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The Toxin of Clostridium Chauvoei.

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CONTENTS.

INTRODUCTION.

Cl. chauvoei is an anomalous member of the group of pathogenic spore-bearing anaerobes. Whilst small amounts (0·1 c.c.) of living cultures, inoculated subcutaneously or intramuscularly into susceptible animals (bovines, sheep, guinea-pigs) may produce gas gangrene and death within 24 hours, yet it requires a large dose (5 to 20 c.c.) of sterile filtrate administered intraperitoneally to guinea-pigs to kill them. One may call such a filtrate " toxin ", but it is hardly to be compared with the exotoxin of *Cl. welchii* and certainly not with that of *Cl. tetani*. Leclainche and Vällée (1900[1]) record that Martin's broth cultures are toxic after 48 hours' incubation and that toxicity increases up to 15 days. Of a filtrate of a 5-day culture, 5 c.c. to 6 c.c. killed a guinea pig in 12 hours, when administered intraperitoneally. They note that the toxin was heat stable. Kelser (1918) states that 1·0 c.c. to 1·5 c.c. of a filtrate of a 10 to 12 days' Martin's broth culture was lethal to guinea-pigs. Kojima (1923[1],[2]) shows that the filtrates of young (18 to 36 hours) cultures in Martin's broth plus 0·1 to 0·2 per cent. glucose and fresh guinea-pig muscle pieces were toxic for mice (intravenous injection) in doses ranging from 0·1 c.c. to 0·7 c.c. The toxin had an acute action, producing symptoms and death in from 2 to 3 minutes up to an hour or two. This toxin (or another constituent of the toxic filtrate) was haemolytic. Both were thermo-labile, resisted storage badly, and were neutralizable by *Cl. chauvoei* antiserum. Eichorn (1918) states that filtrates possess toxic properties and Leclainche and Vallée (1923) record that from the 24th hour, *Cl. chauvoei* secretes a toxin capable of killing, immediately, various species of animals. Basset (1925[1]) shows that the filtrate of serum liver broth cultures kills guinea-pigs if 15 c.c. to 20 c.c. are given intraperitoneally. The lethal properties are practically the same whether the filtrate is fresh, 2 to 4 months old or preserved in the light or in the dark. Scott (1925) and Viljoen and Scheuber (1926) were unable to demonstrate a toxin in *Cl. chauvoei* filtrates. Zeller (1925) used filtrates from three weeks' old peptone liver broth (plus liver pieces) cultures; 10 to 20 c.c. given subcutaneously or intraperitoneally to guinea-pigs did not kill them and swellings only were produced on the subcutaneous injection of 25 c.c. and 50 c.c. into sheep and 100 c.c. into an ox. The work of Grassberger and Schattenfroth (1908[1]) has not been confirmed. They were able to demonstrate a most powerful toxin in filtrates but there would appear to be little doubt that a contaminating toxigenic anaerobe was responsible for this. This view is supported by their own results (1908[2]) in which they show that guinea-pigs immune to the toxin were not immune to the inoculation of virulent black quarter material (e.g. muscle oedema fluid). There is little doubt that St. Ivanič's (1922) cultures were not pure. Of 8 strains investigated, 6 produced a powerful toxin, as little as 0·005 c.c. of filtrate killing rabbits when injected intravenously. The " oedema " serum of the Vienna Serotherapeutic Institute was able to neutralize 10 mouse lethal doses of toxin in a dose of 0·001 c.c. This serum had no neutralizing effect on the cultures of the two non-toxigenic strains. Kerrin (1934) demonstrated toxin and haemolysin in filtrates of

buffered tryptic digest broth cultures. The lysin could haemolyse sheep red cells in a dose of 0·0025 c.c. to 0·005 c.c. Relatively small amounts (0·0025 c.c.–0·05 c.c.) of the sera of man, the ox, the sheep and the horse neutralized 10 minimal haemolytic doses, whilst 1·0 c.c. of the sera of normal guinea-pigs, rabbits and swine did not have this effect. The minimal lethal dose of the toxin (intravenous injection) was from 0·025 c.c. to 0·5 c.c. for mice and 0·5 c.c. for guinea-pigs; 3·0 c.c. did not kill a rabbit. The sera of man, horse, rabbit and guinea pig had a neutralizing effect on the toxin. The effect of the toxin was very quick. Heating the filtrate at 52° C. for 5 to 10 minutes destroyed the lysin and the toxin; however, both resisted storage fairly well.

The writer, in 1930 and 1931, at the Wellcome Physiological Research Laboratories, Beckenham, was able to demonstrate a weak toxin and lysin in the filtrates of young (18 to 24 hours) cultures of *Cl. chauvoei* (unpublished work). The results were similar to those noted above, viz., the intravenous injection into mice of from 0·1 c.c. to 1·0 c.c. of filtrate killed them with a very short incubation period (few seconds to half-an-hour) and from 2·0 c.c. to 5·0 c.c. was necessary to produce death in guinea-pigs. The lysin also was weak, the smallest haemolysing dose for sheep cells being about 0·025 c.c. but usually 0·1 c.c. was necessary. Toxin-antitoxin neutralization tests were unsatisfactory, owing to the small number of killing doses that could be used per test dose, often only one killing dose, and owing to the rapidity with which the toxin killed the animal. However, the experiments showed that an antitoxin made in a rabbit had more protective power than normal rabbit serum and the impression was gained that *Cl. septicum* antitoxin had some neutralizing effect. Unfortunately no definite conclusion could be drawn, as antitoxins made in horses against the toxins of *Cl. welchii*, *Cl. histolyticum* and *Cl. tetani* and the sera of some normal horses, bovines, goats and sheep sometimes neutralized one or two killing doses.

On the writer's arrival at Onderstepoort, the subject was taken up afresh, it being felt that if a " useable " toxin could be obtained, the disease, black quarter, could more easily be studied.

METHODS.

Cultures Used.

Cl. chauvoei.

Strain 64.—Isolated from a muscle of a heifer dead from black quarter in Waterberg (Transvaal) in 1929. This strain has been and is being used for the routine production of black quarter vaccine (anaculture) by the writer's colleague, Dr. J. R. Scheuber.

Strain D.—Brought from England by writer. Originally isolated from the muscle of a bovine, which died of black quarter in Europe.

Strain R. 77.—Isolated by the writer in 1934 from a piece of muscle of a bovine which died of black quarter in the Transvaal.

Strain L.—A strain supplied by Mr. D. A. Lawrence, Director of Veterinary Research, Southern Rhodesia. Mr. Lawrence informed the writer that this strain was being used for vaccine production and was relatively non-pathogenic for guinea-pigs and sheep. The writer was able to confirm this—2·0 c.c. to 5·0 c.c. of a 24-hour meat broth culture injected, intramuscularly, into a guinea-pig produced a swelling but not death.

Cl. septicum.

Strain K.F.—Brought from overseas by the writer, origin unknown. It produced a powerful toxin and had been used at the Wellcome Laboratories for the production of antitoxin.

Purity of Cultures.

Cl. chauvoei 64 and *Cl. septicum* K.F. were " single-celled " by the method described by Mason (1936[2]). The remaining cultures were repeatedly plated and typical colonies picked. As guinea-pig passage cultures of *Cl. chauvoei* were repeatedly used, frequent recourse was had to surface purity and to fermentation tests. Throughout the work, candled or pulped filtrate was injected intravenously into mice, as a check on the presence of, for example, the toxins of *Cl. septicum* or *Cl. oedematiens*. Beyond, very occasionally, the presence of a staphylococcus (when the culture was discarded) no indication was got that the various cultures were other than pure. The fermentation reactions of the various strains were as follows (1 per cent. " sugar " in 1 per cent. peptone water plus 5 per cent. sterile sheep serum, 14 day's incubation in a McIntosh's and Fildes' jar at 37° C.):—

All four chauvoei strains fermented with acid formation and usually with the production of gas, saccharose, galactose, glucose, lactose, laevulose, maltose; in arabinose, a " trace acid " reaction was got; salicine, sorbite, mannite, dulcite, raffinose, rhamnose and inuline were not attacked. In litmus milk (plus 5 per cent. sheep serum) a soft clot and acid was produced. Gelatine was liquified but Loeffler's serum medium and inspissated horse serum were not attacked.

The *Cl. septicum* strain (K.F.) produced acid and gas in galactose, glucose, lactose, laevulose, maltose, salicine, dextrine (±) and inosite. It was without action on saccharose, adonite, dulcite, raffinose, sorbite and mannite. An acid clot was formed in litmus milk, gelatine was liquified but neither Loeffler's medium nor solid serum was attacked.

The morphological, cultural and pathogenic peculiarities of the four chauvoei strains and the one septicum strain were those accepted as typical for these bacteria. K.F. produced a powerful exotoxin after 24–36 hours' incubation (M.L.D. for mouse i.v. between 0·005 c.c. and 0·05 c.c.) whereas, as will be shown in detail later, none of the chauvoei cultures produced a toxin the M.L.D. of which for a mouse was less than 0·1 c.c.

Media.

Horse flesh meat broth (meat broth).—This was a slight modification of Robertson's (1916) medium. The preparation is given in the article by Mason and Scheuber (1936). In all instances, about half the volume of medium in the tube or flask was meat particles. Preparatory to inoculation, the pH of the medium was adjusted to 7·8 with NaOH, and then boiled for from 10 minutes (tubes of 10 c.c. capacity) to 2 hours (500 to 2,000 c.c. flasks) and cooled rapidly.

Pope's "straight line digest" medium.—[Pope and Smith (1932).]

Hartley's digest medium (1922).

Viljoen's and Scheuber's medium 1926 (see Mason and Scheuber, 1936, for preparation).

Von Hibler's medium (1908).

Colebrook's digest liver medium [see Mason and Scheuber (1936) for preparation].

As with meat broth, the pH of all meat-particle-containing media was adjusted to 7·8, and boiled and cooled just prior to inoculation. (This does not apply to volumes of 10 or 20 litres: the pH sometimes was and at other times was not altered but owing to the bulk, boiling was not resorted to.)

Surface cultures were obtained on horse flesh infusion peptone agar, plus 10 per cent. of a mixture of equal parts of sheep serum, sheep haemolysed red cells and a saline extract of guinea pig liver (see Mason 1934 and 1936[2]).

Boiled bacilli (Henderson 1932). It was found that dense suspensions of *Cl. chauvoei* could be obtained by growing the germ in meat broth for 18–24 hours, pulping the culture, discarding the meat and mushing up the pulp, containing the bacteria, in a small quantity of water. One could then express a considerable portion of the bacteria-containing fluid with a press and the organisms could then be washed and spun out in a centrifuge. The dense suspension, so obtained, could then be treated as described by Henderson.

Filter candles.—The Berkefeld N variety was used throughout.

Animals: Mice.—These were supplied by one dealer and weighed between 15 and 18 grammes.

Guinea pigs.—White or predominantly white animals were used and weighed between 250 and 300 grammes. A barium sulphide depilatory was used to remove the hair, for the intradermic titration of toxin.

Sheep.—These were of the Merino breed, varied in weight from 18 Kg. to 30 Kg. and had usually passed through an anthrax and/or blue tongue experiment. Intradermic titration of toxin was carried out on the bare portion of skin of the inner side of the hind leg.

EXPERIMENTAL.

PART I.

The Demonstration and Production of a Toxin.

Preliminary experiments.—In a series of preliminary experiments an attempt was made to demonstrate a toxic action of the supernatant fluid (spun) or of the pulped or candled filtrate of a 6 to 18 hours' *Cl. chauvoei* culture (meat broth plus 5 per cent. haemolysed cells and serum). From 10 c.c. to 500 c.c. of medium was used and particular attention was paid to a thorough boiling to expel dissolved air. In some instances the medium was boiled, cooled, inoculated and then exhausted in an air-tight jar until bubbles ceased to appear. The jar was then washed out 2 or 3 times with hydrogen and finally filled with this gas and incubated. In one experiment, the following substances were added to the medium, prior to inoculation—chalk (excess), saponin (1 in 40 of a 0·4 per cent. solution), cystein (0·2 per cent.) and ferrous sulphate (1 in 40 of a 0·4 per cent. solution). To a number of filtrates, sodium hydrosulphite ($Na_2S_2O_4$) was added to make a concentration of 0·01–0·1 percent., and exhaustion carried out for 2–3 hours. The inoculum in all instances was the heart blood and/or liver of a guinea-pig just dead from an intramuscular inoculation of *Cl. chauvoei* or a meat broth culture once removed from a guinea-pig passage.

The results of the use of these various products (eleven in all) were as follows—guinea-pigs died after receiving 0·5 c.c.–2·0 c.c. intravenously (cardiac puncture). Symptoms usually developed in 2 to 10 minutes, the animal being distressed and breathing in a pumping fashion. It might then die within a further 5 to 30 minutes or might apparently recover to die within 24 hours. Some showed this distress but recovered and remained well. The most striking post-mortem appearance was the presence of petechiae or suggilations in the lungs; in some cases the lungs were a haemorrhagic mass. The serum from a sheep which had received anaculture and then living culture and survived, neutralized the toxic effect of the filtrate whereas normal sheep serum did not do so (filtrate and serum mixed, left for 1 hour at room temperature and injected). However, the antiserum was not able to prevent the appearance of the initial symptoms of distress.

Mice usually survived the injection of 0·5 c.c. intravenously. As a rule, early symptoms of distress appeared, to pass off in from 5 to 30 minutes.

Two sheep, one normal and one immune, received intravenously, 200 c.c. of a pulped filtrate which killed guinea pigs in a dose of 1·5 c.c. No symptoms or disturbances were observed.

No indication was got that any one method of toxin production was better than another (period of growth, exhaustion, reduction, etc.). These preliminary experiments indicated that there was a very weak toxic material in chauvoei filtrates, but that, unless it could be concentrated, no useful work could be carried out. Before attempting concentration, the effect of intradermic injection in guinea pigs was ascertained.

Experiment 1.*

Strain 64 was grown in 3 × 40 c.c. flasks of meat broth plus 5 per cent. serum and haemolysed cells in a MacIntosh's and Fildes' jar. After 18, 48 and 72 hours' incubation a tube was removed and filtrate injected intradermically into guinea-pigs. In addition the supernatant of the spun material or the candled sample of each day's filtrate was reduced with $Na_2S_2O_4$ and exhausted and its intradermic effect ascertained.

The one and the two day filtrates produced small red-blue areas on the skin, apparent after 2–3 hours; the three day filtrate formed a very pale red flush. Anti-chauvoei sheep serum neutralized this reaction, whilst normal sheep serum had little if any effect.

Experiment 2.

As experiment 1 indicated that 18-hour filtrates contained a product which caused a reaction on intradermic injection, an attempt was made to concentrate this material by drying. Strain 64 was grown in meat broth plus serum and haemolysed cells for 18 hours, in an air-exhausted jar. Of the candled filtrate, 0·2 c.c. produced a faint red area on the skin. A dry powder was made by drying down 30 c.c. over H_2SO_4; 30 mg. caused an intense blue mark, only slightly affected by chauvoei antiserum but not by normal serum. The same amount gave rise to a small red reaction in a normal sheep (36859) and to no reaction in an immune sheep (37405). Further experience indicated that part of the reaction in guinea-pigs was due to non-specific material, in great measure, to NaCl. Some batches of dried and powdered broth caused quickly-appearing bloody reactions. However, these tended to disappear quickly and the area was, as a rule, only with difficulty located after 24 hours.

Experiment 3.

