

The Production of Immunity to *Cl. Chauvoei*.

By J. H. MASON and J. R. SCHEUBER, Section of Bacteriology,
Onderstepoort.

INTRODUCTION.

THE purpose of this communication is to place on record a number of experiments, having as end, the production of a vaccine which, when parenterally introduced into sheep, would consistently protect them against naturally-occurring black quarter. We have no intention of reviewing the very extensive literature on the subject; only those investigations having a direct bearing on this piece of research will be quoted.

About ten years' experience in the production of black quarter vaccine has taught one of us (J.R.S.) that a vaccine which so immunizes six experimental sheep that they all withstand one or two lethal doses of *Cl. chauvoei* culture (introduced intramuscularly), will prove satisfactory in the field. On the other hand, when two or three of six experimentally immunized sheep fail to resist the injection of one fatal dose of culture, reports on the lack of protection to the natural disease may be expected.

The work, about to be detailed, was initiated by failure of a number of vaccines to produce satisfactory immunity. The vaccine, issued from this laboratory, was made in a manner similar to that described by Viljoen and Scheuber (1927)*. For a number of years, such a prophylactic gave consistently good results both in experiments at Onderstepoort and in the field. From 1928 onwards the protection afforded by the vaccines (prepared in multiples of 40 litre quantities once or twice per month) slowly began to decrease, to such an extent that some batches had to be discarded in 1931-1932. Although every point in the preparation of the medium, in the culture for inoculation and in the treatment of vaccine was checked up, only one departure from the prescribed procedure was discovered. This was that the medium was autoclaved at 115° C. in 20-litre flasks on two successive days for four hours instead of previously

* See appendix for description.

for one hour on three successive days. The raising of the pathogenicity of the strain by repeated passage through guinea-pigs did not lead to an improvement in immunizing value of the prophylactic. One of us (J.H.M.) who has had some experience in the production of formol-toxoids and anaerobes of *Cl. welchii*, Types A and B* (Wilsdon 1931) and of *Cl. septicum* suggested that the medium used (a liver infusion, peptone, salt and glucose plus liver particles) was unsuitable but against this there was the evidence of the good results in the past. However, in view of the good results obtained with Robertson's† (1916) meat broth in the production of toxins of other anaerobes and of the poor results obtained with liver medium with these other anaerobes (unpublished experiments of J.H.M.) it was decided to test out meat broth with *Cl. chauvoei*.

EXPERIMENTAL.

Strains of Cl. chauvoei used:—

- (1) 64—Isolated from a natural case of black quarter in a heifer in Waterberg (Transvaal) in 1929.
- (2) D—Originally isolated from the muscle of a bovine dead of black quarter, in Europe.

*Media:—*Meat broth was prepared as noted in the appendix.

Sloppy agar was a boiled extract of horse or donkey muscle, plus 1.0 per cent. peptone, 0.5 per cent. NaCl and 0.15 per cent. agar.

The routine liver medium was prepared as noted in appendix, and will be referred to as "routine medium".

The sheep haemolysed red cells serum mixture was prepared in the manner described by Mason (1934).

Small amounts (10 c.c.-2,000 c.c.) of meat broth were autoclaved for half an hour at 115° C., i.e. the temperature was held at 115° C. for half an hour after having reached this point. Twenty-litre quantities of meat broth were autoclaved for 4 hours on each of two successive days. Small quantities were boiled for from 10 minutes to 2 hours and rapidly cooled just prior to inoculation. Large quantities (20 litres) of medium were allowed to cool for 24 hours before being seeded.

The pH of all media was adjusted to 8.2 just prior to autoclaving and usually, but not on all occasions, with all media except the routine, the pH was again adjusted to 7.6-7.8 with N/1 NaOH before boiling, or in the case of 20-litre amounts, before seeding. The reason for this was because the pH of media containing meat particles usually falls on storage.

* Type A=classical *Cl. welchii*.

Type B=the "lamb dysentery bacillus" (Dalling, 1928).

† See appendix for preparation.

The inoculum for meat broth media was, unless otherwise stated, an 18 hours meat broth (plus haemolysed cells and serum) culture and for the routine medium, an 18 hours' von Hibler culture. The amount of the inoculation varied—for one- to two-litre quantities, 10-20 c.c. was added and for 20-litre amounts, about 50-100 c.c. was seeded over.

The length of incubation varied and will be noted under each experiment; the treatment of the culture after growth will also be noted under each experiment.

The virulent material used to test the immunity in sheep was, for meat broth antigens, a young (18 hours') meat broth (plus haemolysed cells) culture. Usually, such a culture was produced by seeding a tube of medium with the heart blood and/or liver of a guinea-pig killed by the intramuscular injection of the germ. In any case, the organisms in the test culture were never far removed from an animal passage. For testing animals vaccinated with routine vaccine, a guinea-pig passage culture in von Hibler's medium was usually employed.

Experiment 1.

To compare the immunizing power of antigens prepared (1) in meat broth plus 5 per cent. of a mixture of equal parts of sheep serum and haemolysed red cells; (2) in 0.15 per cent. sloppy agar plus the same mixture; (3) in meat broth plus the same mixture but using a different chauvœi strain; and (4) by using the 1.5 per cent alum precipitate of the anaculture prepared from (1).

Two litre quantities of media (1) and (2) were adjusted to pH 7.8, boiled for one hour, cooled and, after the addition of haemolysed cells and serum, inoculated with a guinea-pig passage culture of strain 64; to another flask of medium (1) strain D was added. After three days' incubation at 37° C., the meat was removed from those flasks containing it, and to all cultures enough formalin (40 per cent. formaldehyde) was added to make a 0.4 per cent. concentration. (In future this will be referred to as "adding 0.4 per cent. formalin"). The flasks were then incubated at 37° C. for three days. The sloppy agar culture was contaminated with a coccus, the meat broth cultures were not contaminated and all anacultures were sterile.* To 200 c.c. of the anaculture (1) 3 gm. of potash alum was added, the precipitate washed three times in saline and finally suspended in 200 c.c. of 0.5 per cent phenol-saline. Sheep received subcutaneously 10.0 c.c. of the one or the other antigen on 12.4.33 and were tested for immunity on 11.5.33. Table I records the results.

* By "sterile" is meant that 5.0 c.c. of anaculture, in 50 c.c. of meat broth plus haemolysed cells and serum, produced no growth after 7 days' incubation at 37° C. and that 2.0 c.c. introduced, intramuscularly, into guinea pigs caused no more than a slight swelling of the leg.

TABLE I (Expt. S. 5003).

Immunizing power of Cl. chauvoei vaccines, noted in Experiment 1. 10 c.c. vaccine injected s.c. on 12.4.33. Test with culture i.m. 11.5.33.

