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THE BETA TOXIN OF *CLOSTRIDIUM WELCHII* TYPE B, WILSDON, IN RELATION TO THE PRODUCTION OF A VACCINE AGAINST LAMB DYSENTERY

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Economic Importance of the Investigation

The sheep industry in South Africa occupies a very important place in the country's economy as can be seen from the following figures extracted from the 1953 census:—

Revenue from wool	£75 million.
Value of skins produced	£4½ million.
Value of Karakul pelts produced	£1½ million.
Total number of sheep	31·3 million.
Number of sheep slaughtered by butchers	4–5 million.

Exact figures for losses suffered by sheep farmers through disease are not available but a fair estimate would be an amount of several million pounds per annum. Of this vast sum enterotoxaemia-like conditions caused by the *Clostridium welchii* group of organisms account for a considerable portion. Mason (1935a) mentions that E. M. Robinson had isolated *Cl. welchii* Type B from cases of lamb dysentery prior to 1931. Scheuber (personal communication) reports having isolated the same organism from specimens received from the Karoo, the North-Eastern Cape Province and the Orange Free State. Schulz & Sutton (1950) state that lamb dysentery is enzootic in the southern part of the Orange Free State and parts of the Eastern Province.

During 1948 Schulz & McIntyre made a tentative diagnosis of pulpy kidney after investigating extensive losses among sheep in the Eastern Cape Province. This was confirmed when Scheuber demonstrated *Cl. welchii* Type D and its toxin in the intestines of some of these cases. Schulz & Sutton (1950) maintain that this disease is widespread and that it has been encountered on sheep farms in different parts throughout the Union and South West Africa.

In agreement with the experience of Batty, Thomson & Hepple (1954) in Great Britain and Dayus (1938) in New Zealand, Sutton (personal communication) diagnosed pulpy kidney in two-weeks old lambs in South Africa.

The problem of protecting very young lambs against both *Cl. welchii* Type B and Type D infections and older lambs and adult sheep against *Cl. welchii* Type D infections, is therefore posed.

In South Africa where sheep are grazed predominantly on an extensive system they are collected periodically at irregular intervals of up to a month. Since the introduction of jackal-proof fencing, ewes are allowed to lamb in the pastures by preference. Seldom are shepherds used. This arrangement has distinct advantages for the control of internal parasites and diseases favoured by the concentration of sheep but certainly excludes the possibility of using antitoxins for the prevention of lamb dysentery and pulpy kidney in very young lambs as a practical measure. Hence resort must be made to the immunization of pregnant ewes to provide their lambs with a colostral immunity against these diseases during the first few weeks of their lives.

A Brief Consideration of the Toxic Fractions produced by *Cl. welchii* Type B

The welchii group of Clostridia contains six types distinguished one from the other by the toxic fractions produced in artificial media. These toxic fractions have very definite actions by which they can be identified. In Table 1 which was derived from a publication by Oakley & Warrack (1953) the properties and distribution of these antigenic fractions are given.

TABLE 1.—*Properties and Distribution of the Toxic Fractions Produced by* Cl. Welchii *in Culture Media*

Desig-nation	Activity	Occurrence in Filtrates of *Cl. welchii* Types					
		A	B	C	D	E	F
α	Lethal, necrotizing, haemolytic lecithinase C....	***	*	*	*	*	*
β	Lethal, necrotizing.....	–	***	***	–	–	*
γ	Lethal.....	–	*	*	–	–	*
δ	Haemolytic, lethal.....	–	*	**	–	–	–
ε	Lethal necrotizing (activated by trypsin).....	–	**	–	***	–	–
$\varkappa$	Lethal (validity doubtful).....	(*)	–	–	–	–	–
θ	Haemolytic (oxygen-labile, ? lethal.....	**–	*	*	*	*	–
ζ	Necrotizing, lethal (activated by trypsin).....	–	–	–	–	**	–
π	Collagenase (necrotizing gelatinase, ? lethal).....	**	–	*	*–	*	–
λ	Proteinase, disintegrates hide powder but not collagen; gelatinase	–	***	–	*–	*	–
ψ	Hyaluronidase.....	*–	*	–	*–	–	–
υ	Desoxyribonuclease.....	*	*	*	*	*	*

**– or *– = present in some strains.
(*) = limited to very few strains.

From the standpoint of vaccine production the main toxin produced by the *Cl. welchii* Type B strains examined by Glenny, Barr, Llewellyn-Jones, Dalling & Ross (1933) was the beta toxin. The culture filtrates also contained some epsilon toxin in addition to minor fractions such as gamma and delta toxins. Mason (1935a) comments as follows:—

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

The strains isolated by the present author from lambs infected with lamb dysentery produce beta as their main and epsilon as their subsidiary toxic fraction. This was confirmed by Max Sterne of the Wellcome Research Laboratories, Beckenham, England.

In the case of *Cl. welchii* Type D, epsilon toxin is the principal toxin produced (Glenny *et al*; 1933). The South African strains isolated by the author correspond in this respect.

From the above it is obvious that for the immunization of sheep against *Cl. welchii* Type B and Type D separately and in some cases simultaneously, the required level of antitoxin in these animals has to be produced against the different toxic fractions produced by the organisms concerned. The study of this problem was initiated by an investigation into the production of an immunity against *Cl. welchii* Type B and the results obtained form the basis of this thesis.

Evans (1943a) produced experimental evidence that a *Cl. welchii* Type A antiserum with a high content of alpha antitoxin and only a trace of theta antihaemolysin was highly effective in protecting guinea-pigs against infection with *Cl. welchii* Type A. On the other hand, a *Cl. welchii* Type A antiserum with a high content of theta antihaemolysin and containing only a trace of alpha antitoxin was quite ineffective. He further proved (1943b) that *Cl. welchii* Type A antiserum with a high content of alpha antitoxin and no antihyaluronidase was highly effective while an antiserum containing a considerable quantity of antihyaluronidase and only a trace of alpha antitoxin was unable to influence the course of the infection or to enhance the protective action of alpha antitoxin. The three challenge strains of *Cl. welchii* Type A he used produced fatal infections in guinea-pigs; two of these produced hyaluronidase in culture and *in vivo*, but the third did not.