The experiments collected under this heading were continuations of experiment 2. Strain 64 was cultured for 18 hours in meat broth plus serum and haemolysed cells and pulped or candled filtrates were either dried down or saturated with ammonium sulphate (about 60 gm. per 100 c.c.) and the precipitate dried. Such powders, in a dose of from 10 to 30 mg., produced intradermic reactions in guinea-pigs and sheep. Chauvoei immune sheep† either did not react or only very slightly. Chauvoei antiserum reduced the size and the intensity of the reactions (blue-red spots) but did not annul them entirely. Thus, there was reason to hope that the toxin could be further concentrated. With this end in view 2 × 2 litre quantities of meat broth plus serum and haemolysed cells were inoculated direct from a guinea pig, incubated for 18 hours and pulped. The wet precipitate produced by saturating with ammonium sulphate was dialysed, in

* It is to be assumed throughout, unless otherwise stated, that the pH of the medium was rectified and the medium boiled just prior to inoculation.

Further it is to be assumed that the inoculum was either the heart blood and/or liver of a guinea pig just dead from black quarter or a culture not far removed from a passage. When the statement is made that the inoculum was the liver of a guinea pig, it refers to the liver of a guinea pig just dead of an experimental infection.

† Unless otherwise stated, a chauvoei immune sheep means one which has received anaculture and living culture.

parchment paper, for four days against distilled water. This was carried out at 10° C. to minimise any bacterial growth that might occur. The precipitate from 1 litre was placed in the paper, water added and this dialysed against about 16–20 litres of water, which was changed twice daily. The final outside fluid gave no precipitate or only the faintest opalescence when barium chloride was added. (Later on it was found advantageous to carry out the first 4–6 hours dialysis again running tap water). A copious, white, rather gelatinous precipitate formed in the bag. This was spun off and the supernatant dried down, first with fans blowing over shallow layers, and then when nearly dry, in an exhausted desiccator over H_2SO_4. Some experiments with the dry toxin so formed (Toxin 19) will now be detailed.

Toxin 19.—The dry powder was not fully soluble in saline, distilled water or broth, a white, rather sticky precipitate being got after spinning. The pH of the supernatant was 5·5–5·6; on adjusting the pH to 7·4–7·6 nearly all the deposit went into solution. However as this did not render the material more toxic, it was evident that the deposit was inactive, so that, as a routine, the powder was stirred up in the saline, spun and the supernatant used. After several trials, it was found that 100 mg. in 1·0 c.c. gave a solution that was mobile enough for the purposes required.*

Boiling the toxin for half-an-hour produced a considerable coagulum; heating at 60° C. for half-an-hour caused opalescence; 0·1 c.c. of neither produced a recognizable intradermic reaction in guinea pigs.

Minimum Reacting Dose (M.R.D.) in Guinea-pig.—This was found to be in the region of 0·01 c.c. A dose of 0·1 c.c. produced a red flush in half-an-hour, increasing in intensity after 2 hours and being at a maximum in 4–6 hours. After 24 hours, there was usually a tendency to spread with a less intense colouration. Doses around the M.R.D. came up more slowly, being apparent in 2–6 hours and recognizable as small reddish-blue marks after 24 hours.

Toxicity on Intravenous (i.v.) Injection.—A dose of 0·25 c.c. did not kill a mouse and 0·5 c.c. was non-lethal for a 150 gm. guinea-pig.

Reactions in Sheep.—Two immune sheep and one normal sheep received 0·25 c.c. intrademically. In 6 hours, the reaction in the normal animal was manifesting itself and in 24 hours was definite. This consisted of a very roughly circular red-blue area about 20 mm. in diameter, of a more intense hue in the centre. After 48 hours it had faded somewhat and was only a pale flush after 72 hours. No reactions appeared in the two immune sheep.

Specificity.—Ten M.R.D. (0·1 c.c.) were neutralized by 0·005 c.c. (not by 0·0025 c.c.) of the serum of one immune sheep, by 0·1 c.c. (not by 0·05 c.c.) of that of another but not by 0·1 c.c. of the sera of two normal sheep. (Using other toxins, it will be shown that 0·1 c.c. of normal sheep serum does not neutralize one sure M.R.D.).

*Throughout the work with different toxins 100 mg. of dry toxin was dissolved in 1·0 c.c. saline. Therefore, the amounts of toxin injected will be given in c.c. and not in mg.

Comment on Toxin 19.—It appeared reasonably certain that a toxin had been demonstrated in a *Cl. chauvoei* filtrate, and that the intradermic injection into guinea-pigs was a satisfactory method of demonstrating it. One disadvantage was the rapidity with which the reaction faded. Often after two hours, what looked like a forerunner of a strong reaction was visible—a definite dark red area of about 10–15 mm. in diameter—to fade to a faint red flush within 18 hours. The overcoming of this difficulty and the investigation of other methods of concentration was tackled next.

Attempts to Concentrate Toxin.

Experiment 4.

(*a*) *Effect of Dialysing in Parchment Paper and in Cellophane.*—Strain 64 was grown for 18 hours in meat broth plus 10 per cent. of horse serum* and 2 per cent. of guinea-pig liver extract. The pulped filtrate was precipitated with ammonium sulphate and the precipitate dialysed against distilled water for 3 days in parchment paper and in cellophane (commercial variety obtained in South Africa, grade number unobtainable). Both inside fluids were treated as described for Toxin 19. The M.R.D. and Lr of each was approximately the same, with, if anything, the advantage to the "cellophane" toxin (Toxin 35).

(*b*) *The Effect of Dialysing for different lengths of time.*—Strain 64 (direct from a guinea-pig liver) was grown for 18 hours in 500 c.c. flasks of meat broth plus 10 per cent. horse serum. The precipitate produced by saturating the pulped filtrate with ammonium sulphate was dialysed in cellophane for different periods, and the inside fluid dried down after removing insoluble material. The M.R.D. and in some cases the Lr was then established (table 1).

Table 1.

The effect of Dialysis on Cl. chauvoei *toxin* (*Toxin* 34).

Dialysis.	M.R.D. (c.c.).	Lr.
Nil†	? 0·025	—
3 hours	>0·1	—
21 hours	0·025	—
48 hours	0·005–0·01	0·0025
96 hours	0·01–0·025	0·01
144 hours	? 0·1	—
288 hours	? 0·1	—

Similar tests carried out with other brews of toxin confirmed, in large measure, the results of table 1, viz. that a 3 to 5-days' dialysis with removal of insoluble material produced the best results. The questionable reaction got with the non-dialysed precipitate is to be attributed to the high concentration of ammonium sulphate.

* The serum, taken with aseptic precautions, was incubated at 37°C for 48 hours in the presence of 0·1 per cent. formalin prior to its addition to the medium.

† "Nil" means that the crude dried ammonium sulphate precipitate was used. The figures under Lr mean that those amounts in c.c. of a certain antiserum neutralized 0·1 c.c. of toxin, whilst 25 per cent. less antitoxin did not do so. The sign "—" indicates no test.

(*c*) *The Use of Media other than Meat Broth.*—The details of each individual experiment need not be recorded. The following media were used, usually in 500–1,000 c.c. amounts, the inoculum being guinea-pig liver or a culture once removed from it, and the incubation being 18 hours at 37° C.—Hartley's digest broth (plus meat particles and sheep serum and haemolysed cells), Pope's digest broth (plus meat particles and sheep serum and haemolysed cells), Colebrooke's liver digest broth mixed with horse flesh broth and strongly buffered (mixed 1 to 9, 1 to 5, 1 to 1 and 2 to 1, no meat particles) and Viljoen's and Scheuber's medium. Under this heading may be included experiments comparing the toxin-producing power of large volumes (10–20 litres) and small volumes (500 c.c.) of meat broth plus 5 per cent. horse serum, meat broth plus 5–10 per cent. horse serum, 5 per cent. sheep serum and haemolysed cells and guinea-pig liver extract (the saline extract of from half to one liver, added to 1,000 c.c. of medium), meat broth plus 5 per cent. horse serum when the peptone was added right at the start of the preparation of the medium and when it was added in the usual way after the preparation of the muscle extract and meat broth plus 0·25 per cent. glucose.

It can be stated definitely that Viljoen's and Scheuber's medium and large volumes of meat broth gave poor results. It is difficult to submit an opinion on the results with the use of the other media, because sometimes the one and at other times the other gave indications of producing the best toxin. At one time it was thought that the addition of as much as 10 per cent. of horse plasma was very advantageous. Although this was undoubtedly so, there was the disadvantage that only about half of the final dry powder was soluble in water or saline. After repeated comparative tests, meat broth (meat particles about one half by volume) plus 2 to 3 per cent of guinea-pig liver extract (or, if the inoculum was a guinea-pig liver, about a half liver to 500–1,000 c.c. of medium) was adopted as yielding good toxin, with a final powder of which a considerable percentage was soluble.

(*d*) *The effect of the inoculum.*—When the inoculum was a portion of the liver of a guinea-pig killed by the intramuscular injection of culture (or of a culture not more than one meat broth tube removed from it) more potent toxin (in M.R.D.) was obtained than when a culture distant from an animal was used. The following brief summaries of two experiments illustrate this:—

1. (*a*) *Strain* 64: 6 meat broth cultures removed from a guinea-pig passage—grown 20 hours in 1 litre of meat broth plus sheep serum, haemolysed cells and guinea-pig liver extract. M.R.D. of dissolved powder, 0·05–0·1 c.c.

   (*b*) As (*a*) but inoculum direct from a guinea-pig liver. M.R.D. 0·01 c.c.

2. (*a*) Strain L as received from Mr. Lawrence (5·0 c.c. avirulent for a guinea-pig). Grown 36 hours in 1 litre of meat broth plus guinea-pig liver extract. M.R.D., 0·05–0·1 c.c.

   (*b*) As (*a*) but inoculum direct from a guinea-pig liver. M.R.D. 0·005–0·01 c.c.

(*e*) *The effect of the length of incubation.*—The three illustrative experiments to be noted were conducted with strains 64, L and R 77 respectively, in the medium and method of choice noted under (*c*). They were carried out when a means had been found of "stabilising" the intradermic reactions (see page 447 under " adrenalin "); with such a means a definite reaction could be elicited with pulped or candled filtrate. Flasks of 500 c.c. capacity were used. Pulped filtrate and the same, precipitated with ammonium sulphate, dialysed for 4 days in cellophane and then dried were employed. Table 2 records the results.

The results given in Table 2 do not allow the drawing of definite conclusions, although the indication is that, with increasing length of incubation, the toxicity drops. This indication was strengthened by results obtained in media such as meat broth plus horse plasma, where a 20 hours' filtrate was more potent than one of 48 hours' incubation. It will be noted that, in conformity with diphtheria toxin, the antitoxin-binding-power of the toxins need not necessarily bear a close relation to the M.R.D. Although the M.R.D. of the 72 hours' precipitated toxin of 64 is $2\frac{1}{2}$ to 5 times that of the 20 hours' toxin, the Lr has not altered. However, it is realised that the Lr titration was carried out at three-fold limits, so that one cannot be certain that there was no difference.

One difference between the dry toxins from young and old cultures was the relatively clear-cut intradermic reactions produced by the former compared with the somewhat indefinite flushes caused by the latter. Chiefly for this reason, toxins for routine purposes were prepared from 20 hours' cultures.

(*f*) *The Effect of the Strain.*—No indication was got that any one of the four *Cl. chauvoei* strains (64, D, R 77 or L) was a better toxin-producer than the other, when a virulent culture direct from a guinea-pig liver was used and when the medium was meat broth plus serum, haemolysed cells or liver extract and the incubation 20 hours.

(*g*) *The Precipitation of Toxin other than by Saturation with Ammonium Sulphate.*

1. Half saturation with ammonium sulphate: The toxicities of two dry powders obtained by saturating (60 gm. per 100 c.c.) and by half saturating a 20-hours' filtrate with ammonium sulphate with subsequent dialysis and drying were approximately the same.

2. Precipitation with potash alum: (*a*) The filtrate of a 20 hours' meat broth plus horse plasma culture of strain 64 was saturated with ammonium sulphate, the precipitate dialysed for 6 days and the inside fluid spun. Some of the clear supernatant was dried down and to another lot at pH 5·7 enough alum was added to make a 1·5 per cent. concentration. After washing the alum precipitate twice in distilled water, it was dissolved in 2 per cent. sodium citrate and dialysed for 48 hours. The inside fluid was then spun and the supernatant dried down. The M.R.D. of the original dialysed ammonium sulphate precipitate was 0·01 c.c., and of the final alum product 0·1 c.c.

TABLE 2.

The Effect of the Length of Incubation on Toxin Production.

Incubation.	Toxin.											
	Strain 64.				Strain R 77.				Strain L.			
	Pulped.		Precipitated.		Pulped.		Precipitated.		Pulped.		Precipitated.	
	M.R.D.	Lr.	M.R.D.	Lr.	M.R.D.	Lr.	M.R.D.	Lr.	M.R.D.	Lr.	M.R.D.	Lr.
20 hours	0·1	0·0001–0·0003	0·01	0·0003–0·001	0·025	0·0006–0·002	0·015	0·001–0·0015	0·05–0·1	0·001–0·003	0·01	0·006–0·008
48 hours	0·05–0·1	0·0003–0·001	0·01–0·02	0·001–0·003	0·05–0·1	0·0006–0·002	0·01–0·015	0·001–0·003	0·025	0·001–0·003	0·01	0·006–0·008
72 hours	0·05–0·1	0·0001–0·0003	0·025–0·05	0·0003–0·001								
96 hours									?0·025–0·05	0·0003–0·001	0·015	0·001–0·002
120 hours					0·025	0·0003–0·001	0·01	0·003–0·01				
168 hours									0·05	0·0003–0·001	0·015–0·02	0·002–0·004

(All figures = c.c. For the M.R.D. titrations, 100 mg. of dry toxin were dissolved in 1·0 c.c. of saline. Lr. = the first figure is that amount of antitoxin which did not neutralize 0·2 c.c. of pulped toxin or 0·1 c.c. of dissolved toxin and the second is that amount which did neutralize these amounts.)

(*b*) A 20 hours' filtrate was treated with enough alum to make a 1·5 per cent. concentration, and the precipitate dealt with as noted under (*a*). The M.R.D. was 0·025 c.c.

(*c*) Samples of a 20 hours' filtrate were treated with enough alum to make respectively a 1·0, 2·5, 5·0, and a 10·0 per cent. concentration and the precipitates dealt with as noted under (*a*). The smallest M.R.D. (1·0 per cent. alum) was 0·025 c.c.

(*d*) The dry powder of a dialysed ammonium sulphate precipitate was dissolved in saline, the pH adjusted to 5·6 and alum added (1·5 per cent. concentration). The precipitate was treated in the manner noted under (*a*). In addition, the supernatant of the alum precipitate was dialysed for 48 hours and then dried down. The M.R.D. were as follows—ammonium sulphate precipitate 0·005–0·01 c.c., alum precipitate 0·005–0·01 c.c., alum supernatant 0·007–0·015 c.c.

(3) Precipitation with Sodium Sulphate.—Strain 64 was grown for 20 hours in meat broth plus 5 per cent. horse serum. To the pulped filtrate 20 gm. of sodium sulphate per 100 c.c. was added and the precipitate dialysed for 4 days and the resultant fluid dried. To the supernatant of the first precipitate a further 20 gm. per 100 c.c. added, and the precipitate dialysed and the resultant fluid dried. The M.R.D. of the first precipitate was 0·03 c.c. and of the second 0·04–0·05 c.c.

(4) Precipitation with Acetic Acid.—The same filtrate as noted under (3) was used.

Enough acetic acid was added to bring the pH to 5·2 and the precipitate collected (1st precipitate). The supernatant was brought to pH 4·7–4·8, and the precipitate collected (2nd precipitate). The supernatant of this was brought to pH 4·5 and the precipitate collected (3rd precipitate). These three precipitates were dried down *in vacuo* and 100 mg. shaken up in saline (the pH of the suspension was brought to 7·8 with ammonia). A considerable amount did not go into solution; the M.R.D. of each supernatant was more than 0·1 c.c.

(5) Precipitation with Zinc Chloride: Strain 64.—The pulped filtrate of a 20 hours' meat broth plus liver extract culture of strain 64 was treated as follows. One sample was saturated with ammonium sulphate and the precipitate dialysed in the usual way: to another volume enough zinc chloride was added to make a 1 per cent. solution. The precipitate after two washings with distilled water was dissolved in 2 per cent. sodium citrate, dialysed for 4 days, and the inside fluid dried down.