Vaccine.	Sheep. Test with living culture i.m. (c.c.).			
	1·0	2·0	3·0	4·0
1. H.F. meat broth + Hm. cells (strain 64)	1 (35789) L	2 (21756) L	3 (35523) L	4 (35859) L
2. Sloppy agar + Hm. cells (strain 64)....	5 (35857) † 3	6 (35789) † 2	7 (35854) † 1	8 (37021) † 1
3. H.F. meat broth + Hm. cells (strain D)	9 (34478) † 2	10 (35521) L	11 (33611) L	12 (35850) L
4. Alum precipitate of 1	13 (31588) L	14 (37019) L	15 (35794) L	16 (35355) L

Controls : 17 (35517) 0·5 c.c..... † 1
 18 (35537) 1·0 c.c..... † 1
 19 (35542) 2·0 c.c..... † 1

(L = lived ; † 1, † 3 = died 1, 3 days ; H.F. = horse flesh ;
 Hm. cells = sheep serum and haemolysed cells mixture.)

Result.—At the time when this experiment was carried out, the approximate lethal dose of a culture, prepared in meat broth from a guinea-pig passage culture was not known; later tests showed that 0·1 c.c. of such a culture killed a sheep in from twenty-four to forty-eight hours. On the assumption that the M.L.D. of the culture, used in Experiment 1, was of this order, it will be seen that three of the four vaccines so immunized sheep that they withstood about 40 lethal doses. A probable explanation for the failure of the sloppy agar anaculture to immunize is that the culture giving it origin was contaminated with a coccus.

Experiment 2.

To compare the immunizing power of (1) anacultures made in horse-flesh meat broth and ox-flesh meat broth, (2) the alum precipitates of these vaccines, (3) routine vaccine, and (4) the alum precipitate of a routine vaccine.

The horse and ox flesh broths, in 500 c.c. quantities were treated as noted under Experiment 1. The routine medium, in 20-litre amounts, was inoculated with a von Hibler culture of a guinea-pig passage strain and treated as noted in the appendix. Strain 64 was used throughout. Sheep received 5·0 c.c. of each vaccine subcutaneously on 29.5.33 and were tested on 21.6.33 by the intramuscular injection of culture (18 hours' meat broth culture of guinea-pig passage strain of 64). Table II records the results.

TABLE II (Expt. S. 5050).

Immunizing power of Cl. chauvœi vaccines, noted in Experiment 2. Sheep injected s.c. on 29.5.33. Tested with culture i.m. 21.6.33.

Vaccine.	Sheep. Tested with living culture i.m. (c.c.).			
	2·0	4·0	8·0	16·0
1. H.F. meat broth + Hm. cells.....	1 (37549) L	2 (37617) L	3 (35514) † 2	4 (26647) L
2. Alum precipitate of 1	5 (37668) L	6 (36796) L	7 (35778) † 2	8 (32944) † 2
3. Ox flesh meat broth + Hm. cells.....	9 (37456) L	10 (37651) L	11 (35536) † 2	12 (34510) L
4. Alum precipitate of 3	13 (37533) L	14 (37394) L	15 (35550) L	16 (32502) † 2
5. Routine medium. Ana- culture. Flask 552.	17 (37577) † 3	18 (35774) † 2	19 (35551) † 3	20 (33116) † 1
6. Alum precipitate of 5	21 (37638) L	22 (35530) † 1	23 (35512) L	24 (34367) † 4
7. Routine medium, Seitz filtrate. Flask 552..	25 (37556) † 1	26 (35515) † 1	27 (37539) † 1	28 (35024) † 1

Controls : 29 (37524) 0·3 c.c..... † 1

30 (37200) 0·5 c.c..... † 1

31 (35799) 1·0 c.c..... † 1

(† 1, 2 = died after one, two days; L = lived;
Hm. cells = haemolysed sheep cells pus serum.)

Result.—On the assumption that the M.L.D. of the test culture was about 0·1 c.c., it will be seen, from the results given in Table II, that the anacultures prepared in both horse and ox flesh meat broth so immunized some sheep that they withstood 160 lethal doses of cultures. The results do not indicate whether or not the alum precipitates of these anacultures were better or worse immunizing agents than the anacultures themselves. The routine vaccine both as an anaculture and as a filtrate was definitely inferior to the meat broth vaccines. The alum precipitate obtained from the routine anaculture proved to have considerable antigenic power.

Experiment 3.

To test the immunizing power of anacultures made in (1) horse-flesh meat broth plus (a) 5 per cent. sheep haemolysed red cells, (b) 5 per cent. ox haemolysed red cells, (c) 5 per cent. horse haemolysed red cells, and in (2) ox-flesh meat plus (a), (b) and (c). This experiment was carried out because of the difficulty that would be experienced in obtaining sufficient sterile serum for 100-200 litre quantities of medium. The haemolysed cell solution was prepared by adding 80 c.c. of blood to 120 c.c. of distilled water containing 2·0 c.c. of a 10 per cent. pot. oxalate solution; excess ether was then added, the mixture shaken and finally incubated for 24 hours at 37° C. The sterility of the cell solutions was proved before their addition to the media. The media were contained in 500 c.c. flasks and a guinea-pig passage culture of *C. chauvœi*, strain 64, was used.

THE PRODUCTION OF IMMUNITY TO "CL. CHAUVOEI".

The treatment of the cultures and anacultures was as detailed under Experiment 1. Sheep received 5.0 c.c., subcutaneously, of the various vaccines on 7.7.33 and were tested for immunity on 25.7.33 (intramuscular injection of strain 64).

TABLE III (EXPT. S. 5083).

The immunizing power of Cl. chauvoei anacultures made in horse and ox flesh meat broth plus haemolysed red cells of the sheep, ox and horse (no serum). Sheep received 5.0 c.c. of vaccine s.c. on 7.7.33; test 25.7.33.

Vaccine.	Sheep. Test with culture i.m. (c.c.).		
	1.0	2.0	4.0
1. H.F.M.B. plus			
(a) Sheep Hm. cells.....	1 (37155) L	2 (36533) † 1	3 (37303) † 1
(b) Ox Hm. cells.....	4 (37756) L	5 (37229) L	6 (37384) L
(c) Horse Hm. cells.....	7 (37702) L	8 (35793) L	9 (36541) L
2. Ox F.M.B. plus			
(a) Sheep Hm. cells.....	10 (37290) L	11 (37684) L	12 (37894) L
(b) Ox Hm. cells.....	13 (37309) L	14 (37405) L	15 (37431) L
(c) Horse Hm. cells.....	16 (37224) † 2	17 (37335) L	18 (37430) † 2

Controls: 19 (37494) 0.2 c.c..... † 1
 20 (37678) 0.3 c.c..... † 1
 21 (37680) 0.4 c.c..... † 1

(H.F. = horse flesh; Ox. F. = ox flesh; M.B. = meat broth; Hm. cells = haemolysed red cells (no serum); L = lived; † 1 = died 1 day.)

Result.—It is doubtful if the results given in Table III are really significant. It will be noticed that the vaccine made from horse-flesh plus sheep cells was poor and that plus horse cells was good whereas the reverse held good with the use of ox flesh. A repeat test with the same first mentioned two vaccines gave results comparable with those reported in Table III.