Evans (1947) also showed that the anticollagenase value of a serum has no relationship to its protective value against a *Cl. welchii* Type A infection in guinea-pigs. He (1945) further states that the ability of *Cl. welchii* Type A to produce fatal infections in guinea-pigs is related to its alpha toxin production *in vitro* and is independent of the other antigenic elements.

Although the fact that an antitoxin against the main toxic fraction produced by *Cl. welchii* Type A protects against a fatal infection by that organism cannot be applied *mutatis mutandis* to the *Cl. welchii* group in general without further evidence, Glenny *et al.* (1933) maintain that *Cl. welchii* Type C toxin (which contains the beta fraction but no epsilon) has frequently been used at the Wellcome Research Laboratories for the immunization of horses in the production of lamb dysentery antitoxic sera. Such sera have proved effective in protecting against lamb dysentery in the field. In a personal communication J. R. Scheuber informed the author that he had prepared a vaccine against lamb dysentery for many years at the Onderstepoort Research Laboratories using a strain of *Cl. welchii* Type B which produces mainly the beta fraction and no epsilon (*vide infra*). This vaccine was completely satisfactory.

In the present study, therefore, emphasis has been placed on the production of an immunity against the beta toxin. The significance of the other toxic fractions produced by *Cl. welchii* Type B and the value of immunity against them are difficult to assess, since the natural disease is not easily reproducible in the laboratory. It is the author's intention, however, to assess these by means of a long-term field experiment.

Materials and Methods

1. *Antitoxin*

For the production of a laboratory standard beta antitoxin two horses were given two subcutaneous injections of 20 ml. of an alum-precipitated *Cl. welchii* Type C toxoid separated by 30 days. The horses were then rested for one year before they were put on to an intensive course of *Cl. welchii* Type C toxoid followed by toxin injections. The rest period was allowed with the object of obtaining an avid antitoxin (Glenny, Pope & Waddington, 1925; Glenny & Barr, 1932a; Glenny & Barr, 1932b; Jerne, 1951). Four days after the largest dose of toxin 8 litres of blood were withdrawn from each horse and the bleeding repeated after three days. The serum was collected, pooled and part of it preserved with merthiolate in a final concentration of 1 : 5,000 while the rest was refined by the pepsin digestion method of Pope (1939). The refinement was kindly undertaken by J. H. Mason of the South African Institute for Medical Research.

The different antibody fractions contained in the refined serum were determined by Mrs. I. Batty of the Wellcome Research Laboratories and reported to be as follows:—

Beta	9,500 units.
Alpha	1,500 units.
Delta	about 5 units.
Kappa	2,000 units.
Lamda	less than 50 units.
Epsilon	0·02 units.

2. *Determination of the antitoxin titre of the sheep sera*

For the determination of the antitoxin content of the sera of the sheep after injection of toxoid a dried toxin was prepared from a culture filtrate of the " 1930 strain " of *Cl. welchii* Type B. Ammonium sulphate was used for precipitation and again removed from the dried product by chloroform flotation.

The titration was done in white mice weighing about 20 grams and a constant quantity of 0·004 mg. dried toxin was used per mouse. This weight of toxin contained 2 M.L.D.'s and represented 0·04 test dose as determined against serum EX 1254 obtained from the Wellcome Research Laboratories, Beckenham, Kent, through the courtesy of Dr. G. H. Warrack.

The mice were injected intravenously with 0·2 ml. of toxin-serum mixture made up as follows:—

(*a*) 0·004 mg. toxin dissolved in saline to a total volume of 0·1 ml. plus

(*b*) a quantity of sheep serum made up with saline to a total volume of 0·1 ml.

Successive tubes contained increasing amounts of serum. The serum-toxin mixtures were left at room temperature for one hour prior to injection. From each tube sufficient was withdrawn for the injection of two mice and these were observed for 24 hours after injection.

At first the sera were titrated over a wide range to find the approximate neutral point and subsequently within narrower limits to determine the exact point as closely as possible. Control mice were injected regularly to check the lethal effect of the toxin. The titre of the sera was expressed as units per ml. and where 0·5 ml. of serum failed to protect against 0·004 mg. toxin its titre was recorded as 0 units per ml.

A negligible quantity of alpha toxin was present in the 0·004 mg. dried toxin used since its lethal effect was not influenced by the addition of an excess of alpha antitoxin.

3. *The flocculation test*

The Ramon flocculation test as modified by Glenny & Okell (1924) for titrating diphtheria toxin and antitoxin was used. Mason & Widdicombe (1946) showed that the beta fraction of *Cl. welchii* Type B toxin can be titrated by this method and their procedure, slightly modified, was followed. For titrating the high value toxic filtrates it was necessary to maintain the temperature of the bath at 38°C because at 45°C flocculation occurred in less than one minute. Tubes measuring 75 × 8 mm. were used and the pH of the filtrates adjusted to 6·8. One millilitre amounts of toxic filtrate proved convenient.

When toxic filtrate was flocculated with natural serum in a concentration range round the *in vivo* neutral point several zones of flocculation could be detected. In each of these floccules formed in the contents of three or four tubes adjacent to the so-called " indicator tube ". The zones represented the combination of separate antigens with their corresponding antibodies. A toxic filtrate, for instance, which had a value of 76 L+ per ml. showed values of 30, 80, 100 and 150 Lf per ml. when flocculated with natural serum. This confused the issue to the extent that the use of crude serum had to be abandoned. With pepsin-refined antitoxin, however, only one zone of flocculation took place in a range of serum quantities varying from well below to well above the *in vivo* point of neutrality. To prove that this was a specific beta toxin-antitoxin zone the following experimental evidence is offered:—

(*a*) By applying the gel-diffusion precipitin technique of Pope (personal communication) only one precipitin line could be demonstrated. Undiluted antitoxin mixed with an equal volume of 2 per cent gelatin in 0·8 per cent NaCl solution was pipetted to the bottom of a 75 × 8 mm. tube to form a column 1 cm. high. This was left in a refrigerator to solidify and then a 1 cm. layer of clear 1 per cent gelatin in 0·8 per cent NaCl solution was layered on top of the antitoxin-gelatin column and the tube returned to the refrigerator. The toxic filtrate was then layered on top of the gelatin and the tube left in a standing position in a refrigerator at 4°C. A single precipitin line formed after four days and no more appeared in three weeks. Undoubtedly more lines would have formed if the antigen and antibody had been concentrated, but they were used in the same concentrations as in the flocculation test.