The M.R.D. of both toxins was between 0·005 and 0·01 c.c.

(6) Precipitation with Acetone Saturated with Benzoic Acid.—Five grammes of toxin 19 were dissolved in 100 c.c. of distilled water and the solution clarified by spinning. To the chilled supernatant at pH 5·5, 5·0 c.c. of a saturated solution of benzoic acid in acetone was added. A copious white precipitate formed immediately. This was kept cool and washed 3 times with acetone (100 c.c. per washing). The final precipitate was dried *in vacuo*. The powder was white, impalpable and went into solution readily in saline of pH 8·0–8·2.

Intradermically, amounts of toxin between 0·05 and 0·1 c.c. produced quickly-appearing blue spots, which disappeared in a few hours.

(7) Precipitation with Alcohol and Ether.—On a number of occasions, the precipitates obtained by adding from 2 to 6 volumes of alcohol or of ether to a 20 hours' filtrate were dried down and titrated intradermically in guinea-pigs. None of the powders went into solution readily and their toxicities were low.

(*h*) *Adsorption on and Elution from Kaolin.*—To 20 c.c. of toxin 19 at pH 8·5 (dissolved in Universal Buffer*) enough kaolin to make a 10 per cent. suspension was added. The suspension was kept cool and shaken periodically for 5 hours. The kaolin was then spun out and suspended in 10 c.c. of Universal Buffer at pH 5·5. After thorough agitation, the kaolin was again spun off. No toxin could be demonstrated in the supernatant.

(*i*) *Effect of Freezing and Thawing Toxin.*—Salimbeni and Loiseau (1934) state that diphtheria toxin may be concentrated by freezing the fluid and collecting the bottom third, after thawing has taken place at room temperature. In such a test, carried out with one toxin (29) the M.R.D. of the original toxin, the top and the bottom third of the frozen and then thawed fluid was approximately the same.

Comment on Concentration of Toxin.

No definite indication was got that better concentration of toxin could be produced consistently by methods other than the ammonium sulphate-dialysis technique. One experiment might indicate that alum yielded the best product but repeat tests did not necessarily confirm this. For this reason, the ammonium sulphate method was adopted as routine.

Experiment 5.

Attempts to Obtain Clearer Intradermic Reactions.

As previously stated, the intradermic injection of toxin produced, when 5 to 10 M.R.D. were used, a reaction appearing as a tiny flush in 5 to 15 minutes, increasing in size and intensity up to 3 to 5 hours but fading out considerably in 18 hours. Thus, the determination of the M.R.D. and Lr was attended with some difficulty. One gained the impression that if the toxin could be fixed to one spot for a hour or two or if the tissue could be slightly damaged a more persisting and therefore more easily read reaction would be obtained.

(*a*) *Effect of Different Diluents* (Toxin 36).—This toxin was dissolved (100 mg. in 1·0 c.c.) in the following diluents, spun to remove any insoluble material and the supernatants tested–saline (0·85 per cent.) at pH 5·5, 7·4 and 8·0, 1·0 per cent. glucose (in distilled water) at pH 7·4 and 8, 2·0 per cent. starch (pH 8), 1·0, 2·5, 5·0 and 10·0 per cent. peptone (Witte) in distilled water at

* British Drug Houses, London.

pH 7·4, distilled water, salt solutions (NaCl) of 1·0, 2·5, 5·0 and 10·0 per cent. concentrations, 10 per cent. serum (horse) broth and 10 per cent. guinea-pig serum in saline (pH 7–7·2). In most cases an M.R.D. and an Lr test was carried out. No definite indication was got that any one of these diluents at whatever pH gave reactions superior to those obtained when the toxin was dissolved in 0·85 per cent. salt solution of about pH 7–7·2. Quickly-appearing bloody flushes were produced when distilled water and the higher concentrations of salt and peptone were used, but, in these cases, the control fluid, itself, without toxin, produced a similar reaction.

A small amount of insoluble deposit was obtained when toxin 36 was placed in saline, the final pH of the supernatant being about 6. By raising the pH to 7·6–8·0, nearly all of this deposit went into solution but without lowering the M.R.D. or intensifying the reaction.

(*b*) *The Effect of Incorporating Agar in the Toxin.*—When the toxin was dissolved in 0·1 to 1·0 per cent. nutrient agar, the reaction produced was much more definite and persistent than when saline was the diluent. However, a difficulty was experienced in carrying out a toxin-antitoxin titration. It was difficult and, at times, impossible to ensure a thorough mixing of the toxin and antiserum, when 0·25 per cent. agar was employed. For this reason, results varying from day to day were got, depending upon the degree of admixture.

The Effect of Adrenalin.

As adrenalin produces a vasco-constriction, the possibility existed that, mixed with toxin, it would localise it in the injected area, and thus give it the opportunity of producing an easily recognizable reaction. Experiments proved the correctness of this supposition. The adrenalin used was that prepared by Gehe and Co. A. G. Dresden, solution 1/1,000. Of this, 0·1 c.c. produced a very extensive haemorrhagic area in a guinea-pig's skin in 18 hours, the animal dying in 48 hours. Dilutions of 1/10, 1/20, 1/30 in 0·3 c.c. saline nearly always caused spreading haemorrhagic reactions, the animal usually dying or having to be killed. A borderline dose was 0·3 c.c. of a 1/50 dilution; sometimes a dirty-red spreading flush was produced, particularly if the site of injection was near the loose skin of the belly. There was less risk of causing a reaction when the adrenalin was injected into the firmer skin over the ribs. Dilutions higher than 1/50 proved relatively safe and after considerable experimentation 0·3 c.c. of a 1/100 dilution was chosen as being suitable. The toxin was localized for a time sufficiently long to produce a reaction readable after 24 to 48 hours whilst the risk of the non-specific effect of the adrenalin was very slight. In a quite negligible number of occasions, the adrenalin did, *per se*, cause flushes, but very little experience was necessary to distinguish these from toxin reactions and further, a repeat test invariably cleared up any doubt. The practice was adhered to of storing the adrenalin in an amber coloured bottle in a refrigerator and discarding it if it became discoloured in any way or if any deposit was discernible.

The reaction produced by the toxin plus adrenalin will be described under " the actions of the toxin ".

The Effect of Removing Insoluble Material from the Toxin.—No toxin has been prepared which is fully soluble when added to saline at pH 7·0. The pH of the toxin in saline solution is between 5·5 and 6·5 depending on the toxin. By raising the pH of the toxin solution to about 8, most of the undissolved material dissolves but, as has previously been stated, without a corresponding reduction of the M.R.D. An experiment was conducted in which a dry toxin was dissolved in saline, the deposit being removed by centrifugation. The supernatant was dried and the powder again dissolved in saline, and this process repeated three times. On each occasion, a precipitate was obtained on attempting to dissolve the toxin, and further, the final supernatant (toxin dissolved 100 mg. to 1·0 c.c.) was less toxic than the original. Thus, it would appear that each drying denatured some protein and destroyed some toxin.

The Actions of the Toxin.

1. *The Effect of Intradermic Injection.*

(These remarks apply when the toxin is dissolved in a 1/100 dilution of adrenalin and 0·3 c.c. is injected.)

When 5 to 10 M.R.D. are injected into a guinea-pig a flush usually appears within 5 to 15 minutes, to disappear in about half-an-hour. For a further one or two hours the site of injection is recognized by the paleness of the skin, the effect of the adrenalin. This gradually disappears and 2 to 4 hours after treatment a somewhat strippled pale red spot appears increasing in intensity up to 8 hours. After 12 to 24 hours, a typical reaction (caused by 5 M.R.D.) is very roughly circular, from 1·5 to 3·0 cm. in diameter, with an intense red centre and a rather mottled-red radiating periphery. In some instances, 5 to 10 reacting doses produce a small area of necrosis in the centre, but the reaction is, in the main, haemorrhagic in nature. Amounts of toxin, around the M.R.D., produce nothing recognizabe for 6 to 8 hours and are visible after 24 hours as small red flushes measuring 1 to 2 cm. in diameter. (See Fig. 1.)

As much as 10 guinea-pig reacting doses did not cause a lesion in rabbits.

The M.R.D. for the sheep was approximately that for the guinea-pig, the injections being made into the skin of the thigh. All the sheep used at Onderstepoort were docked so that the much thicker and tenser skin of the under surface of the tail could not be used. Experiments carried out by Dalling, Gordon and Mason at the Wellcome Physiological Research Laboratories proved that this was a most suitable site for the intradermic titration of *Cl. welchii*, Type B toxin (unpublished work), and the writer suggests that the same would hold for *Cl. chauvoei* toxin.

2. *Lethal: (Intravenous Injection).*

Guinea-pigs.—(Toxin 37 L. M.R.D. = 0·01 c.c.)

The toxin was dissolved in saline (100 mg. in 1·0 c.c.) the deposit removed, and the dose injected in a total volume of 1·0 c.c.

A dose of 0·5 c.c. often produced a few spasmodic movements, with quickened respirations, in a few minutes. However, these passed off, to be followed in from 2 to 3 hours by weakness and hurried breathing. The animal lay on its side, occasionally uttering a squeak. Death usually supervened in 4 to 6 hours, being preceded by spasms. The minimal lethal dose of this toxin was between 0·1 and 0·15 c.c.

Mouse.—Per gram body weight, this animal was much less susceptible than the guinea-pig, the M.L.D. being 0·1 c.c.

Rabbit.—Twenty guinea-pig fatal doses produced only transitory distress.

Sheep.—The fatal dose proved to be between 10 and 15 c.c. In about half-an-hour the respirations became markedly accelerated and, a thick ropy mucous discharge appeared at the nostrils. In 2 or 3 hours, the breathing was of the pumping variety, strings of mucus hung from the nose, and occasionally diarrhoea was present. Incoordination of movement and later inability to support the body supervened and death occurred in from 6 to 48 hours after the injection.

Post-mortem Appearances.—In the guinea-pig, mouse and sheep, these were entirely of a haemorrhagic nature. The lungs were always the seat of bloody effusions, sometimes only petechiae being present but at other times the lungs were bloody masses. On the pleura and peritoneum petechial spots to suggilations were seen. The heart was usually flabby, with petechiae or suggilations on the endocardium. extensive haemorrhages were present in the wall of the intestine, and in one sheep haemorrhages had occurred in the gut-wall from the abomasum to the rectum. Blood was usually noticeable in the lumen of the intestine. In one sheep, which died half-an-hour after receiving 2 M.L.D., about 1 litre of bloody fluid was present in the pleural cavity, the lungs also being full of blood. This animal obviously drowned in its own exudate.

3. *Haemolytic.*

In conducting the haemolytic test, toxin was added to 1·0 c.c. of a 2·5 per cent. suspension of thrice washed sheep red cells, the total volume made up to 2·5 c.c. with saline, the tubes incubated at 37° C. for 2 hours and, after standing at room temperature for a further 2 hours, were read as complete (C), nearly complete (N.C.), partial (P), trace (T) or negative (O) haemolysis. In table 3 are recorded the minimal haemolytic doses (M.H.D.) and Lhs of a number of toxins. The Lh was arrived at by mixing 0·5 c.c. of the toxin with decreasing amounts of antitoxin, allowing the mixtures to stand for one hour at room temperature, adding 1·0 c.c. of red cell suspension and after adjusting the volume to 2·5 c.c. with saline treating the tubes as described for the M.H.D. test. In these tests, the haemolytic power of the pulped culture and of this pulped filtrate after precipitation, dialysis, drying and re-solution (100 mg. in 1·0 c.c.) was established.

TABLE 3.

The Haemolytic Power of Cl. chauvoei *Toxin.*

Age of Toxin.	Pulped toxin.		Precipitated toxin.	
	M.H.D.	Lh.	M.H.D.	Lh.
		STRAIN L (V).		
20 hours	0·1	0·03–0·1	0·1	0·03–0·1
48 hours	0·1	0·03–0·1	0·05	>0·1
96 hours	0·25	0·03–0·1	0·1	?0·1
168 hours	0·25	<0·01	0·05	0·03–0·1
		STRAIN L (V) (another brew).		
36 hours	0·05	0·03–0·1	—	—
		STRAIN L (AV.).		
36 hours	0·05	0·03–0·1	—	—
		STRAIN D.		
20 hours	—	—	0·1	0·1–0·3
		STRAIN 64.		
20 hours	0·025	0·03–0·1	—	—
		STRAIN 64 (another brew).		
20 hours	1·0	N.D.	0·2	0·03–0·1
48 hours	0·25	0·03–0·1	0·2	0·1 –0·3
72 hours	1·0	N.D.	0·25	0·1 –0·3

(All figures = c.c., precipitated toxin dissolved 100 mg. in 1·0 c.c. saline; N.D. = not done; age of toxin = number of hours that culture was grown; in the Lh titrations, 0·5 c.c. of toxin was used; the first figure is that amount of a certain antitoxin which did not neutralize the lysin contained in this amount of toxin and the

second figure is that amount which did neutralize it; the M.H.D. and Lh titrations were carried out at 100 per cent. limits; the M.H.D. was taken as that amount which produced a " partial " or " large trace " haemolysis).

It will be noticed that neither the original pulped nor the precipitated filtrates of *Cl. chauvoei* are markedly haemolytic, from 0·025 c.c. to 1·0 c.c. being required to cause partial lysis of sheep red cells. Further, large amounts of antitoxin are required to produce neutralization, much more than was necessary to neutralize that portion of the toxin causing skin reactions. For example, 0·008 c.c. of a certain antitoxin (38212) was required to neutralize a test dose (0·1 c.c. = 10 M.R.D.) of strain L precipitated toxin, whereas 0·1 c.c. was necessary for 0·5 c.c. (5 M.H.D.) in the haemolytic test. The sera of different animals (not immunized against *Cl. chauvoei*) had a definite neutralizing power. The sera of normal horses, cattle, sheep, goats, rabbits and guinea-pigs in a dose of 0·1 c.c. could neutralize 5 to 10 M.H.D. of 37 toxin. The indications were that cattle and guinea-pig sera were poorer in this respect than those of the horse. No indication was got that the antitoxins of *Cl. welchii*, Types A, B or D, *oedematiens*, *septicum*, *tetani* (all made in horses), *histolyticum* (rabbit) or *botulinum*, Types A, B, C, and D (goats) contained more antihaemolysin than the sera of the normal animals in which they were made. Later it will be shown that few normal sera have a neutralizing effect on the skin-reacting product of *Cl. chauvoei* toxin. Thus, it is reasonably certain that the lysin and the toxin are serologically distinct.

It is possible that culture medium plays a big part in lysin formation. This may explain the stronger material obtained by Kojima (1923) and Kerrin (1934). However, using Hartley's and Pope's digest media, strong lysins were not obtained by the writer.

(4) " *Aggressive* " *Action of Toxin.*

On subcutaneous injection of the toxin into guinea-pigs or sheep, an appearance similar to that caused by culture, but without gas formation, was produced. Without recourse to smear examination and culture, it would be difficult to distinguish the one condition from the other. With one toxin (37 P) the M.R.D. of which was 0·01 c.c., 0·5 c.c. produced death in a guinea-pig in from 36 to 48 hours, with a dark red oedema from the sternum to the pubis.

The experiment recorded in table 4 shows that the toxin was able to activate spores. A 5-day-old meat-broth culture of strain 64 was spun, the bacillary deposit washed twice in saline and then after reconstitution in saline to one quarter of the original volume was heated at 60° C. for half-an-hour. To different amounts of this suspension, toxin was added, the total volume made up to 2·0 c.c. with saline and injected intramuscularly into guinea-pigs and sheep.

TABLE 4.