Experiment 4.

To test the immunizing power of anacultures made in horse-flesh meat broth plus (a) 5 per cent. sheep serum (b) 5 per cent. of a mixture of equal parts of sheep serum and sheep haemolysed red cells, and (c) 5 per cent sheep haemolysed red cells. The serum was obtained from the clot and the haemolysed cells as noted under Experiment 3. Ether was added to these three products and incubated with them for 24 hours. Sterility was proved prior to their addition to the media. Flasks of 500 c.c. capacity were used, these and the anacultures being treated as noted under Experiment I. Sheep received 5.0 c.c. of vaccine subcutaneously on 16.8.33 and were tested for immunity on 30.8.33 by the intramuscular injection of a guinea-pig passage strain of 64. Table IV records the results.

TABLE IV (Expt. S. 5122).

The immunizing power of *Cl. chauvœi* anacultures made in horse-flesh meat broth plus sheep serum, sheep haemolysed red cells and a mixture of cells and serum. Sheep received 5.0 c.c. of vaccine s.c. on 16.8.33; test 30.8.33.

Vaccine.	Sheep. Test with culture i.m. (c.c.).		
	1.0	2.0	4.0
H.F.M.B. plus			
(a) Sheep serum.....	1 (32951) L	2 (33927) L	3 (33939) L
(b) Sheep Hm. cells.....	4 (35007) L	5 (35019) † 1	6 (36770) † 1
(c) Sheep Hm. cells and serum..	7 (37241) L	8 (37896) † 2	9 (36932) † 2

Controls: 10 (37509) 0.1 c.c..... † 2
 11 (37623) 0.2 c.c..... † 1
 12 (35526) 0.3 c.c..... † 1
 († = died. L = lived.)

Result.—The indication given in Table IV is that the addition of sheep serum to medium produces a better vaccine than the addition of sheep haemolysed red cells.

Experiment 5.

As for Experiment 5, but using horse serum and horse haemolysed red cells. Flasks of 500 c.c. capacity were used, the media and anacultures being treated as noted under Experiment I. The inoculation and the test culture were guinea-pig passage strains of *Cl. chauvœi* 64. Sheep received 5.0 c.c. vaccine subcutaneously on 14.9.33 and were tested for immunity on 13.10.33. The test consisted in the intramuscular injection of 1.0 c.c. of culture; all sheep survived (three per group). A control animal receiving 0.1 c.c. of culture lived and another getting 0.25 c.c. died in twenty-four hours. Thus, no comparison can be made of the immunizing value of the three products. (Experiment S. 5148 and sheep 37134, 37157, 37167, 37236, 37255, 37285, 37534, 37546, 37713, 37830, 37882.)

Experiment 6.

The results recorded in Experiments 1 to 5 show that a potent antigen can be prepared in 500 c.c. quantities of horse or ox flesh meat broth plus serum and/or haemolysed red cells of the sheep, ox or horse. However, experimentation showed the difficulty of obtaining serum or haemolysed cells and of adding them to medium under sterile conditions as a routine measure. For this reason, the antigen producing power of Hartley's (1922) and Pope's and Smith's (1932) digest broths was investigated. In addition, the antigen producing power of horse-flesh meat broth plus 5 per cent. horse serum and plus 5 per cent. horse haemolysed cells [these sterilized by incubating

THE PRODUCTION OF IMMUNITY TO "CL. CHAUVOEI".

them at 37° C. for twenty-four hours in the presence of 0·1 per cent. formalin (40 per cent. formaldehyde) was ascertained. The media used were as follows:—

Medium 1.—500 c.c. of Hartley's digest broth, meat particles one-sixth to one-fifth by volume; pH adjusted to 7·8; boiled and cooled.

Medium 2.—As medium 1, plus 5 per cent. etherised horse serum.

Medium 3.—500 c.c. of Pope's "straight line" digest broth; meat, etc., as medium 1.

Medium 4.—As medium 3, plus 5 per cent. etherised horse serum.

Medium 5.—500 c.c. of Hartley's digest broth (no meat particles) plus 0·15 per cent. agar. Otherwise treated as medium 1.

Medium 6.—20-litre flask of horse-flesh meat broth (broth about 14 litres, meat particles about one-third by volume). Had been prepared for about one week; not boiled and pH not investigated; 2 per cent. of 0·1 per cent. formalised horse plasma added.

Medium 7.—As medium 6, but plus 5 per cent. of 0·1 per cent. formalised horse haemolysed cells.

Medium 8.—As medium 7.

All flasks were inoculated with a meat broth subculture of a three weeks' old guinea-pig passage culture of strain 64. The subsequent treatment was as noted under Experiment I.

Order of growth in the various media.—(4 days 37° C.).

Medium 1.—Rather poor, (as judged by opacity and gas production).

Medium 2.—Fair.

Medium 3.—As (1).

Medium 4.—Fair.

Medium 5.—Excellent.

Medium 6.—Excellent, (contaminated with a coccus).

Medium 7.—Poor, (contaminated with a coccus).

Medium 8.—Good, (contaminated with a Gram + aerobe).

The two flasks comprising media 7 and 8 were treated separately, owing to the difference in growth.

Sheep received 5·0 c.c. subcutaneously of the various vaccines on 27.10.33 and were tested for immunity on 16.11.33 (a recent passage culture used). Table V. records the results.

TABLE V.

*Immunizing power of vaccines detailed in Experiment 6.
 Sheep inoculated on 27.10.33; test 16.11.33 (Expt. S. 5184).*

Vaccine.	Sheep. Test with culture i.m. (c.c.).		
	1·0	2·0	4·0
Medium 1.....	1 (37143) L	2 (37581) L	3 (37688) L
Medium 2.....	4 (37716) L	5 (37732) L	—
Medium 3.....	6 (37767) L	7 (37772) L*	8 (37784) L
Medium 4.....	9 (37785) L	10 (37812) L	11 (37847) L
Medium 5.....	12 (37855) L	13 (37876) L	—
Medium 6.....	14 (37910) L	15 (37911) L	16 (37921) L
Medium 7.....	17 (37980) L	18 (37990) L	19 (37997) L
Medium 8.....	20 (38015) L	21 (38017) L	22 (38059) K*

* Very severe reaction.

K* Killed 24.11.33 owing to reaction.

Controls : 23 (36907) 0·1 c.c..... † 2
 24 (37712) 0·2 c.c..... † 1
 25 (37880) 0·4 c.c..... † 1

Result.—The vaccines prepared in all media produced excellent immunity. Even in those flasks in which growth was poor and in which contamination occurred, good antigen was formed.

Experiment 7.

To ascertain if horse-flesh meat broth plus 5 per cent. formalised (0·1 per cent.) horse plasma could be used as a routine medium for the production of vaccine.