When the toxic filtrate was flocculated with the antitoxin at a level corresponding to the neutral point of the same reagents as determined by intravenous titration in mice and the floccules removed before the solution was put into the gel-diffusion system no line resulted. In the determination of the neutral point in mice the alpha toxin contained in the filtrate (the strain of *Cl. welchii* Type B used produces only two lethal factors, viz. alpha and beta toxins), was neutralized by an alpha antitoxic serum containing no beta antitoxin.

This evidence indicates that the same factor in the toxic filtrates is responsible for the precipitin line, flocculation reaction and neutralization of the beta antitoxin in the refined antiserum used.

(*b*) One hundred ml. of toxic filtrate were flocculated at a level as described in (*a*). The floccules were separated from the liquid by centrifugation, washed three times with physiological saline and then re-suspended in saline which was made up to 100 ml. Each of ten guinea-pigs received subcutaneously 5 ml. of the floccule suspension and the injection was repeated after three weeks. Ten days after the second injection their resistance to injections of beta toxin was tested by the Cumulative Minimal Lethal Dose test as described by Mason, Ross & Dalling (1931). A dried toxin prepared from a culture of the " 1930 variety " of *Cl. welchii* Type B by means of ammonium sulphate precipitation was used as test material. Before injection, however, the lecithinase activity of the redissolved dried toxin was neutralized with an anti-alpha serum, with the result that the beta fraction only was responsible for the mortality. The results are summarized in Table 2.

TABLE 2.—*The Resistance to Beta Toxin of Guinea-pigs having received injections of Suspended Floccules*

Guinea-pig	*M.L.D. withstood*
1	16 but less than 32
2	16 but less than 32
3	16 but less than 32
4	16 but less than 32
5	16 but less than 32
6	16 but less than 32
7	32 but less than 64
8	32 but less than 64
9	32 but less than 64
10	32 but less than 64

These results prove that the injection of the floccules was responsible for a resistance against the lethal effect of beta toxin.

(*c*) The value of each of a series of 33 different toxic filtrates was determined in terms of units by means of the flocculation test. Simultaneously their L+ values were determined against the same antitoxin by titration in mice after neutralization of the alpha toxin. These tests were done while the filtrates were fresh and the following results were obtained:—

Lf values (*Lf per ml.*)	*L+ values* (*L+ per ml.*)	*Lf values* (*Lf per ml.*)	*L+ values* (*L+ per ml.*)
38	34	560	540
33	30	52	46
43	36	38	34
38	38	38	34
67	60	47	46
81	76	38	34
105	105	47	40
105	98	38	38
76	70	33	30
67	60	105	110
38	46	105	114
57	48	123	114
275	260	114	114
199	190	123	114
171	162	85	78
162	154	95	78
170	163		

The value of chi-square for these two series of numbers is smaller than the value at $P = 0{\cdot}05$ (P in the vicinity of $0{\cdot}8$) indicating a satisfactory agreement between these two methods of determining the value of a toxic solution in terms of its antitoxin-binding power.

(*d*) Moloney & Hennessy (1944) used the property of floccules from true toxin-antitoxin combination to absorb toxin and antitoxin as against the inability of floccules from false zones to do the same in the preparation of a single zone tetanus antitoxin. This antitoxin was proved by various other means to measure the true toxin or toxoid content of a filtrate.

On the same basis floccules were prepared from 50 ml. of a clarified toxic filtrate (*vide supra*). The floccules were spun down and the supernatant tested for residual toxic or antitoxic activity; neither could be demonstrated. The packed floccules were washed twice in saline and the final supernatant discarded. Finally half the floccules were suspended in 10 ml. of the original toxic filtrate (277 Lf per ml.) and the rest in 3 ml. of concentrated antitoxin (9,500 units per ml.). After leaving the mixtures at room temperature for one hour the floccules were removed by centrifugation and the values of the toxic and antitoxic supernatants respectively determined. Fifty units of toxin per ml. and 7,125 units of antitoxin per ml. remained.

The conclusion that the floccules represent a true toxin-antitoxin combination seems justified.

Glenny & Okell (1924), working with diptheria toxin and antitoxin, confirmed Ramon's observation that the flocculation test could also be used to assay a toxin modified by chemical means, i.e. a toxoid. Mason & Widdicombe (1946) applied it to the titration of formol beta toxoid using pepsin-refined antitoxin.

To establish the applicability of the flocculation test as a means of titrating the toxoids prepared in the present series of experiments eight toxoids were prepared by formolizing selected toxins of different Lf values. Subsequently the flocculation value of these formol-toxoids was determined by blending with a quick-flocculating toxin and also their total combining power (Mason, 1935b).

The results summarized in Table 3 show that the toxoids are placed in the same order of strength by the two methods.

TABLE 3.—*Comparison between the Total Combining Power and Flocculation Value of Toxoids*

Total Combining Power	*Flocculation Value*
Units	*Lf*
165	57
200	100
350	151
400	160
460	171
480	266
500	276
630	380

Mason & Widdicombe (1946) state that " Formol-toxoid, used to prevent lamb dysentery, may be titrated with some accuracy by ascertaining its antitoxin-binding power. However, the true value of the toxoid is not necessarily obtained because toxin is, as a rule, more avid than toxoid and may throw some toxoid out of combination with the antitoxin and take its place, thus giving the impression that the toxoid has a lower binding value than it really has."

On the strength of the author's experience with the total combining power test, he fully agrees with this statement and feels that the absence of a constant relationship between the values as determined by the total combining power method and the flocculation method for the respective toxoids can be accounted for by the relative inaccuracy of the former method.

In view of the reported experimental evidence and the wide application of the flocculation test it is felt that this test can be used to assay the beta fraction of *Cl. welchii* Type B toxin and its corresponding antitoxin.

