is clear that the value to be assigned to the antitoxins varied greatly depending upon the toxin used for the test. One is hesitant in suggesting the presence of still another toxin to explain this discrepancy, but the existence in the toxic filtrates of two fractions and of their antibodies in the antisera, all in different proportions, would meet the facts.

It comes out clearly from the foregoing experiments that the intradermic method is a most unsuitable one for the titration of the toxins or antitoxins of the D type.

THE TOXIN OF "CL. WELCHII", TYPE A, AND ITS RELATION TO THE TOXINS OF "BLOEDPENS", AND "CL. WELCHII", TYPES B, C AND D.

It has previously been recorded [Mason, Ross and Dalling (1931), Mason (1933)], that as much as 0·1-0·5 c.c. of potent Type A antitoxin will not neutralize 1 M.L.D. or 1 M.R.D. of either Type B or Type C toxin, whereas Types B and C antitoxins neutralize Type A toxin. Further, Glenny *et al.* (1933) have shown that the anti-alpha titre of a serum bears no relation to its anti-beta titre. Bearing this in mind, no attempt was made to titrate accurately the anti-alpha content of the various anti-toxins—whether or not they did contain alpha antitoxin was investigated.

The results may be briefly recorded as follows: Two potent Type A antitoxins (one made in a goat at Onderstepoort, and the other (G.G. 2748) in a horse at the Wellcome Laboratories) were unable, in a dose of 0·1 c.c.-0·2 c.c. to neutralize 1 M.L.D. or 1 M.R.D. of Type B 1930, Type C, Type B, original 5, BP 1, BP 5, Type D 1 and 5 toxins. On the other hand, from 0·01-0·02 c.c. of the antitoxins of these toxins neutralized from 5-15 M.L.D. of a Type A toxin.

Haemolysis.

It is well-known that *Cl. welchii* produces a haemolysin, and Glenny *et al.* (1933) recorded experiments dealing with that of Types B and C.

Table 16 summarises the results obtained in the titration of a number of toxins and antitoxins for haemolytic and anti-haemolytic power respectively.

Little comment need be passed on the data in Table 16. From the ratios, one may conclude that the haemolysins, contained in the various toxins, are serologically identical. Glenny *et al.* (1933) reported that, in certain Type C filtrates, a haemolysin was present which was not neutralizable by antibody contained in either Types A or B antisera. The Type C toxin used for the above test either did not contain it or if so, in undemonstrable amounts. However, it is realised, that many filtrates, produced under various conditions might have to be prepared before such a haemolysin could be demonstrated.

ADDENDUM 1.

Dalling (1932) made brief reference to work done by himself and the writer on *Cl. welchii*, Type D (*B. ovitoxicus*, Bennetts). As this work has a strong bearing on what has just preceded, it will not be out of place to record the experiments in somewhat greater detail.

The germ was received from Dr. Bennetts in a sealed-off tube of meat broth, and was labelled "B.L.D. bacillus strain R2 15.8.30". The meat was very fragmented and of a brown pink colour. No disagreeable odour was noticed on opening the tube and Gram-stained smears revealed bacilli of the *Cl. welchii*-type. Attempts by standard methods to demonstrate more than one germ in the culture failed.

TABLE 16.

Amount of Antihaemolysin required to Neutralize different Haemolysins.

HAEMOLYSIN.	c.c.		T.A. 748.	T.A. 54.	T.B. 1930.	T.B. orig. 5	T.C. pal.	BP 1.	BP 5.	T.D. 1.	T.D. 5.
	M.H.D.	T.D.									
Type B, 1930.....	0·5	N.D.									
Type B, orig. 5.....	>0·5	N.D.									
Type C, pal.....	0·05	0·2	0·0025	0·0025	0·03	0·02	0·006	0·04	0·1	0·04	0·0015
BP 1.....	0·25	0·5	0·0025		0·02		0·007	0·04	0·08	0·04	0·0015
Type D, 1.....	0·05–1	0·5	0·001		0·01	0·008	0·003	0·02	0·04	0·02	0·0007
BP 5.....	0·5	N.D.									
Type D, 5.....	0·1	N.D.									
Type A, (54).....	0·01	0·1		0·0035	0·04		0·009	0·05	0·15		0·0025
Type A, (318).....	0·003	0·1	0·008		0·08	0·05	0·02	0·15	0·4	0·15	0·005
					RATIOS.						
Type C, pal.....			$1\frac{2}{3}$	$1\frac{2}{3}$	20	13	4	26	66	26	1
BP 1.....			$1\frac{2}{3}$		13		$4\frac{2}{3}$	26	53	26	1
Type D, 1.....			$1\frac{3}{7}$		14	12	4	28	56	28	1
Type A, (54).....				$1\frac{2}{5}$	16		4	20	60		1
Type A, (318).....			$1\frac{3}{5}$		16	10	4	30	80	30	1