To seven 20-litre flasks of horse-flesh meat broth (meat particles about one-third by volume) approximately 5 per cent. formalised horse plasma was added. The media stood for three days at room temperature and were then seeded with a von Hibler culture of a recently passaged strain of 64. The pH of the flasks was not investigated. Incubation was for three days at 37° C. and good growth was obtained in each flask, but a purity test revealed a contamination with a Gram + aerobe. To the Seitzed filtrate, 0·5 per cent. formalin was added and incubation carried on for three days. Sterility tests on the final product passed. Sheep received 5·0 c.c. of vaccine subcutaneously on 7.10.33 and were tested for immunity on 19.10.33 by the intramuscular injection of a twenty-four hours' von Hibler culture. (Experience over many years has shown that the M.L.D. of such a culture is between 0·5 c.c. and 1·0 c.c.)

Result.—The control sheep receiving 1·0 c.c. and 2·0 c.c. of culture died within thirty-six hours. Of the vaccinated sheep, one withstood 2·0 c.c. and another 3·0 c.c. of culture; the third succumbed to the injection of 4·0 c.c.

It is difficult to make a fair comparison between the immunizing power of this vaccine and those produced in smaller volumes of medium, where the inoculum was a meat broth culture. However, the indication is that the degree of immunity was less than that produced by the vaccines made from media and 7 and 8 of Experiment 6, where the inoculum was a meat broth culture. (Experiment S. 5168 and sheep 37683, 37771, 37777, 3804004, and 38021.)

Experiment 7 (a).

This was a repetition of Experiment 7 with a few modifications. The pH of the horse-flesh meat broth (20-litres, meat particles one-third by volume) was adjusted to 7·8, 3 per cent. of 0·1 per cent. formalised horse plasma and 0·25 per cent. glucose was added. The inoculum was a meat broth culture of a recently passaged strain of 64. Incubation was for four days at 37° C., the meat was removed and anaculture made by adding 0·4 per cent. formalin and incubating for forty-eight hours.

Sheep received 5·0 c.c. of the vaccine on 9.11.33 and were tested for immunity on 6.12.33, using a meat broth culture of strain 64.

Result.—The control sheep receiving 0·1 c.c. of culture died within forty-eight hours. One immunized sheep which received 1·0 c.c. of culture died, a second which got 2·0 c.c. lived, as did a third into which 4·0 c.c. were injected.

One could conclude that the vaccine prepared as described had a high immunizing value. (Experiment S. 5197 and sheep 35552, 36784, 36879, 36961.)

Experiment 8.

To ascertain the immunizing value of anacultures prepared from one, two and four day cultures.

To a one-litre quantity of donkey-flesh meat broth (meat particles one-third by volume) 3 per cent. horse plasma (formolised 0·1 per cent.) and 0·25 per cent glucose was added, after the medium had been boiled for an hour and the pH adjusted to 7·8. A direct guinea-pig passage culture of strain 64 was used as inoculum. After one, two, and four days' incubation at 37° C., samples of culture were removed, formalised to 0·4 per cent. and incubated for a further forty-eight hours. Sheep received 5·0 c.c. of the one or the other vaccine on 17.11.33 and were tested for immunity on 6.12.33.

Result.—The control sheep were those used in Experiment 7 (a). The sheep receiving the one, two, and four day anacultures (3 per anaculture) survived the intramuscular injection of 1·0 c.c., 2·0 c.c. and 4·0 c.c. respectively of the same culture as used for the controls.

Although no answer could be given as to the respective immunizing values of the different vaccines, the conclusion may be drawn that, under certain conditions, excellent immunity is produced in sheep by the use of anacultures made from one, two or four day cultures. (Experiment S 5201 and sheep 35022, 35528, 36814, 36984, 36991, 36961, 37887, 37092, 37408.)

Experiment 9.

To ascertain if there is any difference in the immunizing value of anacultures made in 10-litre quantities of donkey-flesh meat broth, when the inoculum is a meat broth culture of *Cl. chauvæi* and when it is a von Hibler culture.

The pH of two 10-litre flasks of donkey-flesh meat broth (meat particles one-third by volume) was adjusted to 7·8. The inoculum of flasks A and B was as follows: Flask A, guinea-pig liver (killed by i.m. injection of strain 64)→meat broth→meat broth→flask: Flask B, guinea-pig liver (as above)→meat broth→von Hibler→von Hibler→flask. Incubation was for 3 days at 37° C.: to the meat-particle-free supernatant, 0·4 per cent. formalin was added and incubation carried on for three more days.

Sheep received 5·0 c.c. s.c. of vaccine on 7.3.34 and were tested for immunity on 27.3.34. Table VI. records the results.

TABLE VI (Expt. S. 5117).

To test the immunizing value of anacultures made in donkey-flesh meat broth when the inoculum was a meat broth culture and when it was a von Hibler culture.

Sheep inoculated s.c. on 7.3.34; test 27.3.34.

Vaccine.	Sheep. Test with culture i.m. (c.c.).		
	1·0	2·0	4·0
Meat broth inoculum.....	1 (36555) L	2 (38846) † 2	3 (37129) L
Von Hibler inoculum.....	4 (36996) † 2	5 (33893) L	6 (36680) † o/n

(L = lived; † = died; o/n = overnight.)
Controls: 7 (38629) 0·1 c.c..... † o/n
 8 (38201) 0·2 c.c..... † 1

Result.—The result given in Table VI. allows of no definite conclusion being drawn. As the von Hibler inoculum vaccine so immunized one sheep that it withstood at least 20 fatal doses of cultures, it would not appear that the inoculum played a very large rôle.

Experiment 10.

To determine the effect on immunity production of altering the pH of the medium, and of the addition of glucose.

Three 20-litre flasks of donkey-flesh meat broth (meat particles one-third by volume) were used. To two, 3 per cent. of horse plasma (0·1 per cent. formalised) was added—the pH of one was left at 6·8 and that of the other brought to 8.

THE PRODUCTION OF IMMUNITY TO "CL. CHAUVŒI".

To the third flask of medium at pH 8, 0.25 per cent. glucose was added. The inoculum was a twenty-four hours' old meat broth culture of a recent passage strain of 64. After four days' incubation at 37° C., the meat was removed from each flask, 0.4 per cent. formalin added and incubation continued for a further three days.

Into sheep 5.0 c.c. of vaccine was injected subcutaneously on 2.5.34, the test being carried out on 22.5.34 (intramuscular injection of a twenty-four hours' meat broth culture). Table VII. records the results.

TABLE VII (Expt. S. 5371).

The immunizing power of anaerobes made in meat broth at pH 6.8 and 8.0 plus horse plasma and in meat broth, pH 8 plus 0.25 per cent. glucose.

Sheep inoculated 2.5.34; test 22.5.34.

Vaccine.	Sheep. Test with culture i.m. (c.c.).			
	0.25	1.0	2.0	4.0
M.B. + plasma, pH. 6.8	1 (39493) L	2 (39467) L	3 (39172) L	4 (39307) † 2
M.B. + plasma, pH. 8.0	5 (39398) L	6 (39303) L	7 (39464) L	8 (39398) L*
M.B. + glucose, pH. 8.0	9 (39254) L	10 (39393) L	11 (39160) L	12 (39366) L

Controls : 13 (39953) 0.1 c.c. † 2
 14 (39997) 0.25 c.c. † 2

(L = lived ; † = died ; * = Sheep very ill, lame, oedema of leg, but recovered.)