4. *The Selection of a Strain of* Cl. welchii *Type B Suitable for the Production of Beta Toxin*

In conducting the studies on beta toxin a strain of *Cl. welchii* Type B designated the " 1930 strain " by Mason (1935a) was used. While the standard *Cl. welchii* Type B produces beta toxin as its main antigenic fraction as well as epsilon toxin the " 1930 strain " has lost its power to produce the epsilon fraction. Mason mentioned that this variety did produce alpha toxin as a subsidiary constituent and during the course of its investigation it was found that the organism also produces some hyaluronidase when cultured for five hours in meat broth containing 1 per cent peptone

The advantages of using this strain for the study of beta toxin are the lack of an epsilon fraction which would complicate the experiments, and the absence of proteolytic enzymes which would affect the stability of the filtrates. On cultivation in liquid media, however, a sticky growth resulted due to the production of mucus which considerably impeded filtration and processing of the toxic material.

After 24 hours' growth on the surface of serum-agar in an anaerobic jar the following morphologically different colonies could be distinguished:—

(*a*) *Smooth colonies* (Fig 1).—Dome-shaped with sharp, regular margins. Glistening white, smooth appearance. These colonies are remarkably stringy and, when subcultured into liquid media, a mucoid growth results.

The organisms comprising the smooth colonies are toxigenic and display a wide capsule when stained supravitally in wet films with India ink.

On serial passage of the parent strain in liquid media these mucoid organisms appear in increasing proportions and oust the other types to be described below.

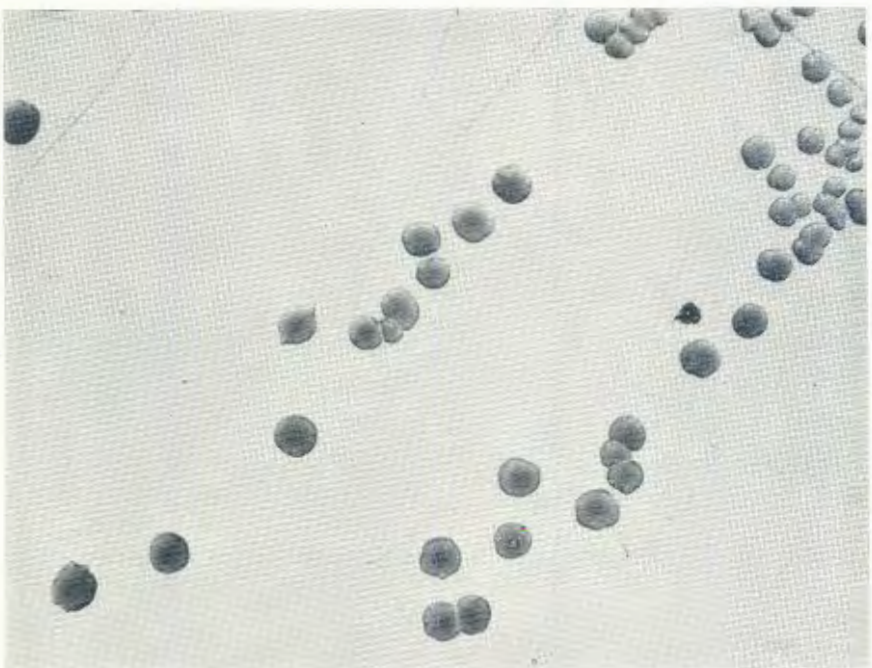

FIG. 1.—Smooth Colonies produced by Organisms isolated from a Culture of *Cl. welchii* Type B (" 1930 Strain ")

(*b*) *Colonies composed of non-toxigenic organisms* (Fig. 2).—These are flatter than the smooth variety and have regular margins. They have a dull white appearance.

The organisms are non-capsulated and, when grown in meat broth, could not be shown to produce beta toxin by blending with a quick-flocculating toxin (Glenny & Okell, 1924). They, however, have the following characteristics which identify them as *Cl. welchii*:—

Short, straight, stout rods with parallel sides arranged singly or in small bundles. Gram-positive. Gelatin is liquified but not coagulated serum. A typical " stormy fermentation " reaction is produced in litmus milk. Glucose, lactose, maltose and inulin are fermented, but not mannitol and dulcitol. Alpha toxin is produced.

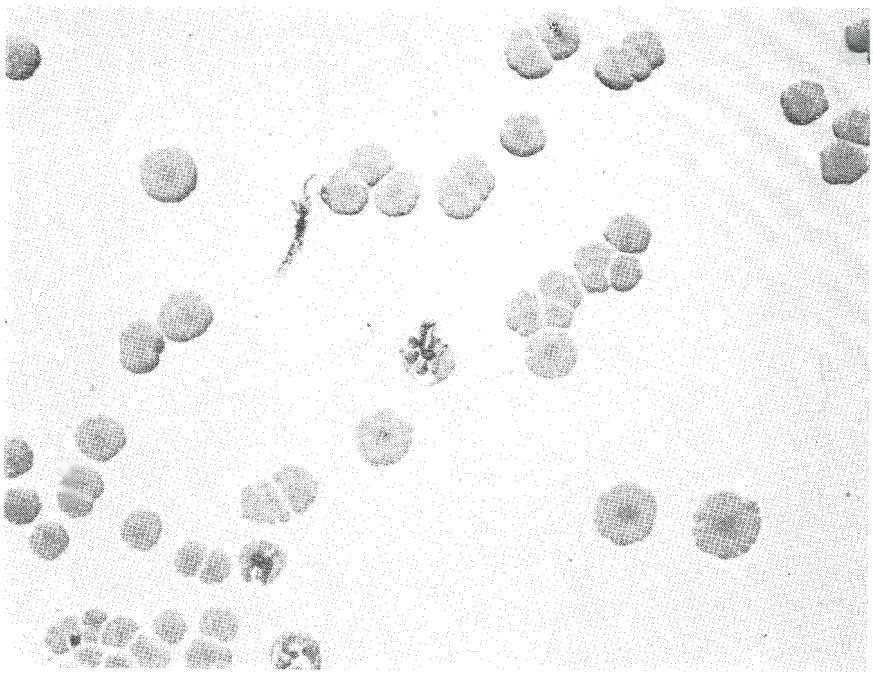

FIG. 2.—Appearance of Colonies produced by Non-toxigenic Organisms isolated from a Culture of *Cl. welchii* Type **B** (" 1930 Strain ")

(*c*) *Rough colonies* (Fig. 3).—Flat colonies with very irregular and roughened margins. Irregular surface and a pronounced mat appearance. The organisms are not-capsulated and produce beta toxin in liquid media. The culture fluid is not sticky.