(M.H.D. = minimal haemolytic dose; T.D. = test dose; N.D. = not done; T.A. = type A; T.B. = type B, etc.)

The morphological and cultural characteristics were those of *Cl. welchii*, Type A. Glycerine was fermented, Loeffler's serum medium partially liquefied, alkaline egg clotted, and solid serum softened but not liquefied.

Simple Animal Test.—The intramuscular injection of 1·0 c.c. of a 24 hours meat broth culture into a guinea pig killed it within 48 hours. The autopsy revealed a pale gelatinous oedema of the subcutaneous tissue, with little or no reddening and reminding one of the picture produced by *Cl. oedematiens*. The small intestines and the adrenals were very congested. A similar post-mortem finding has been obtained in sheep at Onderstepoort.

The Toxin.—A dry (ammon. sulph. precipitate) toxin was prepared from a meat broth culture, incubated at 37° C. for 12 hours.

In the tests to be noted, the mice were observed for only 24 hours after the intravenous injection of the toxin-antitoxin mixtures. It was not known, at that time, that epsilon toxin could produce death as long as 5-7 days after injection. Still, it is felt that the results do prove that the Type D antitoxin contained some of the anti-beta fraction and that the Type B antitoxin contained some anti-epsilon. This statement is based upon the fact that the test dose of Type D used (0·1 c.c., 10 M.L.D.) must have consisted, in a preponderating measure, of the epsilon fraction and would certainly have killed a mouse within 24 hours; in the same way, the Type B test dose consisted chiefly of the beta fraction, and contained sufficient M.L.D. (5) to kill a mouse in a few hours. In table 17, cross-neutralization tests between the toxins and antitoxins of Types B and D are recorded. The Type B antitoxin was prepared in a horse by the injection of formol-toxoids and toxins made from cultures, incubated for 18 hours; the Type D antitoxin was made in a rabbit, by injecting into it the formol-toxoid prepared from a 12 hours' culture.

TABLE 17.

Cross-neutralization Tests between the Toxins and Antitoxins of Types B and D.

Toxin.		ANTITOXIN (CC.)		
	M.L.D. (cc)		Type B (12479).	Type D.
Type B.....	0·001	Test dose of toxin used (cc)=....	(0·1) 0·003	(0·005) 0·08
Type D.....	0·01	Test dose of toxin used (cc)=....	(0·1) 0·015	(0·05) 0·08

(The non-bracketed figures under antitoxin = c.c. required to neutralize the test doses. The bracketed figures = test doses used.)

From the results given in table 17, the following may be deduced:—

(1) The Type B antitoxin was produced at a time when the Type B bacillus was still capable of forming the epsilon toxic fraction.

(2) The toxic filtrate of a 12 hours' Type D culture contained sufficient beta toxin to stimulate the formation of the anti-beta fraction.

(3) The antitoxin detected in both sera by Type B toxin was the anti-beta portion and by Type D toxin, the anti-epsilon portion.

(4) Although the Type B toxin used in the above titration may have contained some epsilon toxin and the Type D toxin some beta toxin, such a deduction cannot be made from the data.

The following antitoxins, in amounts of 0·2 c.c. did not neutralize 2 M.L.D. of Type D toxin—*Cl welchii*, Type A (2853), *Cl. septicum* (2997), *Cl. oedematiens* (2744), *Cl. sordellii*, *Cl. botulinum* (A plus B) and *Cl. histolyticum*. On the other hand, 0·05 c.c. of Type D antitoxin neutralized 7 M.L.D. of a Type A toxin.