Result.—All three vaccines proved to be excellent antigens. The results given in Table VII do not indicate definitely which, if any, was the best.

Experiment 11.

Although excellent vaccine could be made with 20-litre quantities of horse or donkey flesh meat broth containing 0.25 per cent. glucose, a definite practical disadvantage was the inability of this medium to initiate growth with certainty. On several occasions growth either did not occur or was delayed for several days. The addition of serum overcame this difficulty, but one had then to reckon with the possibility of introducing contaminating organisms and in addition, there was the difficulty of obtaining serum in bulk if the method was adopted as routine. One of us had had experience with Colebrooke's liver digest medium* in the cultivation of anaerobes. Excellent growth could be obtained in this medium, without the addition of meat particles, with *Cl. chauvœi*, but a disadvantage was the lowering of the pH that took place with resultant killing of the organisms, and possibly harmful effect upon the antigen produced. To try to overcome this effect, the following modification was tried.

* See appendix for preparation.

Three parts of ox-liver digest and seven parts of horse-flesh peptone broth (no meat particles) were mixed. Salt was omitted and in its place enough phosphate buffer to make a 0.8 per cent. concentration was added. Seven hundred c.c. at pH 7.4 was boiled for two hours, cooled, inoculated with a recently passaged strain of D and grown for twenty hours at 37° C. Excellent growth resulted, the pH being between 6.5 and 6.7. After adjusting the pH to 7.4, 0.4 per cent. formalin was added and incubation carried on for forty-eight hours.

Sheep received 5.0 c.c. s.c. of the vaccine on 20.9.34 and were tested for immunity on 10.10.34. The method of test was changed. Robertson and Felix (1930) and Henderson (1932) in testing the immunity produced in laboratory animals by the injection of washed boiled suspensions of *Cl. septicum* and *Cl. chauvœi* used spore suspensions activated with calcium chloride. Henderson found that *Cl. chauvœi* could be induced to spore copiously when grown on Dorset's egg medium. Although we never obtained copious sporulation on this medium, we were able, after several attempts, to obtain a suspension rich enough in spores for our purpose. This was heated at 60° C. for half an hour to destroy vegetative elements and then stored at +5° C. It was our intention to use this spore suspension for several months and thus be able to compare the results of several tests. Unfortunately, the suspension lost virulence after only a fortnight, so that comparative work could not be carried out. As activator, adrenalin was employed. We found that, in guinea-pigs, as little as 0.02 c.c. of the 1/1000 dilution received from the manufacturers was sufficient, when diluted to 2.0 c.c., to activate one-tenth of a doubtfully lethal dose of spores. For the sheep test 0.5 c.c. of the spore suspension plus 0.05 c.c. of adrenalin, diluted to 2.0 c.c. with saline, was injected i.m. into four control and the four vaccinated sheep.

Result.—All the control sheep died within forty hours; the vaccinated sheep survived. Although this experiment shows that the vaccine produced in the mixture of digest liver medium and horse-flesh broth was antigenic, we do not know how high the immunity was. From other experiments (not noted in this communication) we have gained the impression that the activated spore test is not a severe one, inasmuch as animals immunized with proved low value antigens (test with culture) survived the injection of the activated spores. (Experiment S. 5484 and sheep 38953, 37626, 41049, 40071, 40771, 40585, 40189 and 40901.)

Experiment 12.

To ascertain if a washed alum precipitate of a *Cl. chauvœi* vaccine produces better immunity than the vaccine itself.

Glenny *et al.* (1926), Glenny and Waddington (1928), and Glenny, Buttle and Stevens (1931) have shown that the alum precipitate of a diphtheria toxoid is a better immunizing agent than the toxoid itself. They attribute this improvement to the decreased solubility of the alum precipitate; it is only slowly absorbed by the body,

resulting in increased immunity. One of us (J.H.M.) has had experience of the use of alum precipitate of toxoids of *Cl. welchii*, Type A and B (Wilsdon) and of *Cl. septicum*. The results of immunization with *Cl. welchii* Type B precipitates were disappointing and will be published at an early date. With *Cl. welchii* Type A and with *Cl. septicum*, the definite indication was that alum precipitates were better antigens than the toxoids from which they were prepared.

In Experiment 2, the alum precipitate of a batch of routine medium vaccine (anaculture) proved a better antigen than the anaculture itself. In that instance, the alum was added to the vaccine, and the precipitate washed twice and injected. In this experiment, enough alum was added to make a 1 per cent. concentration, and the precipitate (without being washed) plus the supernatant was injected. As table VIII shows, the experiment is not conclusive inasmuch as the sheep into which the vaccines were injected were not tested at the same time or with the same test culture as were the alum vaccine sheep. However, the immunity of these sheep was so high that, judging from results obtained with other batches made in the same way and about the same time one may justifiably conclude that the alum vaccines were superior antigens. Into two groups of sheep, two batches of vaccines (routine) were injected. One group received batch No. 86 and the other batch No. 87.

To each batch 1 per cent. alum was added and sheep received 5.0 c.c. s.c. The details of the injections and test are recorded in Table VIII.

Result.—As stated above, the experiment allows of no definite conclusions being drawn, but in view of the fact that experience has shown that good value routine vaccines usually produced immunity only to one to four fatal doses of culture (0.3 c.c.—0.5 c.c. of a von Hübner culture was in the region of an M.L.D.), one may be allowed to infer that the alum precipitate was the better vaccine. It will be observed that sheep 11, which received 86 alum precipitate, resisted the inoculation of at least sixty lethal doses of a meat broth culture.

Experiment 13.

To determine if the addition of agar to vaccine would increase the immunizing value.

This experiment was in the nature of a "follow-on" of that detailed above. *Cl. chauvoei*, 64, was grown in 8 × 20 litre flasks of donkey-flesh meat broth (meat particles one-third by volume) plus 0.5 per cent. glucose for three days. Growth was good. The meat was removed and 0.5 per cent. formalin added and incubation carried on for three days. To a quantity of anaculture, enough melted agar was added to give a 0.25 per cent. concentration. Sheep received 5.0 c.c. s.c. of the one or the other vaccine on 11.6.34 and were tested for immunity on 5.7.34 (i.m. injection of a meat-broth culture of strain 64). Table IX records the results.

TABLE VIII.
Comparison of the immunity by routine vaccines and by alum precipitates.