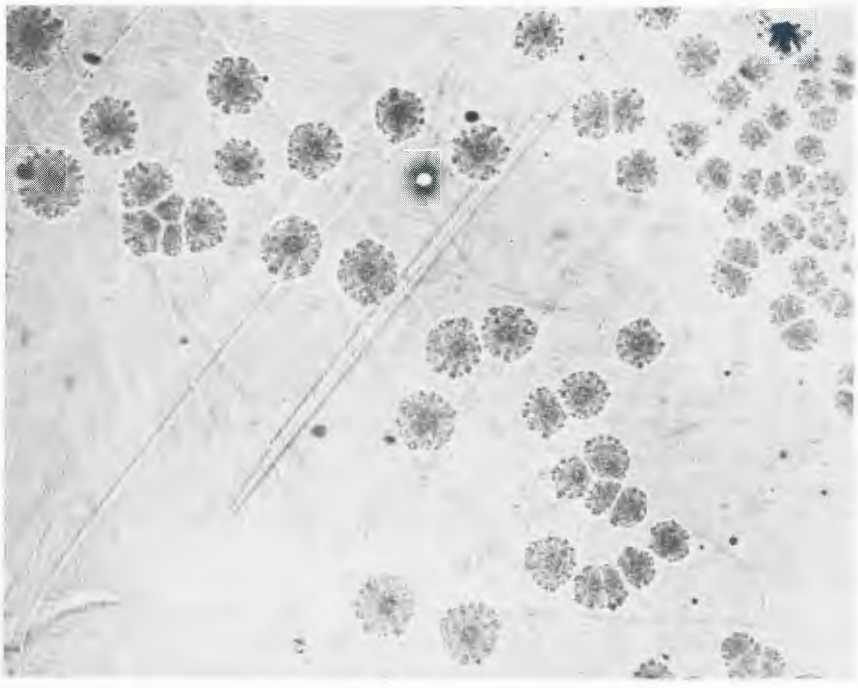

FIG. 3.—Appearance of Rough Colonies produced by Organisms isolated from a Culture of *Cl. welchii* Type B (" 1930 Strain ")

Because the rough variant did not produce sticky capsules it was selected for the production of toxin in these investigations. Its tendency to give rise to further mutants was examined by subculturing it twenty times in Robertson's meat broth containing one per cent glucose. The original colony morphology and toxigenicity were retained. However, after the same number of serial passages in Robertson's meat broth without glucose a mutant did appear when the culture at this stage was grown anaerobically on the surface of serum-agar for 24 hours; these mutants could be detected by their more regular margins and smoother surfaces. These colonies were not stringy when picked while the individual organisms were non-capsulated and toxigenic.

Similar dissociation was described by Stevens (1935) and McGaughey (1933) working with *Cl. welchii* Type A.

In view of the variability of this strain of *Cl. welchii* Type B, stock cultures were prepared by cultivating selected rough colonies in Robertson's meat broth containing 1 per cent glucose and lyophilizing the resulting growth in 0·5 ml. quantities. A fresh ampoule of dried culture was used to start the production of each batch of toxin.

The Production of Beta Toxin

Initially beta toxin was produced according to the methods employed at the Onderstepoort Laboratories for the preparation of lamb dysentery vaccine. The medium consisted of a broth prepared by boiling minced horse meat in tap water for 30 minutes (1 litre of water to 1 lb. of meat). The following ingredients * were added:—

peptone	1·0 per cent
sodium chloride	0·3 per cent
dibasic sodium phosphate	0·2 per cent.

The pH was adjusted to 7·8.

Ten-litre volumes of the liquid portion of the medium were dispensed in twelve-litre flasks and sterilized at 120°C for 2½ hours.

After sterilization the medium was left to cool and then placed in an incubator room at 37°C to attain the desired temperature.

The same medium was used in 30 ml. quantities in tubes for growing the inoculum.

Before inoculation the pH was adjusted to 7·4 with sterile 40 per cent NaOH, since sterilization usually caused a drop in the pH of the medium. A final concentration of 0·5 per cent glucose was added in the form of a sterile 60 per cent solution. After being inoculated with about 30 ml. of actively growing culture each, the flasks were incubated at 37°C for 17 hours.

Values ranging from 38 to 57 Lf per ml. were obtained by this method. Subsequently the following attempts at increasing the yield of toxin were made:—

(*a*) *Increasing the peptone content of the medium*

To test the influence of the peptone content of the medium on toxin production an experiment was planned in which the peptone content of the medium described above was varied from 0·5 to 2·5 per cent. After adjustment of the pH and addition of glucose the flasks were inoculated with actively growing culture and left to grow for 4½ hours. By this time active gas production had ceased.

The values obtained were as follows:—

Peptone content	*Lf per ml.*
0·5 per cent	28·5
1·0 per cent	38·0
1·5 per cent	57·0
2·0 per cent	76·0
2·5 per cent	76·0

The conclusion that an increased peptone content up to 2 per cent leads to an enhanced toxin production seems justified.

(*b*) *Finding the period of growth consistent with maximal toxin production*

For this purpose a flask of the original medium with the incorporation of 2 per cent peptone was inoculated with the experimental strain of *Cl. welchii* Type B in the method described and incubated at 37°C. Commencing at 3½ hours after inoculation,

* When in this thesis an ingredient is added in a concentration of, say, 1 per cent, this must be taken to indicate that sufficient of the ingredient is added to give a final concentration of 1 per cent.

when profuse gas production was obvious, samples of culture were withdrawn at 30 minute intervals, clarified and their Lf value determined. After 5½ hours of growth gas production had almost ceased and sampling was stopped. The following results were obtained:—

Duration of growth	*Lf per ml.*
3½ hours	76·0
4 hours	104·5
4½ hours	76·0
5 hours	66·5
5½ hours	38·0

From the above data it can be concluded that the toxin content of the culture fluid increases up to 4 hours after inoculation but decreases subsequently. This phenomenon was confirmed repeatedly and in the experiments that followed the toxic fluid was regularly harvested after 4 hours' cultivation.