The Activating Power of Toxin on Washed and Heated (60° C.) Cl. chauvoei *Spores. Mixture Injected i.m. into Thigh.*

Animal.	Spore suspension (c.c.).	Toxin (c.c.).	Result.
G.P. 1	1·0	None	Nil.
G.P. 2	0·5	0·1	L.L. √
G.P. 3	0·5	0·25	+ o/n.
G.P. 4	0·25	0·25	+ 2 d.
G.P. 5	0·25	0·1	+ 1 d.
G.P. 6	None	0·25	L.L. √
G.P. 7	None	0·1	Nil.
Sheep 1 (37518)	0·5	0·01	+ o/n.
Sheep 2 (34477)	0·5	0·05	+ 1 d.
Sheep 3 (39745)	1·0	None.	Nil.
Sheep 4 (40751)	None.	0·5	L.L. √
Sheep 5 (40390)	0·5	1·0	L. √
Sheep 6 (40492)	0·5	0·5	Sl.L. √

(G.P.=guinea pig; +=died; √=lived; o/n=overnight; d=day(s); nil=no reaction; L=lame; Sl.L.=slightly lame; L.L.=lame, leg swollen and skin red. Sheep 1, 2, 3 and 4 were normal; sheep 5 and 6 were immune.)

The fact that immunized sheep (Nos. 5 and 6) were able to withstand much larger doses of toxin than normal sheep (Nos. 1 to 4) suggested a method of testing the immunity produced in sheep treated with anaculture. Toxin and a spore suspension were prepared and the smallest activating dose of toxin was ascertained in sheep and guinea-pigs. Between tests the spore suspension was held at about 2° C. Unfortunately the suspension deteriorated rapidly and one month after preparation about ten times more than was used in the first test was required (plus the original minimum activating dose of toxin) to cause the death of the experimental animal. As will be shown later, the dry toxin is stable for at least one year at room temperature.

THE SPECIFICITY OF THE TOXIN.

When methods had been devised for producing toxin regularly and for titrating it reasonably accurately, every batch made was titrated against an immune sheep's serum and the sera of one or more normal sheep. This involved the conducting of an Lr test (5 to 10 M.R.D. were titrated intradermically against decreasing amounts of antitoxin) and the demonstration that 0·1 c.c. of normal serum was incapable of neutralizing 1 to 2 M.R.D. Table 5 records the results of a series of M.R.D. and Lr tests with toxin 37 L and antitoxin 38212.

TABLE 5.

The Accuracy of the M.R.D. and Lr Tests (Toxin 37 L).

M.R.D.

Dose (c.c.).	Number of tests.	Results.		
		+	±	—
0·0025	6	0	1	5
0·005	6	2	3	1
0·01	12	9	3	0
0·015	6	5	1	0
0·02	6	6	0	0

LR.

(Test dose toxin=0·1 c.c.)

Dose Antitoxin (c.c.).	Number of tests.	Result.			
		+	±	tr.	—
0·0025	2	2	0	0	0
0·003	3	3	0	0	0
0·0035	4	2	0	2	0
0·004	4	0	0	1	3
0·0045	4	0	0	0	4
0·005	3	0	0	0	3

(The toxin was dissolved in 1 : 100 adrenalin-saline (100 mg. in 1·0 c.c.); +, ± etc.=degrees of reaction ; readings taken after 24 hours.)

The results just given show that *Cl. chauvoei* toxin and antitoxin may be titrated with a considerable degree of accuracy by the guinea-pig intradermic method and in the writer's opinion the accuracy compares favourably with the intradermic titration of the toxins and antitoxins of *Cl. welchii*, Type B and of *Cl. septicum*. Further, results, almost superimposable on those in tabtle 5 (but with different dosage) could be tabulated for at least 5 other brews of toxin.

The results obtained in titrating 1 M.R.D. (0·01 c.c.) of 37 L toxin against 0·1 c.c. of normal sera and against the antitoxins of other sporulating anaerobes may be summarised as follows:—The sera of the following normal animals had no neutralizing effect—4 horses, 10 sheep, 10 guinea-pigs, and 4 goats. The serum of a two-months-old calf and of two one-year-old bullocks was without effect but that of a 2-year-old bovine neutralized 4 but not 6 M.R.D. One normal rabbit's serum reduced greatly the reaction produced by

1 M.R.D. Using another toxin (36 M.R.D. = 0·015) these results were obtained with 4 other rabbits. The serum of rabbit (1) neutralized 1 to 1½ M.R.D., that of rabbit (2) 3 M.R.D., that of rabbit (3) 2 M.R.D. and that of rabbit (4) 5 to 6 M.R.D. The following antitoxins, prepared in horses, rabbits and goats had no neutralizing power—*Cl. welchii*, Types A, B and D, *oedematiens*, *histolyticum*, *tetani* and *botulinum*, Types A, B, C and D. It is interesting, in view of the results just given for normal rabbit serum, to note that the *histolyticum* antitoxin was prepared in a rabbit. The results with *Cl. septicum* antitoxin will be dealt with separately, because it was capable of neutralizing a considerable amount of toxin.

The Serological Relationship of the Toxins Produced by Four Different Strains.—Toxins were prepared from strains 64 (42), D (37 L), L (44 a) and R 77 (40) and antitoxins made in sheep and goats. In the case of 64 toxin, a sheep received first anaculture, subcutaneously, then living culture intramuscularly, followed by anaculture in increasing bi-weekly doses and finally an injection of about 100 fatal doses of culture intramuscularly. The other antitoxins were made in goats by injecting, subcutaneously, increasing doses of toxoid (formol-filtrate). In table 6, the neutralizing doses of the antitoxins against test doses of the four toxins are recorded.

TABLE 6.

Neutralization of Cl. chauvoei *Toxins, made from Four Different Strains, by Antitoxins prepared in Sheep and Goats. (Guinea-pig Intradermic Method.)*

Toxin.			Antitoxin (c.c.) (neutralizing dose).			
Strain.	M.R.D.	T.D.	D.	64	L.	R 77.
	c.c.	c.c.				
D (37 L)	0·01	0·1	0·03	0·0035	0·008	0·1
64 (42)	0·015–0·02	0·1	0·008	0·0012	0·003	0·04
L (44a)	0·01	0·1	0·06	0·008	0·02	0·16
R 77 (40)	0·015–0·02	0·1	0·012	0·0012	0·0025	0·02
			Ratios.			
			8	1	2	28
			7	1	2½	33
			7	1	2½	20
			10	1	2	16

(T.D.=test dose. Toxin and antitoxin mixed, left for one hour and injected; antitoxin levels varied by 20–30 per cent.)

The results given in table 6 show that, no matter which toxin was used, the antitoxins were placed in the same order of value. It will be noticed that the ratios for antitoxin R 77 vary somewhat. The possibility exists that this antitoxin was very non-avid, as some difficulty was experienced in obtaining a sharp end point. A splay of ± or trace reactions from 0·01 c.c. to 0·02 c.c. was often got, indicating a loose combination of toxin and antibody.

Under appropriate headings, results will be presented showing that the injection into guinea-pigs or sheep of filtrate or formol-filtrate rendered them refractory to the intravenous and intradermic administration of toxin or the intramuscular inoculation of living culture and that the serum of such animals contained neutralizing antibodies. The ability of the serum of a bovine of over two years of age and of rabbits to neutralize 1 to 6 M.R.D. of toxin is in accord with the known insusceptibility of these animals to the natural disease (bovines) or to the inoculation of living culture (rabbit). However, it is recognised that the presence of antitoxin in the blood may not be the only factor in operation. The horse is relatively insusceptible to black quarter, yet the sera of a number of normal horses and of horses immunized against the toxins of other anaerobes had no neutralizing properties. Again, as will be shown later, a sheep may be rendered highly immune to culture (and probably to the natural disease) without there being demonstrable antitoxin in the blood stream.

Thus, one may conclude that the presence of *Cl. chauvoei* antitoxin in the blood of an animal is usually a strong indication of a stimulus with the corresponding toxin (the word " usually " is purposely employed, as it is difficult to conceive of rabbits and not guinea-pigs receiving the stimulus). Further, the toxins of two South African, one European and one Rhodesian strain of *Cl. chauvoei* are serologically indistinguishable.

Flocculation.—A number of attempts to titrate toxin and antitoxin by Ramon's (1922) method failed. After establishing the neutral point intradermically in guinea-pigs, a test was put up using 1·0 c.c. of toxin and amounts of antitoxin varying from 100 per cent. more than to 100 per cent. less than the determined neutralizing dose. The mixtures, at pH 7·0, 7·4 and 7·8, were heated at 37° C. and at 50° C. respectively for 5 hours and then left for 18 to 48 hours at 10° C. No flocculation visible through a ×8 hand lens was discernible.

The Stability of the Toxin.

Dry toxins have retained their original values (M.R.D. and Lr) for at least two years. However, in the dissolved state, the precipitated toxins appear to be no more stable than the liquid filtrates. In Table 7, the effects on toxicity of heating pulped filtrate pH 6·5 (Strain 64, toxin 47) and of allowing it to stand for different periods at 5° C. without preservative are recorded.

TABLE 7.

The Effect of Heat and of Storage on Toxin (Toxin 47).

	M.R.D.	Lr.
	(c.c.).	(c.c.).
Original	0·015	>0·004
1 day 5° C.	0·025	0·002
2 days 5° C.	0·025	N.D.
3 days 5° C.	?0·025	0·001
½ hour 60° C.	?0·1	0·0007
½ hour 75° C.	>0·1	<0·001
½ hour 95° C.	>0·2	<0·001

(For the Lr. titrations, 0·2 c.c. of toxin was titrated against antitoxin, the levels of which differed by 20–30 per cent. The figures given under Lr=the amounts of a certain antitoxin required to neutralize the test dose of toxin.)

The results given in Table 7 show that the toxin is thermolabile and resists storage badly even at a low temperature and in sealed-off tubes. This experiment was confirmed on many occasions when it was necessary to store toxin for 18 hours in case a repeat test was necessary. Very often the M.R.D. rose and the Lr fell several hundred per cent. in this short time.

PART II.

THE PRODUCTION OF IMMUNITY.

PRODUCTION OF IMMUNITY WITH PRECIPITATED TOXIN.

It was anticipated that a relatively small amount of precipitated toxin would produce immunity to the inoculation of a lethal dose of virulent culture, because, if the immunity was brought about only by the soluble toxin, then the injection of a few milligrams of the dry material was equivalent to the administration of one to several cubic centimetres of the liquid filtrate. However, as Henderson (1932) has shown in small animals and Mason and Scheuber (1936) in sheep, immunity to culture does not necessarily depend entirely on the presence of toxin or toxoid in the vaccine injected. Heat-killed bacilli alone produce a good immunity to culture.

Three sheep received, subcutaneously, 0·01 c.c., 0·1 c.c. and 1·0 c.c., respectively of a dissolved dry toxin (" S.C." M.R.D. = 0·015 c.c. to 0·02 c.c.). Three weeks later, one did not withstand 1 M.R.D. of culture, inoculated intramuscularly, the second died from 2½ M.L.D. and the third from 10 M.L.D. (Sheep 38837, 36878, 36856, and control sheep 37583, 37105.) One cannot say, from this result, if any immunity at all was produced, but it is definite that it was not of a high order.

From a number of guinea-pigs which had been used for the intradermic titration of toxin and antitoxin, four were set aside and three weeks later, all received, subcutaneously, 0·5 c.c. of a dry toxin ("26a", M.R.D. = 0·01 c.c.). Sixteen days after this injection 0·4 c.c. of culture was inoculated intramuscularly into all. Three survived, and one died within 36 hours; normal control guinea-pigs which received 0·1 c.c., 0·15 c.c., 0·2 c.c. and 0·4 c.c. of the same culture died in from 1 to 3 days.

In an intravenous titration, sheep 41010 received 1·5 gm. and sheep 41530 2 gm. of toxin 49. Both survived. Prior to the injection, 0·1 c.c. of neither serum neutralized 1 M.R.D. of toxin 37 L. Three weeks later, 41010 received 3 gm. of toxin 49 intravenously; and survived. Two weeks later, 0·02 c.c. of the serum of 41010 neutralized 10 M.R.D. of 37 L toxin and 0·1 c.c. of that of 41530 10 M.R.D. Both survived the intramuscular injection of 10 M.L.D. of culture.

Thus, it is shown that precipitated toxin can stimulate the formation of antitoxin, and in large doses can immunize sheep and guinea-pigs against culture.

Production of Immunity with Formolized Filtrate.

It is well known that filtrate or formol-filtrate is a satisfactory antigen. The literature bears witness that guinea-pigs, sheep and cattle may be solidly immunized against culture or the natural disease by the injection of the one or the other of these antigens. Therefore, no good purpose would be served by presenting data on this point. However, the results given in Table 8 do prove that sheep which have received formol-filtrate develop circulating antitoxin, resist the injection of culture and do not react when toxin is injected intradermically. Strain 64 was grown for 18 hours in meat broth plus guinea-pig liver extract and pulped. To the filtrate at pH 7·4 enough formalin was added to make a 0·3 per cent. concentration and the whole incubated at 37° C. for 48 hours. The details of the experiment are recorded in Table 8.

It will be observed that no sheep had demonstrable circulating antitoxin prior to the injection of the antigen but that 0·1 c.c. of serum was able to neutralize from 2 to more than 6 M.R.D. of a certain toxin (37 L) in from 13 to 25 days after the second injection of formol-toxoid. The M.R.D. test was not carried out prior to immunization but results in many other normal sheep showed that only a very occasional one was able to withstand 1 M.R.D. It will be seen that all 8 sheep tested did not react to the injection of 2 M.R.D. of toxin. Three of four sheep resisted 1 M.L.D. (i.v.) of toxin and two showed only minimal reactions after receiving 10 M.L.D. of culture intramuscularly.

TABLE 8.

Immunization of Sheep with Formol-toxoid (Formol-filtrate). Sheep received 5 c.c. on 12.2.25 and 10 c.c. on 5.3.35 (Experiment 48).

Sheep.	Number M.R.D. toxin neutralized by 0·1 c.c. serum.				Number M.R.D. toxin withstood by sheep.	Number M.L.D. (i.v.) toxin withstood by sheep.		Number M.L.D. (i.m.) culture withstood by sheep.
	11/2/35.	18/3/35.	27/3/35.	30/3/35.	27/3/35.	28/3/35.	30/3/35.	31/3/35.
39777	<1			>6	>2		at least 1	
39782	<1		3–4		>2	<1*		
40098	<1	2–5						>10
40701	<1			3–5	1–2		at least 1	
40899	<1	2–5						
40935	<1	2–5						>10
40941	<1	2–5						
41003	<1		4–5		>2	at least 1		
41065	<1			>6	>2			
41082	<1			>6	>2			
41522	<1			3–6	>2			
41582	<1			>6	>2			

* This sheep was alive and well at the 30th hour after injection, when it suddenly started to blow, to die in 6 hours. At post-mortem, the lungs were oedematous with a bloody effusion in the pleura-cavity.

These results prove that the injection of formol-toxoid stimulates the formation of antitoxin, resulting in the sheep being able to withstand toxin administered intradermically or intravenously. Further, such sheep resist culture, inoculated intramuscularly. The results of some dozens of tests have shown that the presence in 0·1 c.c. of serum of sufficient antitoxin to neutralize a 1 to 2 M.R.D. of 37 L toxin will render a sheep insusceptible to the administration of 1 to 10 M.L.D. of culture. However, as results will presently be presented showing that sheep may be solidly immunized against culture and yet have no circulating antitoxin (immunization with boiled bacilli), one cannot be certain that their high degree of immunity to culture is necessarily dependent on the antitoxin.

PRODUCTION OF IMMUNITY WITH WASHED BOILED BACILLI.