Vaccine. 86 (S. 5478)	Sheep..... Dose injected (3/9/34) c.c..... Test (25/9/34)— (v. Hib. cult.) c.c..... Controls.....	1 (41058) 2-0 0-5 L 7 (40726) 0-5 † 1	2 (40414) 2-0 0-5 L	3 (40444) 5-0 0-5 L 8 (40838) 0-5 † 1	4 (40531) 5-0 0-5 L	5 (40538) 20-0 0-5 L	6 (40617) 20-0 0-5 L
86 alum ppt. (S. 5515)	Sheep..... Dose injected (25/10/34) c.c..... Test (20/11/34)— (M.B. culture) c.c..... Controls.....	9 (41123) 5-0 2-0 L 12 (41046) 0-1 † 36 hours.	10 (41038) 5-0 4-0 L	11 (41111) 5-0 6-0 L			
87 (S. 5485)	Sheep..... Dose injected (18/9/34) c.c..... Test (9/10/34)— (v. Hib. culture) c.c..... Controls.....	13 (40654) 2-0 0-5 L 19 (40666) 0-5 † 1	14 (40850) 2-0 0-5 L	15 (40904) 5-0 0-5 L 20 (41044) 0-5 † 1	16 (41010) 5-0 0-5 L	17 (41015) 20-0 0-5 L	18 (41034) 20-0 0-5 L
87 alum ppt. (S. 5506)	Sheep..... Dose injected (16/10/34) c.c..... Test (6/11/34)— (M.B. culture) c.c..... Controls.....	21 (41026) 5-0 0-5 L 29 (41022) 0-1 † 10/h.	22 (41124) 5-0 0-5 L	27 (41043) 5-0 1-0 L 30 (41047) 0-25 † 1.	28 (41001) 5-0 1-0 L		

(† = died; o/n = overnight; 1 = 1 day; hrs. = hours; L = lived; M.B. = horse flesh meat broth culture; v. Hib. cult. = von Hübner culture.)

TABLE IX (Expt. S. 5474).

The immunizing power of anaculture compared with anaculture plus 0.25 per cent. agar. Sheep inoculated on 11.6.34: test 5.7.34.

Vaccine.	Sheep. Test with culture i.m. (c.c.).			
	0.3	0.5	0.5	0.5
Anaculture.....	1 (40174) L*	2 (39944) † 1 0.3	3 (40558) † 1	4 (40520) L*
Anaculture plus agar...	5 (40128) L	6 (40033) L	7 (40157) L	8 (40175) L

Controls: 9 (40131) 0.15 c.c..... † 1

10 (40399) 0.5 c.c..... † o/n

(† = died; L = lived; * = Very ill, lame, leg swollen, recovered.)

Result.—The addition of agar increased the immunizing value of the anaculture to a considerable extent, probably due to decreasing the *in vivo* rate of absorption.

Experiment 14.

To ascertain the immunizing power of washed, boiled *Cl. chauvoei* bacilli. Robertson and Felix (1930) and Henderson (1932, 1933, 1934) have shown that boiled suspensions of *Cl. septicum* and *Cl. chauvoei* are capable, when inoculated subcutaneously into guinea-pigs, of immunizing them against the injection of spores activated with calcium chloride. Henderson suggested that the immunity to *Cl. chauvoei* is produced, in the main, by the "O" somatic antigen of the bacilli. Results, of a confirmatory nature, which will form the subject matter of another communication, have been obtained by one of us (J.H.M.). Roberts (1933) was not able to obtain satisfactory results with Henderson's technique.

The following experiments were carried out to test the method:—

(a) Strain 64 was grown in 1 per cent. glucose broth (horse-flesh) plus haemolysed cells and serum for 18 hours. The bacilli were spun out, washed twice in distilled water, boiled for two hours and enough salt added to make a 0.85 per cent. concentration. About 400 c.c. of medium was required to produce 70 c.c. of a suspension corresponding in opacity to tube 10 of a Burroughs Wellcome nephelometer. Into sheep, 10.0 c.c. of the suspension was injected s.c. on 12.4.33; the test took place on 11.5.33 (the same test as noted in Experiment 1). Table X records the results.

TABLE X.

The immunizing power of boiled bacilli (Expt. S. 5003).

Sheep inoculated on 12.4.33; test 11.5.33.

Sheep.	Test. Culture i.m. (c.c.).	Result.
1 (35333).....	1·0	L
2 (35527).....	2·0	L
3 (35773).....	3·0	L
4 (35783).....	4·0	L
Controls.		
5 (35517).....	0·5	† 1
6 (35537).....	1·0	† 1

(L = lived; † = died.)

Result.—On the assumption that 0·1 c.c. of the test culture was a lethal dose, it will be seen that boiled bacilli produced a very high degree of immunity.

(*b*) As (*a*), but bacilli obtained from meat broth plus serum. The opacity of the suspension corresponded to tubes 5–6 of the B.W. nephelometer.

These sheep received 5·0 c.c. subcutaneously on 6.3.34. Tested twenty-one days later with culture i.m., the M.L.D. of which was 0·1 c.c. for control sheep, these animals reacted as follows: that receiving 5 M.L.D. lived as did that receiving 20 M.L.D., whilst one which got 10 M.L.D. died. (Experiment S. 5117 and sheep 34043, 37354 and 24543.)

(*c*) A group of six sheep received eight subcutaneous injections of the suspension noted in (*b*); in all, each sheep got 180 c.c. of material, over a period of thirty days. Tested 9 days after the last injection with culture i.m., the following result was got. Two sheep withstood 50, one 100, one 200 and two 300 M.L.D., whilst a non-treated died after receiving one lethal dose (0·1 c.c.). (Experiment S. 5432, sheep 38847, 37889, 37014, 37362, 34130, and 36583.)

(*d*) A suspension prepared as noted under (*b*). The opacity correspond to that of tube 10 of the B.W. nephelometer.

Two sheep which received 5·0 c.c. of suspension s.c. withstood, three weeks later, 5 and 10 M.L.D. respectively of culture i.m. The control sheep died when 1 M.L.D. (0·1 c.c.) was injected. (Experiment S. 5588, sheep 41018 and 39749.)

(*e*) The bacilli contained in 200 c.c. of a twenty-four hours' meat-broth culture were spun out, washed twice in distilled water, re-suspended in 200 c.c. of distilled water, boiled for two hours and enough salt added to make a 0·85 per cent. concentration. The opacity was about that of the tube 1 of the B.W. nephelometer.

THE PRODUCTION OF IMMUNITY TO "CL. CHAUVOEI".

Three sheep received 5.0 c.c. s.c.; twenty-seven days later, a meat-broth culture was injected i.m. with the following result:—

Control sheep (39771) 0.1 c.c. † o/n.
 Sheep 1. (41561) 0.2 c.c. ,,
 Sheep 2. (41535) 0.5 c.c. ,,
 Sheep 3. (41542) 1.0 c.c. ,,

(Experiment S. 5565.)

(f) The bacilli from a 24 hours' culture in 25 litres of routine medium were removed. This was accomplished by passing the culture through a paper pulp, washing the pulp in distilled water, squeezing the fluid out of the pulp in a press and finally spinning this material. After two washings, 200 c.c. of a dense suspension was obtained. This was frozen and thawed five times. The freezing chamber of a General Electric Company's refrigerator was used for freezing purposes (-15° C.). This five-times frozen suspension was spun for two hours at 4,000 revolutions per minute, a slightly opalescent fluid being obtained. A portion of it was boiled for two hours, a slight turbidity being produced in the process.