(*c*) *The presence of meat particles in the medium*

The production of toxin in liquid medium with and without the addition of meat particles was compared. To prepare the former sufficient meat particles, which had been cooked for the preparation of the medium as described, were placed in flasks to fill one-fifth of their total volume. Then 8 litres of liquid medium were added. The control flasks contained ten litres of liquid medium only. In both cases twelve-litre production flasks were used and the medium contained 2 per cent peptone.

Samples were withdrawn after 4 hours' growth at 37°C and the Lf value of the toxin determined. From Table 4, which summarizes the results of four separate comparisons, it can be seen that higher yields of toxin were consistently obtained in medium containing meat particles.

TABLE 4.—*The comparison of Toxin Production in Medium with and without Meat Particles*

Medium without meat particles Lf per ml.	*Medium with meat particles Lf per ml.*
104·5	275
76·0	199
66·5	171
38·0	161

(*d*) *The addition of glucose to the medium*

To follow the effects on toxin production of the addition of glucose to the medium a batch was prepared containing 2 per cent peptone and meat particles were added to the contents of the production flasks as described in (*c*) above. Four flasks of medium were used containing, respectively, 0, 0·5, 1·0 and 2·0 per cent glucose. A sterile 60 per cent glucose solution was added to the medium after sterilization to give the final concentrations required. The flasks were inoculated with 30 ml. of actively growing culture each and incubated for 4 hours at 37°C. The flocculation value of the cultures was then determined.

As is shown in Table 5 the addition of glucose stimulates toxin production and the maximal effect is reached with one per cent.

TABLE 5.—*The influence of Glucose on Toxin production*

Percentage Glucose added	*Lf per ml. attained*
0	285
0·5	380
1·0	427·5
2·0	427·5

(*e*) *The sterilization of the medium*

The complete medium as developed in the steps mentioned, except for the addition of glucose, was made up in twelve-litre flasks and the contents of different flasks sterilized at 120°C for progressively increasing periods. After sterilization 1 per cent glucose, which had been sterilized by tyndallization, was added and inoculation and incubation carried out as before. The values of the toxic culture in the different flasks are given in Table 6.

TABLE 6.—*The influence on Toxin production of Sterilization of the Medium at 120°C for varying periods*

Period of sterilization	*Lf per ml.*
30 minutes	427·5
1 hour	332·5
2 hours	332·5
3 hours	332·5
4 hours	237·5

A feasible explanation for the decrease in toxin production with increased sterilization of the medium would be that some essential growth factors are destroyed by prolonged heating.

In the experiments described below sterilization was carried out routinely at 120°C for 1½ hours since a shorter period did not consistently yield a sterile product.

(*f*) *Varying the hydrogen-ion concentration of the medium*

Medium containing 2 per cent peptone was prepared and meat particles were included in the flasks. The pH of the medium in three flasks was adjusted to 6·6, 7·0 and 7·4 respectively. One per cent glucose was added and inoculation of the medium was carried out as usual. After one hour's incubation at 37°C. the pH of the three cultures was measured and readjusted to the original level where necessary. This was repeated every 30 minutes until four hours of growth had been completed. In every case the pH dropped to the acid side during growth. The Lf values of the cultures were determined and recorded in Table 7.

TABLE 7.—*The production of Beta Toxin at different pH Levels*

pH level	*Toxin value*
6·6	350 *Lf. per ml.*
7·0	522 *Lf. per ml.*
7·4	427 *Lf. per ml.*

Because of these results the effect of buffering the medium at pH 7·0 was investigated.

According to Glasstone (1953) a mixture containing a proportion of 0·62 mole of Na_2HPO_4 to 1 mole of KH_2PO_4 will provide a buffer solution of considerable buffer capacity around pH 7·0. The strength of the buffer is dependent on the concentrations of the salts. As a high concentration of buffer salts (Na_2HPO_4 and KH_2PO_4) can inhibit the growth of a microbe, an experiment was carried out to find the maximal amount of these salts in a culture medium that could be added to permit good growth and maintain the desired pH.

Increasing amounts of the two salts in the same proportion were added to twelve-litre flasks each containing 10 litres of medium which was prepared without the addition of any electrolytes. Two per cent peptone was included and the pH checked after sterilization. After the addition of 1 per cent glucose the flasks were inoculated and the growth harvested after four hours. The value of the toxic filtrate in terms of Lf per ml. was determined for each flask.

TABLE 8.—*Value of Toxin produced in the presence of Increasing concentrations of Buffer Salts*

Flask No.	$Na_2HPO_4.12H_2O$	KH_2PO_4	Molarity $Na_2HPO_4.12H_2O$ / Molarity KH_2PO_4	Floc. value (Lf/ml.)	pH after growth
	gm.	gm.			
1.....	35·1	17·0	·0077/·0125	427	6·2
2.....	28·1	13·6	·0062/·01	475	6·2
3.....	21·0	10·2	·0046/·0075	427	6·1
4.....	14·0	6·8	·0031/·005	427	6·0
5.....	7·0	3·4	·0015/·0025	380	5·8
Control...	—	—	—	285	5·4

From Table 8 can be seen that the addition of 28·1 gm. $Na_2HPO_4 \cdot 12H_2O$ and 13·6 gm. KH_2PO_4 to 10 litres of medium produced the best results.

To investigate if the addition of buffer as well as adjustment of the pH during growth would produce even better results a similar experiment was planned differing only in so far as the pH value of the growth was adjusted to pH 7 at 2, 2½, 3 and 3½ hours respectively after inoculation of the flasks.

TABLE 9.—*The effect on Toxin production of the presence of Buffer Salts in the Medium and repeated adjustment of the pH to* 7·0

Flask No.	$Na_2HPO_4.12H_2O$	KH_2PO_4	Floc. value (Lf/ml.)
	gm.	gm.	
1.....	35·1	17·0	475
2.....	28·1	13·6	522
3.....	21·0	10·2	522
4.....	14·0	6·8	560
5.....	7·0	3·4	475
Control.....	—	—	360

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From the results obtained with flask No. 4 (see Table 9) it can be concluded that a higher yield of toxin can be obtained by maintaining the hydrogen-ion concentration of the culture as close to neutrality as possible.