This experiment was one of the last to be carried out, but is inserted at this point so that the section on the titration of toxoids by the total-antitoxin-combining-power test may be made clearer. Several workers, Robertson and Felix (1930), Green (1929), but in particular Henderson (1932, 1933, 1934), have shown that mice, guinea-pigs and sheep may be immunized against *Cl. chauvoei* bacilli and/or spores by the subcutaneous injection of the killed organisms. Henderson tested the immunity of his immunized mice and guinea-pigs by injecting washed spores activated with calcium chloride. Reasoning on hypothetical grounds, the writer considered that the immunity produced by toxin-(aggressin) free killed germs would be

broken down with culture. When activated spores are used for test purposes the antibodies to the bacteria would be sufficient to destroy most of the spores (or the vegetative forms resulting therefrom) but when culture is inoculated, the bacteria could continue to multiply under the activating influence of the toxin, against which no antitoxin is available. As reported by Mason and Scheuber (1936) tests in sheep showed that the injection of killed, washed bacilli produced a powerful immunity against culture, but only when a dense suspension of such bacilli was given. Usually 5·0 c.c. of formolized-whole culture, injected subcutaneously, is sufficient to immunize a sheep against 1 to 20 M.L.D. of culture; the injection of the washed boiled germs of 5·0 c.c. of culture did not immunize against 1 sure M.L.D.

The experiment, the results of which are recorded in Table 9, was conducted along with that noted under "the production of immunity with formolized-filtrate" (Table 8). Strain 64 was grown in the same lot of medium and under the same conditions as noted, and the bacilli from 3 litres were washed 3 times in distilled water, boiled for 2 hours and enough NaCl added to make a 0·85 per cent. concentration. The opacity of the suspension corresponded to tube 9 of a Burroughs, Wellcome and Company's nephelometer. The details of the injections and tests are recorded in Table 9, and should be compared with those given in Table 8.

TABLE 9.

Immunization of Sheep with Washed, Boiled Bacilli. Sheep received 10 *c.c. on* 12.2.35 *and* 10 *c.c. on* 5.3.35 (*Experiment* 48).

Sheep.	Number M.R.D. toxin neutralized by 0·1 c.c. serum.				Number M.R.D. toxin withstood by sheep.	Number M.L.D. (i.v.) toxin withstood by sheep.		Number M.L.D. (i.m.) of culture withstood by sheep.
	11/2/35.	18/3/35.	27/3/35.	30/3/35.	29/3/35.	28/3/35.	3/4/35.	28/3/35.
34301	<1		<1			<1		
39741	<1	<1						>10
39761	<1			<1	<1			
39748	<1	<1						>10
40681	<1			<1				
40684	<1				<1		? miss L*	
40711	<1		<1			<1		
40951	<1			<1	<1			
40952	<1			<1	1 not 1½		<1	
41018	<1	<1						
41070	<1			<1	<1			
41110	<1	<1		<1	<1			

* It is doubtful if this sheep received a full dose. It survived, after the injection of ? 1 M.L.D.

The results are quite different from those recorded in Table 8. Although the sheep were immune to the intramuscular inoculation of living culture, no anti-toxin was present in the blood and the sheep did not resist the intradermic or intravenous injection of toxin. Thus it is obvious that immunity to culture (and most probably to the natural disease) is not necessarily dependent on the presence of circulating antitoxin. In the two foregoing experiments, only two sheep in each group were tested with culture. However, hundreds of examples could be given of the ability of *one* injection of formol-filtrate to immunize against 1 to 40 M.L.D. of culture and sufficient data were given in the paper by Mason and Scheuber to show that a single stimulus with boiled bacilli produces solid immunity. Further, in these experiments the reaction of all 4 sheep to the administration of 10 M.L.D. was minimal. Normal control sheep were included in each immunity test. The M.L.D. toxin (i.v.) test was not absolutely satisfactory. The lethal dose of the toxin (48) used was 1·5 gm., and from the symptoms shown almost immediately by most sheep, it would appear that the toxin contained a considerable amount of non-specific material. There would appear to be no reason why the further purification of the toxin should not eliminate the appearance of these early symptoms.

In Table 10 are recorded some further tests on the " boiled bacilli " sheep.

TABLE 10.

Development of Antitoxin in, and Resistance to Toxin by Sheep First Treated with Boiled Bacilli (12.2.35 *and* 5.3.35) *and then ith Culture* (*i.m.*) *or Toxin* (*i.v.*)

Sheep.	Previous treatment.			30.3.35	No. M.R.D. toxin neutralized by 0·1 c.c. serum.		No. M.R.D. toxin withstood by sheep, 15.4.35
	12.2.35 5.3.35	22.3.35	28.3.35		12.4.35	15.4.35	
39476	Nil	0.5 gm. 48 toxin i.v.				1½	
39741	Boiled bacilli as in Table 9.		10 M.L.D. culture i.m.		·40		
39748			10 M.L.D. culture i.m.		2–3		
39761				1 gm. 48 toxin i.v.		7–10	5
40951				1 gm. 48 toxin i.v.		2–5	5

NOTE.—Sheep 39476 was a " toxin control " sheep.

As would be expected, the intravenous injection of toxin stimulated the formation of antitoxin. It is remarkable that sheep 39741, which received culture, intramuscularly, responded so well, 0·025 c.c. of its serum neutralizing 10 M.R.D. of 37 L toxin (end point not reached).

Production of Immunity against *Cl. chauvoei* with *Cl. septicum* toxoid.

Under "The specificity of the toxin" it was stated that *Cl. septicum* antitoxin neutralized *Cl. chauvoei* toxin. The relationship of *Cl. septicum* and *Cl. chauvoei* has occasioned discussion and even controversy from the time they were first isolated right up to the present day. Mihailesco (1934) discusses the literature and the *pros and cons* very fully. In the writer's opinion, a considerable amount of the confusion has been caused by workers relying upon morphological and cultural characteristics and failing to conduct a toxin-production test. The supernatant fluid or filtrate of a 24 to 48 hours' meat broth culture of *Cl. septicum* will kill a mouse in from 1 to 24 hours after the intravenous injection of from 0·01 c.c. to 0·1 c.c. No chauvoei filtrate of the writer's has behaved in this way. Further, and of great importance, such septicum toxin is specifically neutralizable by the homologous antitoxin, whereas chauvoei antitoxin has no effect on it. As will be shown shortly, the reverse does not hold good.

Leclainche and Vallée (1900[2]) found that *Cl. chauvoei* antiserum administered to guinea-pigs did not immunize them against *Cl. septicum* culture and those actively immunized against *Cl. chauvoei* were not resistant to *Cl. septicum*. They state that the antiserum (protective and agglutinative) of each germ is rigorously specific. Goss *et al.* (1921) note that chauvoei antiserum protects against *Cl. chauvoei* but not against *Cl. septicum* and that chauvoei agglutinating serum is without effect on septicum bacilli. Basset (1925) noted no cross immunity, and Gins and Hussein (1927, 1928) could not demonstrate cross agglutination. Weinberg and his co-workers (Weinberg and Mihailesco 1929, Weinberg, Davesne, Milhailesco and Sanchez, 1929, Weinberg and Davesne, 1935[1], [2]) maintain that *Cl. chauvoei* and *septicum* are allied micro-organisms. They say that *Cl. chauvoei* produces variants and possesses receptors in common with *Cl. septicum*. Anti-septicum serum nearly always neutralizes *Cl. chauvoei* and although a relatively large volume is necessary, this is usually less than for normal serum. Further, they state, there is a correlation between the amount of chauvoei antitoxin in septicum antiserum and the power of this to neutralize chauvoei culture. Also, they noted cross complement fixation between the two germs.

Experiments with Cl. septicum *antitoxin.*

Using chauvoei toxin 27, it was found that 0·1 to 0·15 c.c. of a certain septicum antitoxin (G.G. 2756, prepared in a horse at the Wellcome Laboratories) neutralized 10 M.R.D. A goat (35451) was immunized at Onderstepoort by injecting septicum anaculture subcutaneously over a period of two and a half months, using the same

strain of *Cl. septicum* (K.F.) as was employed to prepare G.G. 2756. Between 0·15 c.c. and 0·2 c.c. of this animal's serum neutralized 10 M.R.D. of chauvoei toxin 27. About 15 mouse (i.v.) M.L.D. of a certain septicum toxin (Batch E) was neutralized by between 0·005 c.c. and 0·01 c.c. of each of these sera. A rather low value septicum antitoxin prepared in a goat by the writer's colleague, Dr. Scheuber, with a local strain (R 27) also neutralized 27 toxin (0·1 c.c. neutralized 3 to 5 M.R.D.). On these results, an experiment was set up to check the matter thoroughly.

Strain K.F. was " single-celled " twice in the manner described by the writer (1936[2]). The morphological, cultural and pathogenic properties of the resultant culture were those given under " purity of cultures ". It was then grown for 36 hours in meat broth, plus serum, haemolysed cells and liver extract. After removal the meat particles, enough formalin was added to make a 0·45 per cent. concentration, and then incubated for 48 hours at 37° C. The value of the toxin and the toxoid (anaculture) was as follows:—

M.L.D. of toxin (mouse i.v.) = 0·005 c.c. to 0·01 c.c.
M.L.D. of toxoid (mouse i.v.) = >0·5 c.c.
Lv of toxin = 0·1 c.c. bound 0·01 c.c. of antitoxin G.G. 2756.
Total antitoxin-binding value of toxoid = 0·1 c.c. bound 0·0075 c.c. of antitoxin G.G. 2756.

One certain fatal dose of the toxin (mouse i.v.) was not neutralized by 0·1 c.c. of the antitoxins of *Cl. welchii, histolyticum, oedematiens, botulinum* (A, B, C and D), *sordellii* and *chauvoei* (serum of sheep 37281).

Experiment in Sheep(1).—Eight normal sheep were set aside and their sera tested for neutralizing power against the above septicum toxin and against chauvoei toxin (37 L). One M.L.D. of septicum toxin and one M.R.D. of chauvoei toxin was not neutralized by 0·1 c.c. of the serum of any sheep. The animals were then treated as noted in Table II, which also gives the results of various tests.

The spore suspension was prepared from a Dorset's egg medium culture as described by Henderson (1932). The adrenalin was used as an activator of the spores because of the results obtained by the writer (1936[1]) with the spores of *Cl. welchii*, Type B. In the second test, the adrenalin was omitted because it was found that toxin, alone, was a suitable activator and did not introduce a non-specific factor.

The results show that two subcutaneous injections of *Cl. septicum* anaculture stimulated the formation of considerable amounts of septicum antitoxin and small amounts of chauvoei antitoxin. After three injections, chauvoei antitoxin was demonstrable in the sera of all five sheep tested. No indication was got of a correlation between the amount of septicum and chauvoei antitoxins in the various sheep. However, there is the definite indication that the amount of circulating chauvoei antitoxin bore a close relation to the amount of toxin (contained in the activated spore suspension) tolerated by the sheep.

TABLE 11.

Production of Immunity against Cl. chauvoei *by Injections of* Cl. septicum *anaculture.*

Sheep.	17/7/34.	7/8/34. 0·1 c.c. serum neutralized		20/8/34. 0·1 c.c. serum neutralized		21/8/34.	3/9/34. 0·1 c.c. serum neutralized		11/9/34. Test with spore suspension i.m.*	12/9/36.	25/9/34. 0·1 c.c. serum neutralized		6/10/34. Test with spore suspension i.m.**
	5·0 c.c. septicum anaculture s.c.	Septicum. M.L.D.	Chauvoei. M.R.D.	Septicum. M.L.D.	Chauvoei. M.R.D.	5·0 c.c. septicum anaculture s.c.	Septicum. M.L.D.	Chauvoei. M.R.D.			Septicum. M.L.D.	Chauvoei. M.R.D.	
39412		? 1	<1	? 1	<1		5–10	? 1		10 c.c. s.c.	5–10	5–10	L.
40116		? 1	<1	<1	<1		3–4	? 1		septicum	>15	? 1	+ 36 h
40390		? 1	<1	<1	<1		1–2	1		anaculture	2–5	>10	L.
40398		<1	<1	<1	<1		4–5	<1	+ o/n	—	—	—	—
40439		<1	<1	<1	<1		5–10	1½	+ 24 h	—	—	—	—
40463		<1	<1	<1	<1		5·10	1½	+ 36 h	—	—	—	—
40492		1	<1	? 1	<1		5–10	<1		10 c.c. s.c.	2–5	1–2	L.
40598		<1	<1	<1	<1		4–5	<1		septicum anaculture	5–10	1½–2	L.

(+ =died; L=lived. *, ** :—the details of these tests are given in Table 11a. o/n=overnight. h=hours.)

(The septicum toxin used to detect antitoxin was that noted in the text; for the detection of chauvoei antitoxin, 37 L was employed.)

Sheep 40116, 0·1 c.c. of the serum of which barely neutralized 1 M.R.D. of 37 L toxin succumbed to the injection of the spore suspension plus 10 mg. of toxin, whereas sheep 40390, 0·1 c.c. of the serum of which neutralized at least 10 M.R.D. of 37 L toxin, withstood spore suspension plus 100 mg. of toxin.

TABLE 11 (*a*).

Details of the Immunity Tests (*against* Cl. chauvoei) *noted in Table* 11.

Sheep.	Spore suspension.	Adrenalin.	Chauvoei toxin (37 L).	Result.
		11/9/34.		
40398	1·0 c.c.	0·1 c.c.	1·0 mg.	+ o/n.
40439	1·0 c.c.	0·1 c.c.	1·0 mg.	+ 24 h.
40463	1·0 c.c.	0·1 c.c.	1·0 mg.	+ 36 h.
Controls.				
40458	1·0 c.c.	0·1 c.c.	1·0 mg.	+ o/n.
40443	1·0 c.c.	0·1 c.c.	1·0 mg.	+ o/n.
		6/10/34.		
39412	0·5 c.c.		100 mg.	L.
40116	0·5 c.c.		10 mg.	+ 36 h.
40390	0·5 c.c.	not.	100 mg.	L.
40492	0·5 c.c.		50 mg.	L.
40598	0·5 c.c.	used.	50 mg.	L.
Controls.				
34477	0·5 c.c.		5·0 mg.	+ 24 h.
37518	0·5 c.c.		1·0 mg.	+ o/n.
39745	0·5 c.c.		Nil.	L.

Experiment in Sheep (2).—Two sheep received, subcutaneously, in the course of six weeks, 10 injections of a *Cl. septicum* (K.F.) anaculture. Prior to injection, 0·1 c.c. of the serum of neither animal neutralized 1 M.L.D. of a dry septicum toxin or 1 M.R.D. of a dry chauvoei toxin (37 L). At the end of the course of immunization, the following results were obtained:—

	Sheep 37322.	*Sheep* 35540.
Septicum antitoxin ...	0·015 c.c. of serum neutralized 20 M.L.D. of a septicum toxin (Batch E).	and 0·01 c.c. to 0·015 c.c.
Chauvoei antitoxin ...	0·025 c.c. to 0·05 c.c. of serum neutralized 5 M.R.D. of 37 L toxin.	and 0·025 c.c. to 0·05 c.c.
Resistance to i.m. inoculation of chauvoei culture...	5 M.L.D. (0·5 c.c.) lived.	5 M.L.D. (0·5 c.c.) lived.

Controls to culture: Normal Sheep 33849 1 M.L.D. (0·1 c.c.) died 36 hours.
Normal Sheep 37734 2½ M.L.D. (0·25 c.c.) died 24 hours.

(As pointed out by Mason and Scheuber (1936), 0·1 c.c. of an 18 hours' meat-broth culture of *Cl. chauvoei* is usually an M.L.D. for a sheep.)

Experiment in Sheep (3).—Two sheep received 12 subcutaneous injections of septicum anaculture (strain R 27), at bi-weekly intervals. Prior to commencing immunization, no septicum or chauvoei antitoxin was demonstrable in 0·1 c.c. of the serum of either. At the end of the course the following results were obtained:—

	Sheep 37457.		*Sheep* 37913.
Septicum antitoxin ...	0·015 c.c. neutralized 20 M.L.D. of Batch E toxin (made from strain K.F.).	and	0·015 c.c.
Chauvoei antitoxin ...	0·07 c.c. to 0·1 c.c. neutralized 5 M.R.D. of 29 toxin.	and	0·06 c.c. to 0·07 c.c.
Resistance to i.m. inoculation of chauvoei culture...	10 M.L.D. (1·0 c.c.) lived.		10 M.L.D. (1 0 c.c.) died.