Two groups of sheep were immunized, one with the spun and the other with the spun and boiled supernatant. Table XI records the results.

TABLE XI.

The immunizing power of the supernatant of frozen and thawed bacilli and of this material after boiling for two hours. (Expt. S. 5595.)

Material Injected.	Date and Amount.		Test: culture i.m. (c.c.).	
			13/3/35.	
			0.1	0.25
Supernatant.....	22/2/35, 5.0 c.c.	Sheep...	1 (41523) L	2 (41555) L
Boiled supernatant..	22/2/35, 5.0 c.c.	Sheep...	3 (41532) † 2	4 (37385) † 1
		Controls.	5 (41541) † 1	6 (41520) † o/n
			4/4/35.	
			0.25	0.5
Boiled supernatant..	22/2/35, 5.0 c.c.	Sheep...	7 (38891) L	8 (37397) L
	14/3/35, 5.0 c.c.	Controls.	9 (40679) † 1	—

(† = died; L = lived.)

Results.—In sufficient concentration, the boiled bacillary bodies of *Cl. chauvæi* produced a high degree of immunity in sheep, the test material being a young meat-broth culture, injected intramuscularly. However, when the boiled germs were suspended in an amount of saline equal in volume to that of the culture from which they were obtained, the immunity produced was poor (or absent).

The supernatant of a five-times frozen and thawed dense living suspension was of high antigenicity. The boiling of such a supernatant, whilst reducing greatly the antigenic value, did not entirely destroy the antigen.

THE CHOICE OF A ROUTINE VACCINE.

The following points were carefully considered before a decision was made on the medium to be employed in the preparation of vaccine:—

1. The routine medium and method of production had proved satisfactory on past occasions.
2. A potent product could be produced in horse or donkey flesh meat broth plus serum. As before mentioned, there was the difficulty of obtaining serum in quantity and of ensuring its sterile addition to medium. Without serum or haemolysed red cells, there was the definite possibility that growth would not be initiated in all flasks.
3. A tryptic digest muscle medium could be employed or a mixture of Colebrooke's liver digest and horse-flesh infusion broth. These were very seriously considered, but finally rejected owing to the difficulty of preparing a large batch in a working day.
4. Unfortunately Henderson's boiled bacilli vaccine could not be considered owing to the large amount of medium that would be required for its production and to the difficulty that would be experienced in separating the germs from the culture.

Finally it was decided to retain the routine medium, but to introduce some variations. It had been the custom of one of us (J.R.S.) to maintain the *Cl. chauvæi* strain in von Hibler's medium, passing a culture through a guinea-pig just prior to the inoculation of a batch of medium. Instead, the strain was held in horse-flesh meat broth, care being taken that the pH of the medium was 7·6 just prior to seeding. From a tube, which was a direct culture from the heart blood of a guinea-pig, a subculture was made into von Hibler's medium, at pH 7·6, and after eighteen hours' incubation, flasks of medium were inoculated from this. The von Hibler's medium was used to ensure an inoculum, containing a large number of organisms. The pH of the medium in the flasks (20 litres) was adjusted to between 7·6 and 8·0 just prior to inoculation. Care was taken that the amount of liver particles in the flasks was about one-third of the total volume. After thirty-six hours' incubation at 38° C., when growth and gas formation were vigorous, the liver

pieces were removed by sieving, 0·5 per cent. formalin added and incubation carried on for three more days. The final product passed adequate sterility tests and was innocuous for sheep in 20 c.c. amounts subcutaneously.

The results of a year's use of vaccines prepared as just described have been satisfactory.

As an example of the type of vaccine now being prepared after a little more than one year's use of the method just mentioned, the following experiment (15) may be quoted. Six sheep received 5·0 c.c. of vaccine (prepared from 20-litre quantities of medium) subcutaneously and were tested three weeks later by the intramuscular injection of an 18 hours' von Hibler culture. Two withstood 10 M.L.D., two, 20 M.L.D. and two, 40 M.L.D. (Experiment S. 5713 and sheep 43388, 43401, 43417, 43488, 43685, 43934, 43586 and 43111.)

COMMENT.

It is difficult to say if only one change was responsible for the improvement in the vaccine. Whilst retaining the original medium the following alterations have been made:—(1) The maintenance of the *Cl. chauvoei* strain in meat broth instead of von Hibler's medium, (2) the ensuring that the initial pH of all media was 7·6–8·0, (3) a shortening of the incubation time, (4) the leaving out of the re-additions of glucose to the medium and (5) anaculture instead of filtrate used.

The experiments detailed have shown that excellent vaccine may be produced after twenty-four hours' incubation of culture but that no significant difference in immunity production was got with vaccines prepared from media of initial pH 6·8 and 8·0 respectively. However, one may justifiably conclude that better growth could be expected from a medium the pH of which was 8 than from one where it was 6·8. The strain was maintained in meat broth, because experience showed that the virulence did not decrease in this medium whereas it did so in brain medium. Actually after one year's maintenance in meat broth the virulence of strain 64 increased four times. Further, meat broth cultures were usually five to ten times more pathogenic than those from von Hibler's medium.

Although the addition of glucose (with the accompanying adjustment in pH to 7·8) on several occasions, as originally carried out, did lead to the production of a dense growth of the germ with a possible extra formation of antigen, the definite possibility existed that the long incubation period and the metabolic products formed during these "re-growths" might destroy antigen already formed.

Finally, one result that comes out clearly is the consistently good results obtained with small quantities (500 c.c. to 2,000 c.c.) of meat broth medium (Experiments 1, 2, 3, 4, 5 and 8); when 10 to 20 litre volumes were used, satisfactory vaccine was produced but not always of the same high value as with small amounts of medium [Experiments 6, 7 (a) and 10 gave good vaccines, and Experiments 7, 9 and 13 vaccines of a lower value]. The probable explanation

for this was thought to be the procedure in autoclaving. The small amounts were heated for half an hour at 115° C. whereas to ensure sterility, the large volumes were heated at 115° C. for four hours on two successive days.

An experiment (16) set up to investigate this question did not yield absolutely satisfactory results. The batch of medium used was noted under "choice of a routine vaccine" (Experiment 15). Flasks of 500 c.c. capacity were used—one was autoclaved twice (one day interval) at 110° C. for one hour and the other at 115° C. (on two occasions) for four hours. The vaccines (anacultures) prepared from these media were injected subcutaneously in 5.0 c.c. amounts into two groups of four sheep each. The test was carried out along with that conducted for Experiment 15 with the following results:—

Vaccine from flask sterilized for 2 × 1 hour at 110° C.—number of fatal doses withstood by sheep—(1) 5, (2) 10, (3) 20, (4) <40.

Vaccine from flask sterilized for 2 × 4 hours at 115° C.—number of fatal doses withstood by sheep—(1) <5, (6) 10, (7) <20, (8) <40.