The haemolysins of *Cl. welchii*, Types A, B and D proved to be identical serologically.

Cross-immunity Tests between Types B and D in Guinea Pigs.

The formol-toxoids, used to produce the immunity, were made from toxic filtrates of meat broth cultures incubated for 16 hours.

Experiment 1: *Type D formol-toxoid.*

13.12.30.—Guinea pigs received 2·0 c.c. of toxoid s.c.

29.12.30.—Guinea pigs received 2·0 c.c. of toxoid s.c.

13. 1.31.—*Test*: 3 Guinea pigs received 1 M.L.D. Type B toxin i.v.—all died.
2·0 c.c. of toxoid injected into the remaining guinea pigs.

20. 1.31.—*Test*: 2 Guinea pigs received 1 M.L.D. Type B toxin i.v.—both died.

Experiment 2: *A different batch of Type D Toxoid used.*

20.1.31.—Guinea pigs received 2·0 c.c. of toxoid s.c.

2.2.31.—Guinea pigs received 2·0 c.c. of toxoid s.c.

10.2.31.—Guinea pigs received 2·0 c.c. of toxoid s.c.

16.2.31.—Guinea pigs received 2·0 c.c. of toxoid s.c.

23.2.31.—*Test*:

Number of M.L.D. of Toxin Tolerated by the Animal.

G.P.	Type B Toxin.	G.P.	Type D Toxin.
1.....	at least 1	7.....	1–3
2.....	at least 1	8.....	at least 1
3.....	at least 1	9.....	at least 1
4.....	1–3	10.....	at least 1
5.....	1–3		
6.....	1–3		

The results just given show that it is not easy to immunize guinea pigs against Type B toxin when the immunizing agent is Type D toxoid. However, the rather meagre results do not indicate that Type D toxoid conferred a high degree of immunity against the homologous toxin.

Experiment 3: *Type B formol-toxoid.*

A number of guinea pigs were immunized with Type B formol-toxoid (2 s.c. injections of 2·0 c.c., 14 days' interval). When tested with Type B toxin, 10 days after the second inoculation, some withstood 32 M.L.D. These were put aside and, one week later, again received 2·0 c.c. of toxoid. Seven days later, 2 guinea pigs received 1 M.L.D. of Type D toxin, by i.v. injection—both died. In view of this result, two further injections of 3·0 and 4·0 c.c. were given at a week's interval to the four remaining animals. When tested with 1 M.L.D., i.v., one week after the last injection, all died.

One may conclude from this experiment that the Type B toxoid used contained none of the epsilon fraction, or contained it in such small amount that insufficient was present to stimulate the formation of antibodies.

The conclusion to be drawn from the work reported in this addendum is as follows:—

(1) The Type B toxin used to produce Type B antitoxin 12479 contained a considerable amount of epsilon toxin (0·015 c.c. neutralized 10 M.L.D. of Type D toxin).

(2) At some time prior to the end of 1930, the Type B bacillus lost the power of forming this toxic fraction (or to form it in anything but minimal amounts), because guinea pigs, highly immune to Type B, did not withstand 1 M.L.D. of Type D.

(3) Under certain conditions, at present unknown, Type D bacilli are capable of producing the beta toxic fraction. Experiment 2 proves this.

ADDENDUM 2.

On reviewing, in 1935, the work just reported in addendum 1, the writer was struck by the fact that the Type D toxin which stimulated the formation of beta antitoxin in a rabbit, was prepared

from a twelve hours' culture. Knowing that the beta toxic fraction is produced early and the epsilon fraction late, in Type B cultures, the possibility existed that very young Type D filtrates might contain sufficient beta toxin to bring about the formation of the antitoxin. On this assumption, goats were immunized with the filtrates of two, four and eight hour cultures. Each animal received 1,500 c.c. of toxin in seven weeks, was then bled and the anti-beta content of its serum ascertained.

The two hour filtrate was relatively non-toxic (M.L.D. mouse i.v. —0·1 c.c.); the four and the eight hour filtrates killed mice in 0·01 c.c. (not tested lower). Type B antitoxin (1930 variety) did not, in a dose of 0·1 c.c. reduce the amount of Type D antitoxin necessary to neutralize any of the three filtrates.