Controls to culture: Normal Sheep 38066 1 M.L.D. died.
Immune Sheep 36552 10 M.L.D. lived.

The results of the three experiments just presented prove that in *Cl. septicum* anaculture, there is an antigen capable of stimulating the formation in sheep of *Cl. chauvoei* antitoxin and of rendering these animals insusceptible to the inoculation of several lethal doses of Cl. chauvoei culture (or activated spore suspension).

Having shown that *Cl. septicum* antigens immunized against *Cl. chauvoei*, it became necessary to prove or disprove that the reverse held good.

Experiment 1.

Eight sheep which had received subcutaneously from 2 to 20 c.c. of *Cl. chauvoei* anaculture and had survived the intramuscular inoculation of one or more M.L.D. of culture were bled and their sera titrated for the presence of septicum antitoxin. One sure fatal dose (0·01 c.c.) of a certain septicum toxin (Batch E) was not neutralized by 0·1 c.c. of any serum, and a doubtful lethal amount, 0·007 c.c., was neutralized by only 2 sera. In a more detailed test, it was found that the sera of four of these sheep had no more neutralizing effect upon fractional M.L.D. of septicum toxin than had the sera of 4 normal sheep.

Experiment 2.

Three sheep were treated as follows. One (37534) received chauvoei anaculture and then living culture (1 to 2 M.L.D.) and two others (37827, 36552) filtrated black quarter oedema fluid (from sheep) and then living culture (10 M.L.D.). Between 5.4.34 and 21.6.34 each received 9 gm. of a dry chauvoei toxin and 1,550 c.c. of a formol-filtrate subcutaneously. On the 4.7.34, 0·1 c.c. of the serum of no sheep neutralized 1 M.L.D. of septicum (Batch E) toxin. Of the pooled sera of the three animals 0·005 c.c. neutralized 10 M.R.D. of chauvoei 37 L toxin. On the 10.7.34 each sheep received a lethal dose

of septicum toxin intravenously, and all died within 12 hours. The amount given was 3·5 mg. of dry toxin per kilogram body weight. Using the same batch (E) of toxin, Mason (1935) found that the lethal dose was about 3 mg. per kilogram body weight.

These two experiments and especially the second one show that sheep highly immune to *Cl. chauvoei* have no more immunity than normal sheep to the toxin of *Cl. septicum*.

PART III.

THE TITRATION OF CL. CHAUVOEI ANTIGENS.

It is accepted in immunology that, with a few exceptions, the more antitoxin a toxin or toxoid binds, the greater is its antigenic power. With diphtheria toxoid, the flocculation titre is closely related to immunizing power and the writer (1936[1]) showed that the total-antitoxin-binding-power of *Cl. welchii*, Type B toxoid bears a close relation to its power of immunizing guinea-pigs. Thus, there was considerable *a priori* grounds for assuming that the same would hold good for *Cl. chauvoei* formol-toxoid or anaculture.

Scott (1923[1]) evaluated filtrates by their power of activating non-lethal doses of *Cl. chauvoei* spores or bacilli. Another method he employed (1923[2]) was to estimate the value of the filtrate in terms of antitoxin. Filtrate and serum were mixed, and living spores added, and the whole inoculated into guinea-pigs. A " good " filtrate would bind more antitoxin than a " bad " one and thus less antitoxin would be left over to " neutralize " the spores and the animals would die. Apart from the fact of whether or not this is a suitable method of titrating filtrates for antigenicity (and the results to be presented do not support the contention), the writer (unpublished work) was unable to obtain consistent results with Scott's method; the guinea-pigs died in a most irregular fashion, irrespective of the amounts of antiserum or filtrate given. In a later article, Scott (1930) states that guinea-pigs are unsuitable test animals since they vary in susceptibility to *Cl. chauvoei*. He also says that the addition of formalin to filtrate increases its potency, but Zschokke (1932) shows that this is due to the irritating effect of the formalin, itself.

At these laboratories, the method of testing the immunizing value of black quarter vaccines is to inject 2 c.c. to 20 c.c. into sheep and to test with living culture after 14 to 21 days. This method gives a result, sufficient for practical purposes—a vaccine which so immunizes a group of six sheep that they withstand 1 to 5 lethal doses of culture is suitable for field use. However, only the roughest of comparisons between two vaccines is possible, unless an unwieldly number of animals is used for the test. One does not know the value of the antigen prior to injection so that it is mere chance if the correct amount of culture to show up a difference is inoculated at the test. This point was brought home forcibly to the writer and his colleague, Dr. Scheuber, in a series of experiments with black quarter vaccines.

About all that the method does tell is whether a vaccine is good or bad, i.e. it is good if 5 or 6 of 6 injected sheep withstand 1 to 10 M.L.D. of culture and bad if 4 to 6 of 6 sheep die after the inoculation of 1 or 2 M.L.D.

To use guinea-pigs instead of sheep would not help, if the method of test was to be the inoculation of living culture. However, on the assumption that immunity production was bound up with the amount of antitoxin an antigen could stimulate into formation, the guinea-pig promised to be a handier, but no better, animal than the sheep.

Experiment 1.

An experiment was carried out in the following way:—One group of guinea-pigs received, as their primary stimulus, the injection of a dry toxin, and as their secondary stimulus, a good or a bad vaccine. A second group received, as their primary and their secondary stimulus, the good or the bad vaccines and a third lot, the dialysed ammonium sulphate precipitates of these vaccines. (For the meaning of "good" and "bad", see above.) The results of the tests are given below.

Group 1:

Scheme. Primary stimulus—30 mgm. of chauvoei toxin (S.C.) subcutaneously.
Secondary stimulus—2·5 c.c. of good or bad vaccine subcutaneously.

5.1.34: S.C. toxin injected. All animals developed a swelling with reddening and 3 died.

25.1.34: 6 guinea-pigs bled and the sera pooled; 0·1 c.c. of serum did not neutralize 1 M.R.D. of toxin 29. Two-guinea pigs each received 1 M.RD. of toxin 29; both reacted.

26.1.34: Half of the group received the good vaccine and the other half the bad vaccine.

5.2.34: The guinea-pigs of each sub-group were bled and sera pooled; 0·1 c.c. of serum was titrated against toxin 29. From 3 to 6 M.R.D. were neutralized by the serum of the "good vaccine" group and 2 to 3 M.R.D. by the "bad vaccine" group. Each guinea-pig now received intradermic injections of toxin 29, 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$, $3\frac{1}{2}$ and 5 M.R.D. There was no significant difference as groups: most animals resisted 2 M.R.D. and some 5 M.R.D. If a difference was to be read into the reactions, it was in favour of the "bad vaccine" group.

Group 2:

Scheme. Primary and secondary stimulus—good or bad vaccine.

5.1.34: Guinea-pigs received subcutaneously 2·5 c.c. of vaccine.

25.1.34: Animals bled as sub-groups and sera pooled; 0·1 c.c. of the "good" sera almost neutralized 1 M.R.D. of 29 toxin and 0·1 c.c. of the "bad" sera gave a doubtful reaction. Two guinea-pigs from each sub-group which received 1 M.R.D. of 29 toxin reacted.

26.1.34: Vaccine re-injected into the guinea-pigs (2·5 c.c.).

5.2.34: Animals bled as sub-groups and 0·1 c.c. of serum tested against 29 toxin; between 2 and 3 M.R.D. were neutralized by the " bad group " serum and 3 to 4 M.R.D. by the " good group " serum. The four guinea-pigs of each sub-group now received 1, 1½ and 2 M.R.D. of 29 toxin intradermically. Three of the " bad " lot reacted to 1 M.R.D. and one doubtfully; two of the " good " lot reached to 1 M.R.D., one resisted 1 M.R.D. and reacted doubtfully to 2 M.R.D. and the fourth guinea-pig reacted doubtfully to 1 and 2 M.R.D.

Group 3.

Scheme: Primary and secondary stimulus—the dried dialysed ammonium sulphate precipitates of the bad and the good vaccines. The same volumes of anacultures were precipitated. The yield of powder from the good vaccine was 6 gm. and of the bad vaccine 0·6 gm.

9.1.34: Guinea-pigs received, subcutaneously, 40 mg. of dry vaccine.

25.1.34: Animals bled as sub-groups and sera pooled; 0·1 c.c. of the " bad " sera partially neutralized 1 and 1½ M.R.D. of 29 toxin and 0·1 c.c. of the " good " sera did not neutralize 1 M.R.D. Two guinea-pigs from each group were tested intradermically, with 1 M.R.D. of 29 toxin—the two bad vaccine animals partially resisted this amount and the good vaccine guinea-pigs did not.

26.1.34: Re-injections of vaccine (40 mg.) carried out.

5.2.34: Guinea-pig bled as sub-groups and sera pooled; 0·1 c.c. titrated against 29 toxin. The " bad " sera neutralized 5 M.R.D. and the " good " sera 1 M.R.D. On testing each animal with toxin intradermically, one of the " bad " group resisted 2 M.R.D., another doubtfully 2 M.R.D. and the third doubtfully 1 M.R.D.; not one of the four animals of the " good " group resisted 1 M.R.D.

Comment.—The results of this experiment are unsatisfactory. Apparently, the primary stimulus of toxin (Group 1) produced such a high basal immunity that the relatively weak antigenic vaccines did not stimulate the production of antitoxin to an extent sufficient for differences to be shown up. Two injections of the anacultures (Group 2) showed up no significant differences in antitoxin production. A significant difference was got when the dialysed ammonium sulphate precipitates of the good and the bad vaccines were used, the bad vaccines stimulating the production of more antitoxin than the good one. The reason for this lies probably in the yields of dry powder obtained. Although equal volumes were precipitated, the weight of the " bad " dry vaccine was ten times less than that of the good. Thus, the probability exists that, as equal amounts of each dry vaccine were injected, 10 times more " bad " than " good " antigen was administered. However, no indication was got that an anaculture

which immunized sheep well against the inoculation of culture stimulated the production in guinea-pigs of more antitoxin than did an anaculture which immunized sheep badly.

Experiment 2.

Four vaccines were chosen on the basis of their having produced bad, fair, fairly good and good immunity (to culture) in sheep. Into 4 groups of guinea-pigs, these vaccines were injected (2·5 c.c. subcutaneously, twice, at 21 days' interval). Ten days after the second injection, the animals were bled as groups and each individual tested intradermically with toxin. Significant differences were not got. Further, 4 groups of 3 sheep each (12 sheep in all) received subcutaneously 5·0 c.c. of one or the other vaccine (twice at 16 days' interval). Ten days after the second injection the sheep were then bled as groups, their sera pooled, and titrated against toxin 27, intradermically in guinea-pigs. The results of these titrations and of the final immunity test with culture are given in Table 12.

TABLE 12.

The Amount of Antitoxin Produced by Sheep after Two Subcutaneous Injections of Bad, Fair, Fairly Good and Good Vaccines. Also the resistance of these Sheep to the i.m. Inoculation of Culture.

Sheep.	13/12/34.	29/12/34.	8/1/35. Group serum pooled 0·1 c.c. neutralized 27 toxin M.R.D.	16/1/35. Resistance to culture i.m.	
				Dose c.c.	Result.
Bad vaccine—					
37000	5·0c. c. vaccine s.c.	5·0 c.c. vaccine s.c.	1–1½	2·0	+ 24 hr.
37703				2·0	+ 5 d.
37731				2·0	+ o/n.
Fair vaccine—					
37877			4	6·0	Lameoedema,L
38183				6·0	+ o/n.
38188				4·0	+ 3 d.
Fairly good vaccine—					
38189			<2	2·0	L.
38196				2·0	L.
38198				4·0	L.
Good vaccine—					
38212			10	4·0	L.
38214				6·0	L.
38216				4·0	L.
Controls to culture injection—					
34732				0·2	+ o/n.
34721				0·1	+ 20 h.

(+=died, L=lived, hr.=hours, d=days. o/n=overnight.)

If only the good and the bad vaccines had been used, the result of the test would have been considered highly satisfactory; 0·1 c.c. of the " bad " serum neutralized only 1 to $1\frac{1}{2}$ M.R.D. of toxin and not one of the 3 sheep was immune to 20 M.L.D. of culture whereas 0·1 c.c. of " good " serum neutralized 10 M.R.D. of toxin and the sheep resisted 40 to 60 M.L.D. of culture. However, the introduction of the fair and fairly good vaccines shows that a correlation between the amount of circulating antitoxin and the amount of culture resisted does not exist. The pooled serum (0·1 c.c.) of the sheep immunized with the fairly good vaccine did not neutralize 2 M.R.D. of toxin (and, from the intensity of the reaction, probably would not neutralize 1 M.R.D.), yet the sheep withstood 20 to 40 M.L.D. of culture. It should be stated that the values (" good ", " bad ", etc.) placed upon the vaccines were arrived at from the results of a preliminary culture immunity test on sheep. Further, the reason why the same amount of culture (see Table 12) was not inoculated into each sheep was because it was anticipated that the antitoxin titration would run parallel to the culture-resistance test.

Experiment 3

If immunity to *Cl. chauvoei* culture depended on the presence of circulating antitoxin, then one could, with justification, assume that the amount of antitoxin a formol-toxoid (anaculture) bound would bear a relation to the immunity produced by it. Attempts to ascertain the total-antitoxin-combining-power (T.C.P.) of liquid toxoids were not altogether satisfactory, because, in many cases, too little antitoxin, to allow of accurate results, was bound. However, by using dialysed ammonium sulphate precipitates, values could be placed on the toxoids. The technique was as follows. The Lr of a dry toxin was accurately established, and this toxin and the antitoxin were used throughout the tests. The dry toxoid, dissolved in saline, was left in contact for one hour at room temperature with a certain amount of antitoxin. Thereupon, to a series of tubes containing this mixture, fractions of an Lr dose of toxin were added and after a further half-hour at room temperature, the toxoid-antitoxin-toxin mixtures were injected, intradermically, into guinea-pigs. In a typical titration, those mixtures containing small amounts of toxin produced no reactions, whereas those with larger amounts did so. Knowing the Lr of the toxin, a simple calculation showed how much antitoxin the toxoid had bound. Further, knowing the volume of toxoid originally precipitated and the weight of the dry powder, one could calculate the amount of antitoxin that 1·0 c.c. of liquid toxoid would bind. In the first half of this experiment one lot of sheep received, subcutaneously, 5·0 c.c. of anaculture, 86 C, and the other lot 5·0 c.c. of anaculture 87. The details of the test are given in Table 13.

Toxoid 86 C bound more antitoxin than toxoid 87 and stimulated the formation of more antitoxin *in vivo*. However, the immunity test with spores allows of no conclusion being drawn. All the sheep survived the test.

TABLE 13.

Experiment to Ascertain if there is a Correlation between the T.C.P. of a Toxoid, the Tolerance to Toxin and to Culture (Activated Spores) of Sheep Immunized with it and the Amount of Circulating Antitoxin in the Sheep.

Toxoid.	Sheep.	Value of toxoid.	Tolerance to toxin (37 L) i.d. M.R.D.	No. M.R.D. of 37 L toxin neut. by 0·1 c.c. serum.	Resistance to toxin-activated spore suspension.	
					Amount toxin (37 L).	Result.
86 C	39309	1·0 c.c.	Not done.	1	50 mg.	L.
	39684	liquid		5	100 mg.	L.
	39964	toxoid bound		5	Not done.	
	40543	0·0008 c.c. A.T. 38212		2	100 mg.	L.
87	40402	1·0 c.c.	<1	<1	50 mg.	L.
	40527	liquid	? 1	1	100 mg.	L.
	40779	toxoid bound	1	? 1	100 mg.	L.
	41004	0·0004 c.c. A.T. 38212	<1	<1	50 mg.	L.
Controls	34477		<1	<1	1 mg.	+ o/n.
	37513		<1	<1	5 mg.	+ 24 h.