It should be noted that the same medium sterilized in bulk (Experiment 15) produced excellent vaccine even although subjected to 2 × 4 hour periods at 115° C. This apparently is at variance with the results noted, but since over-sterilization is detrimental to medium (shown up when small quantities are used) one can conclude that the same detrimental effect occurs when large volumes are employed. However, in this case, all the medium is not subjected all the time to the high temperature and therefore the sterilization of 500 and 20,000 c.c. quantities is not to be compared.

Although it is possible and even probable that over-sterilization played a part in the production of low value vaccines, it is obvious that it is not the whole explanation. Experiments destined to clear up this point are at present under consideration.

CONCLUSIONS.

1. Potent *Cl. chauvæi* vaccine (anaculture) may be made in horse-flesh infusion broth plus 1.0 per cent. peptone, 0.85 per cent. salt and 2—5 per cent. of serum and/or the haemolysed red cells of the horse, ox and sheep (meat particles one-third by volume).

2. Tryptic digests of horse muscle media produce satisfactory vaccines.

3. Boiled, dense suspensions of *Cl. chauvæi* injected subcutaneously into sheep produce a high degree of protection to culture injected intramuscularly.

4. The medium and method finally decided on as a routine measure was: ox-liver infusion broth (liver particles one-third by volume) plus 1.0 per cent. peptone, 0.85 per cent. salt and 1 per cent. glucose added after sterilization, pH 7.8, 36 hours' incubation, and 0.5 per cent. formalization for three days at 37° C.

REFERENCES.

- DALLING, T. (1928). "Lamb Dysentery." *Nat. Vet. Med. Assoc. Congress Rpt.*, Newcastle-on-Tyne.
- GLENNY, A. T., BUTTLE, G. A. H., AND STEVENS, F. (1931). "Rate of Disappearance of Diphtheria Toxoid injected into Rabbits and Guinea Pigs: Toxoid precipitated with Alum." *J. Path. Bact.*, Vol. 34, p. 267.
- GLENNY, A. T., POPE, C. G., WADDINGTON, H., AND WALLACE, U. (1926). "Immunological Notes, XXII. The Antigenic Value of Toxoid precipitated with Alum." *J. Path. Bact.*, Vol. 29, p. 38.
- GLENNY, A. T., AND WADDINGTON, H. (1928). "The Immunity Index Method of Testing Antigenic Values." *J. Path. Bact.*, Vol. 31, p. 403.
- HARTLEY, P. (1922). "The Value of Douglas's Medium for the Production of Diphtheria Toxin." *J. Path. Bact.*, Vol. 25, p. 479.
- HENDERSON, D. W. (1932). "Studies on *Cl. chauvoei* I. The Analysis of the H and O antigens of *Cl. chauvoei*." *Brit. J. Exp. Path.*, Vol. 13, p. 412.
- HENDERSON, D. W. (1933). "Studies on *Cl. chauvoei* III. Passive protection with Pure 'O' Sera." *Brit. J. Exp. Path.*, Vol. 14, p. 108.
- HENDERSON, D. W. (1934). "Experiments with the 'O' Antigen of *Clostridium oedematis maligni* (*Vibrio septique*)." *Brit. J. Exp. Path.*, Vol. 15, p. 166.
- MASON, J. H. (1934). "A Note on the Cultivation of Anaerobes." *Onderstepoort Jnl. Vet. Sc. and Anim. Ind.*, Vol. 2, p. 29.
- POPE, C. G., AND SMITH, M. L. (1932). "The Routine Preparation of Diphtheria Toxin of High Value." *J. Path. Bact.*, Vol. 35, p. 573.
- ROBERTS, R. S. (1933). "The Practical Value of the Heat Stable Antigen of *Cl. chauvoei* as an Immunizing Agent." *J. Comp. Path. and Therap.*, Vol. 46, p. 56.
- ROBERTSON, M., AND FELIX, A. (1930). "Serological studies in the group of spore-bearing anaerobes II. *In vitro* experiments with an 'O' immune serum to *Vibrio septique* devoid of antitoxin content." *Brit. J. Exp. Path.* Vol. 11, p. 14.
- VILJOEN P. R., AND SCHEUBER, J. R. (1927). "Black Quarter in South Africa: With special reference to improved methods of inoculation." *11th and 12th Report Dir. Vet. Ed. and Res. Union of South Africa*, p. 501.
- WILSDON, A. J. (1931). "Observations on the classification of *Bacillus Welchii*." *Sec. Rept. Univ. Camb. Inst. Anim. Path.*, p. 53.

APPENDIX.

PREPARATION OF ROUTINE MEDIUM.

Boil one part of minced ox liver with two parts of tap-water for half an hour.

Fill into flasks of 20 litres capacity and add 1 per cent. peptone, 0.3 per cent. sodium chloride and 0.2 per cent. dibasic sodium phosphate (Na_2HPO_4).

Sterilize at 115° C. for 4 hours on each of two successive days.

Add enough of a saturated watery solution of glucose (sterilized at 110° C. for 1 hour on each of two successive days) to make a 1 per cent. solution and adjust pH to 8.4.

Incubate to test sterility.

PREPARATION OF ROUTINE VACCINE.

Inoculate flask with 50-100 c.c. of a 20 hours' von Hible culture and incubate at between 37° C. and 39° C. After 3-4 days, gas formation having almost ceased, the pH is readjusted to 8.4 and enough sterile saturated glucose solution is added to make a 1 per cent. concentration. Vigorous gas formation again occurs. The addition of glucose and the readjustment of the pH is repeated on one or two further occasions at 3-4 days interval. The culture is clarified by passing it through paper pulp and sterilized by filtration through a E. K. Seitz filter.

To the sterile filtrate enough formalin is added to make 0.4 per cent. concentration and incubated for 4-6 days.

PREPARATION OF MEAT BROTH. (Volumes of 10-2,000 c.c.)

To one pound of finely minced horse muscle add one litre of 0.5 per cent. saline solution.

Boil for one hour and filter.

Neutralize and put in cold room over-night.

Refilter.

Add 1 per cent. Witte peptone.

Adjust pH to 8.5.

Boil and filter.

Fill into tubes or flasks containing enough washed cooked minced meat to make one-third by volume.

Autoclave for half an hour at 15 pounds pressure.

In the case of 10-20 litre quantities autoclave for 4 hours on each of two successive days.

The washed meat is obtained by adding to distilled water the minced meat from which the extract was made. The mixture is brought to the boil and strained through muslin. The meat is then pressed until no more fluid exudes.

PREPARATION OF TRYPTIC DIGEST LIVER BROTH.

Mince up ox liver and to every pound add one litre of water. Heat to 45° C. and add 1 per cent. trypsin and make alkaline. Keep at 45°-50° C. and allow to digest for 1½ hours. Then make acid with acetic acid and boil for 5-10 minutes. Filter, make alkaline and autoclave for half an hour at 15 pounds pressure. Filter through paper, tube and re-autoclave.