In Table 8 the medium containing 28·1 gm. Na_2HPO_4 and 13·6 gm. KH_2PO_4 has produced the highest yield of toxin while in Table 9 the medium containing exactly half this concentration of salts has given the highest yield. This difference is explained by the fact that addition of NaOH to the medium containing a high concentration of salts results in an excessive concentration which is unfavourable to bacterial growth.

On the evidence that the addition of glucose stimulated toxin production but simultaneously lowered the pH of the medium due to acid end-products it was felt that the addition of concentrations higher than 1 per cent to the buffered medium should be investigated. Horse meat broth was used with the addition of the following ingredients:—

peptone	2 per cent
$Na_2HPO_4.12H_2O$	14 gm. per 10 L.
KH_2PO_4	6·8 gm. per 10 L.

Meat particles were added to the contents of the flasks as described.

Four flasks of medium were used containing, respectively, 0·5, 1·0, 2·0 and 3·0 per cent glucose. During incubation the pH of the cultures was readjusted to 7·0 after 2, 2½, 3 and 3½ hours. After 4 hours' growth the Lf values of the cultures were determined. The degree of toxicity in the culture containing 1 per cent glucose was the same as in the cultures containing higher concentrations. No advantage was therefore gained by adding more than 1 per cent glucose.

By applying the results of the experiments reported above the following procedure was adopted for the production of beta toxin for experimental purposes:—

Meat extract is made by boiling minced horse flesh in water for 30 minutes (1 lb. of flesh to 1 l. water). To 10 l. of extract are added—

peptone	200·0 gm.
$Na_2HPO_4.12\ H_2O$	28·1 gm.
KH_2PO_4	13·6 gm.

This medium is siphoned in 10 l. quantities into twelve-litre flasks containing the boiled meat particle residue (about one-fifth by volume). The medium is sterilized at 120°C for 1½ hours. After cooling the medium is transferred to an incubator at 37°C to attain the desired temperature.

Tubes containing about 30 ml. of the same medium are also prepared. They are used for growing the inoculum.

Before the production medium is inoculated its pH is adjusted, if necessary, to 7·0. Then a sterile 60 per cent glucose solution is added to give a final concentration of 1·0 per cent.

Each flask of medium is inoculated with 30 ml. of actively growing culture and incubated at 37°C for 4 hours. The liquid portion of the culture is collected, preserved with 1: 5,000 merthiolate and stored at 4°C pending further processing.

In spite of meticulous adherence to the described procedure some variation in total yield of toxin from batch to batch was experienced.

The Purification of Beta Toxin

Toxic cultures were clarified in a Sharple's centrifuge and subsequent filtration by suction through a layer of diatomaceous earth in a Buchner funnel. The toxin in the filtrate was then precipitated by the addition of 40 gm. dry ammonium sulphate to 100 ml. filtrate, and leaving the mixture overnight. The protein precipitate settled to the bottom under these conditions and a large portion of the clear supernatant liquid could be siphoned off without disturbing the precipitate. The residual supernatant and the precipitate were spun and the packed material in the bottom of the centrifuge tubes dialyzed against distilled water at 4°C. until free of ammonium sulphate. The dialyzed toxin concentrate was centrifugalized to remove insoluble material and its Lf value determined. The protein-nitrogen content was determined by the Kjeldahl method using trichloracetic acid as precipitant.

At this stage the toxin was ready for further purification by one of the two methods described below.

(1) *Ammonium sulphate fractional precipitation*

In each of 6 × 100 ml. samples of concentrate at pH 5·5, 6·0, 6·5, 7·0, 7·5 and 8·0 respectively 15 gm. ammonium sulphate was dissolved. The pH was, where necessary, adjusted to the levels desired by the addition of N NaOH or N HCl. The precipitates were collected 18 hours later and 2·5 gm. ammonium sulphate added to the supernatants. The pH was again adjusted where necessary. This fractionation was continued until the further addition of ammonium sulphate produced no precipitation.

All precipitates were dialyzed free of ammonium sulphate, the volume of the concentrates measured, and the Lf and nitrogen values determined.

An example of the results obtained by this method of fractionation is illustrated in Table 10.

TABLE 10.—*Results of Ammonium Sulphate Fractional Precipitation of Toxic Filtrate*

Volume of original toxic filtrate	14 l.
Value of original toxic filtrate	251 Lf per ml.
Protein-nitrogen	0·5 mg. per ml.
Lf per mg. protein-nitrogen	503.
Volume of concentrated toxin adjusted to 1 litre after dialysis.	
Volume of samples fractionated	100 ml.

Each sample represents a total of 351,400 Lf.

pH 5·5

Percentage $(NH_4)_2SO_4$	Volume after dialysis	Lf per ml.	P.N. per ml.	Lf per mg. P.N.
	ml.		mg.	
15·0	8·0	40	0·061	670
17·5	10·0	101	0·310	324
20·0	6·0	905	0·300	3,000
22·5	18·0	1,006	0·270	3,723
25·0	16·0	137	0·211	650
27·5	14·0	50	0·170	295

Total Lf recovered: 27,760.

TABLE 10 (*continued*)

pH 6·0

Percentage $(NH_4)_2SO_4$	Volume after dialysis	Lf per ml.	P.N. per ml.	Lf per mg. P.N.
	ml.		mg.	
15·0	8·0	12	0·028	426
17·5	12·0	24	0·028	851
20·0	8·0	180	0·225	800
22·5	10·0	719	0·351	2,047
25·0	9·0	240	0·37	647
27·5	14·0	48	0·324	148
30·0	13·0	24	0·33	72
32·5	8·0	24	0·292	82
35·0	10·0	12	0·171	70
37·5	3·0	12	0·086	138

Total Lf recovered: 12,269

pH 6·5

Percentage $(NH_4)_2SO_4$	Volume after dialysis	Lf per ml.	P.N. per ml.	Lf per mg. P.N.
	ml.		mg.	
15·0	24·0	121	0·304	397
17·5	13·0	121	0·225	536
20·0	4·4	62	0·291	213
22·5	8·5	2,098	1·66	1,264
25·0	20·0	1,357	0·979	1,385
27·5	10·0	741	0·390	1,898
30·0	24·0	121	0·460	263
32·5	12·0	49	0·280	175
35·0	32·0	25	0·121	206
37·5	14·0	12	0·058	206
40·0	8·0	12	0·048	247