(L=lived, +=died, o/n=overnight, h=hours, neut.=neutralized, T.C.P.=total antitoxin-combining-power.)

The toxoid was injected subcutaneously, and the toxin tolerance and the serum neutralization tests carried out 3 weeks later. The resistance to activated spore test was carried out after a further 10 days.

Experiment 4.

The details of this test are given in Table 14. It was carried out as noted under Experiment 13, with the difference that culture, instead of activated spores, was used to test immunity.

TABLE 14.

Experiment to Ascertain if there is a Correlation between the T.C.P. of a Toxoid, the Tolerance to Culture of Sheep Immunized with it and the Amount of Circulating Antitoxin in the Sheep.

Toxoid.	Sheep.	Value of toxoid.	No. M.R.D. 37 L toxin neutralized by 0·1 c.c. serum.	Resistance to culture c.c.
88	36881	1·0 c.c. of liquid toxoid bound 0·0005 c.c. of A.T. 38212	? 1	0·5 L.
	38934		1	1·0 L.
	38937		? 1	0·5 L.
	38940		1½	1·0 L.
89A	38881	As 88.	<1	0·5 L.
	40241		>1½	1·0 L.
	41000		>1½	0·5 L.
	41092		>1½	1·0 L.
91	41008	1·0 c.c. of liquid toxoid bound 0·00016 c.c. A.T. 38212.	<1	1·0 L.
	41028		<1	0·5 L.
	41112		<1	2·0 L.
	41131		<1	2·0 L.
Controls	41022		<1	0·1 + o/n.
	41047		<1	0·25 + 24 hr.

(See notes under Table 13.)

The results do not indicate that there is a correlation between the amount of circulating antitoxin, the T.C.P. of the toxoid and the amount of culture borne by the sheep. Toxoid 91 bound about one-third of that of toxoid 89A, circulating antitoxin was not demonstrated in the immunized sheep, yet these sheep resisted 5 to 20 M.L.D. of culture. It is realized that all the sheep resisted the culture inoculation and thus a true comparison cannot be made. However, some years' experience with toxoids as noted in Table 14 has shown that it is unusual to find them immunizing against more than 20 M.L.D., the usual amount being between 2 and 10 M.L.D.

EXPERIMENT 5.

To be certain of having toxoids of widely differing values (T.C.P.), a liquid formol-filtrate was used in the following manner. Sheep received, subcutaneously, 5·0 c.c. of the toxoid, unheated, heated (60° C. half-an-hour), boiled (95° C. half-an-hour) and autoclaved (120° C., 15 pounds pressure, half-an-hour). The T.C.P. was worked out in the manner described. Table 15 records the experiment.

TABLE 15.

The Correlation between the T.C.P. of Toxoids and the Production of Antitoxin by, and the Resistance to Culture of Sheep Immunized with them.

Toxoid.	Sheep.	Value of toxoid.	No. M.R.D. 37 L toxin neutralized by 0·1 c.c. serum.	Resistance to culture c.c.
Not heated	39354	1·0 c.c. bound 0·0005 c.c. A.T. 38212	<1	0·5 oed. L.
	41099		<1	1·0 oed. L.
	40949		? 1	2·0 + 5 d.
	41058		<1	4·0 + 2 d.
Heated 60° C, ½ hour	40945	1·0 c.c. bound 0·0001 c.c. A.T. 38212.	? 1	0·5 L.
	40993		<1	1·0 L.
	40729		<1	2·0 + 5 d.
	41050		<1	4·0 L.*
Heated 95° C, ½ hour	41087	1·0 c.c. bound <0·0001 c.c. A.T. 38212	<1	0·5 + 2 d.
	41097		<1	1·0 L.*
	40959		<1	1·0 + 1 d.
	40976		<1	2·0 + o/n.
Autoclaved, ½ hour	41063	As 95° C. toxoid.	<1	0·5 + 2 d.
	41020		<1	1·0 + 2 d.
	39652		<1	1·0 + o/n.
	40514		<1	2·0 + o/n.
Controls	41125		<1	0·1 + 1 d.
	41103		<1	0·25 + 1 d.

(L=lived, +=died, d=days, oed.=oedema.)

* Sheep 41050 and 41097 had oedema of the leg (the site of inoculation) down to the fetlock, the skin was blue in places and a haemorrhagic fluid exuded from the skin. Careful nursing prevented their death.

The sheep received 5·0 c.c. of toxoid subcutaneously, the serum neutralization test was done 21 days after and the culture test 2 days after this.

The results given in Table 15 show that the antitoxin-binding power of a toxoid has no bearing on its ability to immunize sheep against the inoculation of culture. The heating of the toxoid at 60° C. reduced its binding power 5 times, yet its immunizing power (to culture) was not altered. Further, it is of definite significance that sheep 41087, 41063 and 41020 died after 48 hours and that sheep 41097 survived. A dose of 0·1 c.c. of culture killed a control animal in 24 hours; 5 to 10 times this amount would kill, with certainty, overnight. Unfortunately, the antitoxin production, even by the unheated toxoid, was so feeble that a comparison cannot be made. The experiment indicates that, in *Cl. chauvoei* formol-filtrates, there is an antigen much more stable than toxin capable of immunizing against culture. (It will be recalled that toxin is destroyed after half-an-hour's heating at 60° C.).

Experiment 6.

In this experiment, the L strain of *Cl. chauvoei* was used. As previously stated, this culture, as originally received, was nearly non-pathogenic for guinea-pigs but by passing it through guinea-pigs (culture activated with adrenalin) a virulent strain was obtained. Toxin was made from a 36 hours' culture of the avirulent and of the virulent strain. Toxoids were made from each by adding 0·4 per cent. formol and incubating for 48 hours. The T.C.P. of the toxoids were established, sheep received 5·0 c.c. subcutaneously, were bled 21 days later and 2 days later still were tested with culture. Table 16 records the results.

TABLE 16.

The Correlation between M.L.D. and Lr of the Original Toxin, the T.C.P. of the Formol-toxoid Produced from it, the Amount of Antitoxin formed in Sheep into which the Toxoid is Injected and the amount of Culture Resisted by the Sheep.

Toxoid.	M.R.D. toxin.	**Lr toxin.	Value of toxoid.	Sheep.	No. M.R.D. 37 L toxin neut. by 0·1 c.c. serum.	Resistance to culture M.L.D.
From avirulent culture	? 0·1 c.c.	<0·0003 c.c.	1·0 c.c. bound 0·0001 c.c., A.T. 38212	41510	<1	5 + 2 d.
				41054	<1	10 + 2 d
				41513	<1	20 L.
				41521	<1	20 L.
From virulent culture	at least 0·01 c.c.	>0·003 c.c.	1·0 c.c. bound 0·0045 c.c., A.T. 38212	41527	<1	10 + 2 d.
				41534	? 1	20 + 1 d.
				41500	>1½	30 + 1 d.
				41088	>1½	40 L.*

(+=died, L=lived, d=days.)

* The leg of this sheep was oedematous, the skin blue and oozing a haemorrhagic fluid.

** Liquid toxin used; test dose=0·2 c.c.; the figure given is the amount of antitoxin 38212 required to neutralize this amount.

The results show that there is no correlation between the antitoxin-binding value of a toxoid and the resistance to culture produced by it in sheep. The toxoid from the virulent culture bound 45 times more antitoxin than that from the avirulent, 3 of 4 sheep had demonstrable circulating antitoxin, yet (although the doses of culture inoculated are not exactly the same) the sheep were no more immune to culture than those into which the " avirulent " toxoid was injected.

Experiment 7.

In Table 13 (experiment 3) it is shown that toxoid 86 C, 1·0 c.c. of which bound 0·0008 c.c. of antitoxin 38212, stimulated the formation of more antitoxin, in sheep, than toxoid 87, 1·0 c.c. of which

bound 0·0004 c.c. of the same antitoxin. Also in experiment 6, it is shown that the toxoid of the virulent strain bound 45 times more antitoxin than that of the avirulent strain and stimulated the formation, in sheep, of more antitoxin. In the experiment now to be noted, 3 precipitated toxoids were used. Their T.C.P. were established, guinea-pigs received 2 injections (200 mg. each time) subcutaneously (23 days' interval), their pooled sera (as groups) titrated for antitoxin 14 days after the second injection and three days after this, all guinea-pigs received a lethal dose of toxin intravenously. The object was to ascertain if there was a correlation between the binding-value of a toxoid, the amount of antitoxin produced, and toxin tolerance. Table 17 records the results.

TABLE 17.

The Correlation between the T.C.P. of a Toxoid, Antitoxin Formation and Toxin Tolerance.

Toxoid.	* Value.	No. M.L.D. 37 L toxin neut. by 0·1 c.c. pooled serum.	Toxin (37 L) tolerance 1 M.L.D.
90 autoclaved	<0·0001 c.c.	<1	4 + 1 L.
91	0·0001 c.c.	<1	3 + 1 L.
46	0·0035 c.c.	5	0 + 5 L.

(+ =died, L=lived.)

* Value=20 mg. toxoid bound the amount of antitoxin 38212 noted.

This experiment proves that the total-antitoxin-combining-value of a toxoid is closely bound with the amount of antitoxin stimulated into formation and with the toxin tolerance of animals immunized.

Experiment 8.

This experiment concerns the production of immunity in sheep with oedema fluid, the so-called natural aggressin. It is well known that the oedematous fluid produced by inoculating *Cl. chauvoei* into cattle, is a powerful immunizing agent.

In this experiment, oedema fluid was collected from a number of sheep, just dead from experimental black quarter. After filtration though a Berkefeld N candle, an attempt was made to carry out a binding-power test. No antitoxin was bound. The dialysed ammonium sulphate precipitate bound no antitoxin. No antitoxic property was detected in the fluid. However, as was to be expected, both the oedema fluid and its precipitate immunized sheep—of 8 sheep which received the one or the other material, two withstood 5 M.L.D. of culture, three 10 M.L.D., one 20 M.L.D., one died from 20 M.L.D. and one from 10 M.L.D.

Experiment 9.

At the time this experiment was started, it was known that boiled bacilli produced a solid immunity, that heated toxoid produced a certain degree of immunity and that the binding power of a toxoid and the amount of circulating antitoxin in an animal had no connection with the amount of culture that would be borne. It was difficult to apportion to the toxin (or its atoxic modification) and the heat stable antigen in a toxoid their respective antigenic values but the indications were that the heat stable fraction was the more important. Therefore, an attempt was made to titrate the stable antigen. The bacilli from 25 litres of a 24 hours' culture were obtained. The culture was passed through a pulp, the pulp soaked in a small volume of saline and the bacilli squeezed out in a press. The bacilli were then washed by spinning three times (with changes of saline solution) at 4,000 revolutions per minute. The dense washed suspension, 200 c.c. in amount, was frozen (−15° C.) and thawed (20°–26° C.) 5 times in 5 days, and spun until clear. A slightly opalescent fluid was obtained. No reaction was produced when 0·1 c.c., in 0·3 c.c. of 1/100 adrenalin, was injected, intradermically, into guinea-pigs nor did 0·5 c.c. kill a mouse on intravenous injection. The fluid was fanned down to 80 c.c. Of this 0·2 c.c. produced no reaction in a guinea-pig, nor did 0·5 c.c. kill a mouse. Reduction with sodium hydrosulphite did not produce a reacting or toxic product. No antitoxin-binding-power could be demonstrated. Using an antitoxic serum (38212) no specific precipitation could be shown up. This antitoxin probably contained plenty of the antibody to the heat stable antigen. No precipitation was got on mixing the fluid with sera produced in sheep by injections of boiled bacilli.

An immunity test was carried out in sheep. One lot of sheep received the supernatant fluid, and the other lot the supernatant which had been boiled for 2 hours.

No precedent existed for the amount of culture that a sheep immunized with supernatant fluid would withstand. Thus, it is possible that sheep 41523 and 41555 would have survived more than $2\frac{1}{2}$ and 1 M.L.D. respectively. Further, since one injection produced a solid immunity, the testing of the immunity after 2 injections was not carried out. One injection of the boiled supernatant did not so immunize 2 sheep that they withstood $2\frac{1}{2}$ and 1 M.L.D. of culture respectively. However, 2 injections produced a solid immunity. One injection of neither antigen stimulated the formation of antitoxin; two stimuli with the unboiled supernatant led to the formation of a considerable amount of antitoxin but with the boiled material, two injections did not have this effect.

Thus, from this experiment one may infer the presence, in the frozen and thawed supernatant fluid of a washed *Cl. chauvoei* suspension, of two antigens, viz., one which is heat labile and capable of stimulating the formation of antitoxin and the other, heat stable, incapable of inducing the formation of antitoxin but capable of immunizing sheep against culture. The possibility exists that the heating of the antigen destroyed some of the heat stable material.

TABLE 18.

The Immunizing Power of the Unboiled and the Boiled Supernatant of Frozen and Thawed Cl. chauvoei *Bacilli.*

Sheep.	Material.	22/2/35.	13/3/35. No. M.L.D. 37 L toxin neut. by 0·1 c.c. serum.	13/3/35. No. M.L.D. of culture resisted.	14/3/35.	4/4/35.. No. M.R.D. 37 L toxin neut. by 0·1 c.c. pooled serum.	5/4/35. No. M.L.D. of culture resisted.
41523	Supernatant.	5·0 c.c. s.c.	<1	2½	5·0 c.c. s.c.	5–10	N.T.
41538			<1	N.T.			
41555			<1	1	5·0 c.c. s.c.	5–10	N.T.
41563			<1	N.T.			
37385	Boiled Supernatant	5·0 c.c. s.c.	<1	<1	5·0 c.c. s.c.	<1	5
37397			<1	N.T.			
38891			<1	N.T.	5.0 c.c. s.c.	<1	2½
41532			<1	<2½			

(s.c.=subcutaneously, N.T.=not tested.)

At the time of both culture immunity tests control sheep were inoculated. The M.L.D. is based on the smallest amount required to kill them.

It is to be noted that although unheated supernatant stimulated antitoxin formation, no toxin or toxoid was demonstrable in it by the T.C.P. titration. An absolutely satisfactory explanation for this cannot be given. It may be that the amount of antigen was too small to allow of its being detected by the method of test. Some little support for this is offered by the fact that after one injection, not a trace of antitoxin was detectable in any one of the four sheep.

DISCUSSION.

The results presented in Part I, prove that, in the filtrates of 18 to 24 hours' cultures of *Cl. chauvoei*, a toxin is present. This toxin has been demonstrated on previous occasions, in particular by Kojima and by Kerrin, but their method of titrating it, viz. the intravenous injection of mice, was, in the writer's opinion, unsatisfactory. When from 0·1 c.c. to 0·5 c.c. is necessary to cause death in mice and when death may occur in a few seconds or minutes, great difficulty is experienced in conducting neutralization tests. The intradermic titration method in guinea-pigs, using dried dialysed ammonium sulphate precipitates, allowed of the obtaining of a satisfactory minimum reacting dose and further, a conveniently sized test dose, containing 5 to 10 reacting doses, could be used. However, satisfaction was not obtained until adrenalin was added to the toxin or the toxin-antitoxin mixtures. The effect of this substance appeared to be the localization of the toxin in the tissues for a time sufficiently long for it to produce visible damage. Without it, the toxin caused an early-appearing intense reddening of the dermis, this disappearing to a greater or less extent in a few hours, so that after 18 or 24 hours, only a red flush marked the site of injection. With adrenalin, the reaction was clearly readable as an intense red mark. That this toxin was the product of *Cl. chauvoei* was shown by its being neutralizable by homologous antitoxin. The specificity of the toxin is not detracted from because the sera of some normal rabbits and bovines were able to neutralize a few M.R.D. Weinberg and his colleagues have already noted this fact. It is possible and even probable that in the case of bovines their antitoxin is the result of stimuli with *Cl. chauvoei* but with rabbits this explanation can hardly be invoked since guinea-pigs, living under the same conditions do not develop antitoxin. However, it is difficult to understand why bovines receive stimuli and sheep do not. It may be that it is an inherent characteristic of the animal to produce antitoxin and not the effect of specific stimuli but this, it is agreed, is merely a hypothetical statement and not an explanation.