Not one of the three antitoxins prepared with the Type D filtrates, was able, in an amount of 0·2 c.c. to neutralize 1 M.L.D. of Type B, 1930 toxin. The two hour filtrate antitoxin (0·1 c.c.) did not neutralize 1 M.L.D. of Type D toxin, 0·1 c.c. of the eight hour serum neutralized 2 M.L.D. and 0·025 c.c. of the four hour antitoxin neutralized 10 M.L.D.

The writer has had no opportunity of checking up the results noted in addendum 1, but he has no reason to believe that any mistake was made. Therefore, it would appear that the toxin-producing power of *Cl. welchii*, Type D, has undergone a change, inasmuch as it has lost the power to form beta toxin.

DISCUSSION.

The work detailed in this paper has confirmed many of the findings already reported in the literature. The " bloedpens " bacillus, a germ responsible for lamb dysentery (bloedpens) in South Africa appears to be identical serologically with *Cl. welchii*, Type B (the " lamb dysentery bacillus ") as originally isolated by Gaiger and Dalling. It produces two main toxins; young (18 hours) filtrates consist chiefly of Glenny's beta toxin and old (5 days) filtrates of epsilon toxin. Both young and old filtrates contain alpha toxin, and there was evidence that the former contained gamma toxin.

Toxic filtrates of *Cl. welchii*, Type B (1930 variety), and of *Cl. welchii*, Type C (*B. paludis*), were shown to contain beta toxin as their main constituent and alpha as their subsidiary, but were devoid of epsilon. Evidence of the existence of gamma toxin was got but the presence of delta in the Type C filtrate could neither be demonstrated or inferred.

Filtrates of *Cl. welchii*, Type D (*B. ovitoxicus*), both young and old, and of old filtrates of the original Type B strain and of the bloedpens bacillus contained as major toxic constituent the epsilon fraction, alpha being present as subsidiary fraction. The beta toxin was not demonstrated. That gamma was present was possible but was not proved.

The intradermic method proved to be a most unsatisfactory way of titrating toxic filtrates containing, as their chief constituent, the epsilon fraction, or of antitoxins consisting mainly of epsilon antibody. With two toxins this could possibly be accounted for by the small number of reacting doses per test dose. However, with two other toxins, the test doses of which contained a sufficiency of reacting doses, the values to be placed upon antitoxins varied greatly, depending on the toxin used for the titration. Whether or not this has to be accounted for by the existence of two or more serologically different fractions in the toxins and of their antibodies in the antitoxins, all in varying proportions, must await the results of future research. However, owing to the difficulty, experienced throughout all the intradermic work on the titration of toxins and antitoxins containing the epsilon and the anti-epsilon fraction respectively, of obtaining consistent results, the writer feels that the discrepancies may be inherent to the method of the test itself.

Future work will show whether or not a true Type D strain can produce beta toxin. The results given in addendum 1 prove that on one occasion it did do so. As, however, this was not confirmed some four years later with a subculture of the same strain, final judgment must be withheld until a considerable number of recently isolated organisms has been examined for their toxin-producing power.

Finally, the possibility exists that Type C should not be placed in a separate group. At the present moment, the "1930" variety of Type B and the Type C germ cannot be easily differentiated on the antigenic "make-up" of their toxins. As Type B, 1930 did, at one time, form epsilon toxin, the possibility exists that Type C may also, at one time, have been capable of forming it and has, like Type B, lost this power. However, as many workers throughout the world are at present engaged on the study of the welch group of germs or on disease caused by them, it behoves no one to pass a dogmatic opinion, but rather to await the result of this research.

CONCLUSIONS.

(1) The "bloedpens" strain of *Cl. welchii*, Type B (the "lamb dysentery bacillus") has been examined. Its toxin has been found to be serologically identical with that of *Cl. welchii*, Type B, as originally described by Dalling.

(2) The main differences and similarities of the toxins of *Cl. welchii*, Type A (classical *Cl. welchii*), Type B, Type C (*B. paludis*) and Type D (*B. ovitoxicus*) have been confirmed.

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