Weinberg has insisted for many years that *Cl. septicum* antitoxin may neutralize *Cl. chauvoei* culture. The results presented confirm this, in as much as septicum antitoxin neutralizes chauvoei toxin and sheep may be solidly immunized against *Cl. chauvoei* culture by the use of septicum antigens.

Whilst a haemolysin was demonstrable in filtrates, it was usually of low titre, whereas other workers, for example Kerrin, obtained much higher values. Possibly this is to be explained by the use of different media. However, the writer could not show that the

haemolysin was the same as the toxin—more antiserum was required to neutralize the same volume of lysin as toxin. However, it is admitted that the same attention was not given to the study of the lysin as of the toxin.

The study on the production of immunity was disappointing because a method of titrating antigens apart from their immunizing power was not obtained. The writer had the preconceived idea that the toxin (or toxoid) of *Cl. chauvoei* would be, in great measure, responsible for the production of immunity. If such were the case the filtrate or toxoid binding most antitoxin would produce the strongest immunity. Experiments showed that the total-antitoxin-binding-value of a toxoid had a direct bearing on the amount of antitoxin produced in an animal into which it was injected but was not correlated with the resistance produced to living culture. Boiled, washed *Cl. chauvoei* which bound no antitoxin produced a high degree of immunity in sheep when they were tested with culture, but no antitoxin was demonstrable nor were the sheep resistant to toxin injected intradermically or intravenously. Thus one could conclude that two antigens were involved in the production of immunity, one a heat labile toxin and the other a heat stable antigen. Basset (1925[2]) pointed out that the heating of formol-toxoids at 60° C. for one hour did not destroy their antigenic value and Viljoen and Scheuber (1926) found that artificial aggressin (filtrate) does not lose all of its immunizing value even when exposed to 95° C. for half-an-hour. Ramon (1928), Van Gerderen (1933) and Povitzky (1935) show that tetanus and diphtheria toxoids may be heated at 60° C. to 70° C. without demonstrable loss in immunizing value. This has been borne out in the present investigation with *Cl. chauvoei* formol-toxoids. Heating at 60° C. for half-an-hour had no detrimental effect on immunity production and even boiled toxoid had a slight immunizing value. As toxin and toxoid after half-an-hour at 60° C. bind no or only a trace of antitoxin, it would appear that the heat stable antigen is of great importance in the production of immunity to *Cl. chauvoei*. Lourens (1935) expressed the interesting view that, as the old spore vaccines for black quarter were made from heated infected muscle, the immunity produced was due to the aggressins in the dry powder. The present work would tend to confirm this view. The relatively small number of killed germs in the dry muscle powder would hardly be sufficient to set up immunity and it is a very moot point if the immunity was due to the *in vivo* germination of an odd, so-called attenuated spore.

Finally, the virulence of a *Cl. chauvoei* culture need not necessarily have a bearing on the antigenicity of a formol-filtrate prepared from it. The immunity produced by the toxoids of a virulent and of a relatively avirulent culture of the same strain was of about the same order. This is supported by Haslam and Lamb (1919) and Gräub and Zschokke (1910) who stated that the immunizing power of filtrates was independent of their toxicities.

It would appear that no finality can be reached on the production of immunity to *Cl. chauvoei* until a method is devised for titrating the heat stable antigen and the antibody produced by it. Since (1) antigens, such as boiled bacilii and heated toxoids free from

demonstrable toxin or toxoid can produce a high degree of immunity to culture, (2) since there is no correlation between the antitoxin-binding power of an antigen and its ability to produce resistance to culture and (3) since the absence of circulating antitoxin is no indication of the inability of an animal to resist culture, it is probable that the heat stable antigen is the important one.

Until toxin (aggressin) or toxoid free from the heat stable antigen is obtained, one cannot say whether it plays any part in the production of immunity to the inoculation of living culture or to the natural disease.

Although the writer has gained the impression, from a study of the literature, that many workers suspected the presence of a heat stable antigen, credit must go to Henderson for having so clearly shown that the antigen in boiled bacilli is such a powerful immunizing agent.

CONCLUSIONS.

(1) In culture filtrates of *Cl. chauvoei* two antigens have been demonstrated (*a*) a toxin, and (*b*) a heat stable antigen. The toxin can best be demonstrated by injecting filtrate intradermically into guinea-pigs, particularly if adrenalin is incorporated in the filtrate. The presence of the heat stable antigen is proved if toxin-free (heated) bacilli are injected into sheep; after a suitable interval these are immune to the inoculation of living culture.

(2) The toxin, or its atoxic modification, stimulates the formation of antitoxin when injected into animals, whereas the heat stable antigen does not do so.

(3) The toxin is neutralizable by homologous antitoxin, by *Cl. septicum* antitoxin and to a slight extent by the sera of some normal bovines and rabbits.

(4) The toxins of four different strains of *Cl. chauvoei* have been found to be indistinguishable serologically.

(5) There is no correlation between the amount of circulating antitoxin in an animal and the power of the animal to resist the inoculation of living culture.

(6) There is a correlation between the amount of circulating antitoxin in an animal and its power of resisting toxin, injected intravenously or intradermically.

(7) The evidence points to the heat stable antigen being the important one in the production of immunity to *Cl chauvoei* culture.

REFERENCES.

BASSET, J. (1925[1]). Immunisation des bovidés par la toxine symptomatique. *Rec. de Méd. Vét.*, Vol. 101, p. 549.

BASSET, J. (1925[2]). Atténuation de B. chauvoei. Anatoxine symptomatique. *Comp. Rend. Soc. Biol.*, Vol. 93, p. 172.

EICHORN, A. (1918). Studies in blackleg immunization, with special reference to blackleg filtrate. *J. Amer. Vet. Med. Assoc.*, N.S., Vol. 5, p. 653.

GINS, H. A., AND HUSSEIN, K. (1927). Beiträge zur serologischen Differenzierung der Rauschbrand- und Pararauschbrandbazillen. *Centralbl. f. Bakt.* 1, ref., Vol. 86, p. 379.

GINS, H. A., AND HUSSEIN, K. (1928). Ueber die serologische Differenzierung der Rauschbrand- und Pararauschbrandbazillen. *Centralbl. f. Bakt.* 1, Orig., Vol. 107, p. 96.

GOSS, L. W., BARBARIN, R. E., AND HAINES, A. W. (1921). Some characteristics of B. chauvoei. *J. Inf. Dis.*, Vol. 29, p. 615.

GRASSBERGER, R., AND SCHATTENFROTH, A. (1908[1]). Das Rauschbrandgift. *Handbuch d. Tech. u. Meth. d. Immunitätsfsch.*, Vol. 1, p. 161.

GRASSBERGER, R., AND SCHATTENFROTH, A. (1908[2]). Die Rauschbrandschutzimpfung. *Handbuch d. Tech. u. Meth. der Immunitätsfsch.*, Vol. 1, p. 894.

GRÄUB, E., AND ZSCHOKKE, W. (1920). Die Immunisierung gegen Rauschbrand mit keimfreien Filtraten. *Schw. Arch. Tierheilk.*, Vol. 62, p. 52.

GREEN, W. J. B. (1929). Immunization against anaerobes of the gas gangrene type in South Africa by means of "Anatoxins". *15th Ann. Rpt. Dir. Vet. Serv., Union S.A.*, p. 193

HARTLEY, P. (1922). The value of Douglas's medium for the production of diphtheria toxin. *J. Path. Bact.*, Vol. 25, p. 479.

HASLAM, T. P., AND LAMB, J. W. (1919). Immunization with Blackleg aggressin. *J. Immunol.*, Vol. 5, p. 539.

HENDERSON, D. (1932). Studies on *Cl. chauvoei* 1. The analysis of the H and O antigens of *Cl. chauvoei*. *Brit. J. Exp. Path.*, Vol. 13, p. 412.

HENDERSON, D. (1933). Studies on *Cl. chauvoei* 111 . Passive protection with pure "O" sera. *Brit. J. Exp. Path.*, Vol. 14, p. 108.

HENDERSON, D. (1934). Experiments with the "O" antigen of *Clostridium edematis maligni (Vibrion Septique)*. *Brit. J. Exp. Path.*, Vol. 15, p. 166.

IVANIČ, ST. (1922). Ueber die Erreger des Rauschbrandes der Rinder. *Ztschr. f. Hyg. u. Infektionskht.*, Vol. 97, p. 330.

KELSER, R. A. (1918). Improved methods of immunisation against symptomatic anthrax (blackleg). *J. Agric. Res.*, Vol. 14, p. 253.

KERRIN, J. C. (1934). The haemolytic and toxic activities of filtrates of *Cl. chauvoei*. *J. Path. Bact.*, Vol. 38, p. 219.

KOJIMA, K. (1923[1]). Beiträge zur Erforschung der Rauschbranderreger. 1 Mitteilung. Die Toxinbildung des Types Foth und die toxikologischen, immunisatorischen und biochemischen Eigenschaften des Toxins. *Ztschr. f. Immunitätsfsch.*, Vol. 37, p. 170.

KOJIMA, K. (1923[2]). Beiträge zur Erforschung der Rauschbranderreger. 11 Mitteilung. Ueber Toxin und Antitoxin der Typen Foth und Kitt und ihre besonderen Verschiedenheiten. *Zschr. f. Immunitätsfsch.*, Vol. 37, p. 185.

LECLAINCHE, E., AND VALLÉE, H. (1900[1]). Recherches expérimentales sur le charbon symptomatique. *Ann. Instit. Pasteur*, Vol. 14, p. 202.

LECLAINCHE, E., AND VALLÉE, H. (1900[2]). Étude comparée du vibrion septique et de la bactérie du charbon symptomatique. *Ann. Instit. Pasteur*, Vol. 14, p. 590.

LECLAINCHE, E., AND VALLÉE, H. (1923). Sur la vaccination contre le charbon symptomatique par les toxines. *Comp. Rend. Acad. Sci.*, Vol. 176, p. 207.

LOURENS, L. F. D. E. (1935). Boutvuurfiltraat—Boutvuurformolvaccin. *Tijdschr. v. Diergeneesk.*, Vol. 63, p. 408.

MASON, J. H. (1934). A note on the cultivation of anaerobes. *Onderstepoort Jnl.*, Vol. 2, .p 29.

MASON, J. H. (1935). The lethal dose of the toxins of some anaerobes for sheep. *Onderstepoort Jnl.*, Vol. 5, p. 61.

MASON, J. H. (1936[1]). The production of immunity against *Cl. welchii*, Type B, Wilsdon (The " lamb dysentery bacillus "). *Onderstepoort Jnl.*, Vol. 5, p. 391.

MASON, J. H. (1936[2]). The isolation of single bacterial cells. *Onderstepoort Jnl.*, Vol. 7, p. 133.

MASON, J. H., AND SCHEUBER, J. R. (1936). The production of immunity to *Cl. chauvoei*. *Onderstepoort Jnl.*, Vol. 7, p. 143.

MIHAILESCO, M. (1934). La problème des oedèmes gazeux des bovidés. *12th Internat. Vet. Cong. Rpt., New York*, p. 201.

POPE, C. G., AND SMITH, M., LL. (1932). The routine preparation of diphtheria toxin of high value. *J. Path. Bact.*, Vol. 35, p. 573.

POVITZKY, OLGA R. (1935). The effect of temperature on the antigenic value of diphtheria toxoid. *J. Immunol.*, Vol. 28, p. 209.

RAMON, G. (1922). Floculation dans un mélange neutre de toxine-antitoxine diphterique. *Comp. Rend. Soc. Biol.*, Vol. 86, p. 661.

RAMON, G. (1928). L'ánatoxine diptheérique. Ses propriétés—ses applications. *Ann. Inst. Pasteur*, Vol. 42, p. 959.

ROBERTSON, M. (1916). Notes upon certain anaerobes isolated from wounds. *J. Path. Bact.*, Vol. 20, p. 327.

ROBERTSON, M., AND FELIX, A. (1930). Serological studies in the group of spore-bearing anaerobes 11. *In vitro* experiments with an " O " immune serum to vibrion septique devoid of antitoxin content. *Brit. J. Exp. Path.*, Vol. 11, p. 14.

SALIMBENI, A. AND LOISEAU, G. (1934). Concentration de la toxine et de l'anatoxine diphtérique an moyen de la congélation. *Comp. Rend. Acad. Sci.*, Vol. 199, p. 242.

SCOTT, J. P. (1923[1]). Blackleg vaccines. *Kansas State Agric. Coll. Tech. Bull.*, No. 10.

SCOTT, J. P. (1923[2]). Potency tests for Blackleg filtrate and aggressin. *J. Amer. Vet. Med. Assoc.*, N.S., Vol. 17, p. 67.

SCOTT, J. P. (1925). Studies on certain characteristics of Clostridium chauvoei and Clostridium edematis. *J. Bact.*, Vol. 3, p. 265.

SCOTT, J. P. (1930). Action of formaldehyde on the aggressive substance of blackquarter filtrate, bacterin and aggressin. *J. Inf. Dis*, Vol. 46, p. 460.

VAN GERDEREN, A. J., V. D. HOVEN (1933). The influence of heat on the antigenic properties of diphtheria and tetanus formol-toxoids. *J. Path. Bact.*, Vol. 36, p. 319.

VILJOEN, P. R., AND SCHEUBER, J. R. (1926). Black-quarter in South Africa: With special reference to improved methods of inoculation. *11th and 12th Report of the Director of Vet. Educ. and Res., Union of S.A.*, Part 1, p. 501.

VON HIBLER, E. (1908). Untersuchungen über die pathogenen Anaëroben. G. Fischer. Jena.

WEINBERG, W., AND MIHAILESCO, M. (1929). Recherches sur le charbon symptomatique et le B. chauvoei. *Ann. Inst. Pasteur*, Vol. 43, p. 1408.

WEINBERG, W., DAVESNE, J., MIHAILESCO, M., AND SANCHEZ, C. (1929). Etude comparative du vibrion septique et du B. chauvoei par la reaction de fixation. *Comp. Rend. Soc. Biol.*, Vol. 101, p. 907.

WEINBERG, W., AND DAVESNE, J. (1935[1]). Anticorps du sérum normal vis-a-vis du Bacillus chauvoei et Vibrion Septique. *Comp. Rend. Soc. Biol.*, Vol. 118, p. 1412.

WEINBERG, W., AND DAVESNE, J. (1935[2]). Titre antitoxique et pouvoir anti-infectieuse des sérums thérapeutiques. *Comp. Rend. Acad. Sci.*, Vol. 200, p. 1074.

ZELLER, H. (1925). Die Schutzimpfung gegen Rauschbrand mit Rauschbrandkulturfiltraten. *Berlin. Tierärztl. Wschr.*, Vol. 41, p. 385.

ZSCHOKKE, W. (1932). Beitrag zur Frage der Steigerung des Aggresingehaltes durch Zusatz von Formaldehyd zur keimfreien Rauschbrand-Impstoffen. *Schw. Arch. Tierheilk*, Vol. 74, p. 539.

Intradermic Reaction in Guinea Pig
2 M.R.D. Toxin, 24 hours.

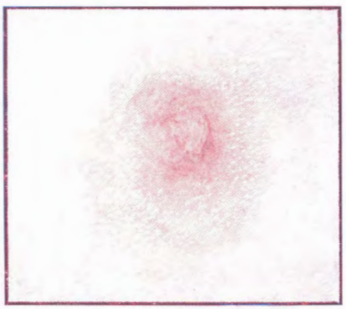

Fig. 1.