Total Lf recovered: 61,653

pH 7·0

Percentage $(NH_4)_2SO_4$	Volume after dialysis	Lf per ml.	P.N. per ml.	Lf per mg. P.N.
	ml.		mg.	
15·0	26·0	121	0·264	457
17·5	11·0	121	0·238	509
20·0	6·0	93	0·160	579
22·5	9·0	2,467	1·210	2,039
25·0	10·0	1,110	0·579	1,914
27·5	13·0	37	0·924	40
30·0	16·0	37	0·370	100
32·5	24·0	25	0·423	59
35·0	14·0	12	0·097	123
37·5	16·0	12	0·089	134

Total Lf recovered: 40,642

TABLE 10 (*continued*)

pH 7·5

Percentage $(NH_4)_2SO_4$	Volume after dialysis	Lf per ml.	P.N. per ml.	Lf per mg. P.N.
	ml.		mg.	
15·0	4·0	24	0·289	83
17·5	11·0	48	0·089	547
20·0	9·0	216	0·460	468
22·5	12·0	477	0·140	3,406
25·0	12·0	178	0·217	818
27·5	16·0	60	0·343	175
30·0	16·0	48	0·320	149
32·5	14·0	48	0·294	163
35·0	10·0	24	0·050	477
37·5	3·0	12	—	—

Total Lf recovered: 13,104.

pH 8·0

Percentage $(NH_4)_2SO_4$	Volume after dialysis	Lf per ml.	P.N. per ml.	Lf per mg. P.N.
	ml.		mg.	
15·0	20·0	101	0·098	1,029
17·5	10·0	101	0·090	1,117
20·0	8·0	603	0·380	1,588
22·5	10·0	1,106	0·342	3,254
25·0	20·0	201	0·160	1,251
27·5	8·0	251	0·232	1,092
30·0	6·0	40	0·180	223

Total Lf recovered: 25,182.

From the results in Table 10 the following points become clear:—

(*a*) The toxic fraction of the filtrate is not precipitated at one particular level of ammonium sulphate concentration, but over a range from 15 to 40 gm. per 100 ml. This excludes ammonium sulphate fractional precipitation from being a practical method of producing a toxin of higher purity on a large scale.

(*b*) The highest yield of total toxin recovered was in the case of the sample fractionated at pH 6·5. This low yield of 17·55 per cent of the original further excludes this method from practical application.

(*c*) Taking the purification factor as—

$$\frac{\text{Number of Lf per mg. P.N. in purified fraction}}{\text{Number of Lf per mg. P.N. in parent filtrate}}$$

the highest figure attained was at pH 5·5 in the fraction 20 to 22·5 gm. ammonium sulphate per 100 ml., viz. 7·4.

(2) *Purification of beta toxin by employing an acetic acid–sodium acetate buffer*

In the course of an investigation into the effect of low pH values in solutions of known ionic strength on beta toxin it was found that a high degree of purity was obtained at certain levels.

By utilizing a nomogram for acetate buffers (Boyd, 1945) and the buffer tables drawn up by Green (1933) the following sets of conditions were effected in samples of the dialyzed concentrated toxin solution:—

(1) pH 4·8; ionic strength 0·2.
(2) pH 4·9; ionic strength 0·35.
(3) pH 5·0; ionic strength 0·6.
(4) pH 5·1; ionic strength 1·1.
(5) pH 5·2; ionic strength 1·5.
(6) pH 5·3; ionic strength 2·0
(7) pH 5·4; ionic strength 2·4.
(8) pH 5·5; ionic strength 5·6.

Analytical grade chemicals were used. The protein-nitrogen content of the concentrated toxin was adjusted to a constant level in the whole series, viz. 2·5 mg. per ml., and the distilled water used for the dilution to this level was simultaneously employed for dissolving the calculated quantities of sodium acetate and acetic acid before addition to the toxin.

After the addition of the acetate and acetic acid the pH was checked and, if necessary, adjusted to the required level with 40 per cent NaOH solution or concentrated acetic acid.

The samples of toxin solution were then left at 4°C overnight during which time a precipitate formed. After centrifuging, the precipitate was discarded and the pH of the supernatant adjusted to 7·0. The flocculation value and the protein-nitrogen content of the supernatant were then determined. This purification procedure is illustrated by the following example:—

Value of crude toxin	251 Lf per ml.
Degree of purity of crude toxin	393 Lf per mg. P.N.
P.N. content of samples	2·5 mg. per ml.

pH	Ionic strength	Lf per mg. P.N.
4·8	0·2	827
4·9	0·35	1,233
5·0	0·6	1,372
5·1	1·1	2,857
5·2	1·5	6,286
5·3	2·0	5,029
5·4	2·4	4,373
5·5	5·6	479

At pH 5·2 and ionic strength 1·5 the purest product was obtained, viz. 6,286 Lf per mg. P.N. showing a purification factor of 15·9 over the crude toxin.

In this particular case the ammonium sulphate precipitation which preceded the purification was responsible for a loss of 23 per cent of the original toxin. The purification by means of the buffer at 4°C, however, did not cause any reduction in the flocculation value of the concentrated toxin. The conclusion appears to be justified that at pH 5·2, ionic strength 1·5, a large proportion of the non-toxin protein was precipitated, leaving the toxin in solution. Hence the purification.

The advantages of this method over fractional ammonium sulphate precipitation as a method for large scale production of a purer toxin are obvious.

By starting with a crude toxic filtrate of higher purity, i.e. 1,036 Lf per mg. P.N. an end-product containing 7,396 Lf per mg. P.N. was obtained by this method.

The keeping qualities of Beta Toxin

For the determination of the stability of liquid beta toxin a toxic culture was clarified as described (*vide supra*) and its Lf value established.

One hundred ml. samples were withdrawn and their pH adjusted to 5·0, 6·0, 7·0 and 8·0, respectively. After the addition of 1 per cent toluol the samples were left in an incubator at 37°C and at weekly intervals their Lf values were determined. The results are recorded in Table 11.

TABLE 11.—*The keeping qualities of Beta Toxin at different pH levels*

pH	Original value Lf/ml.	Value after 1 week	Value after 2 weeks
5·0	427·5	38·0	38·0
6·0	427·5	228·0	152·0
7·0	427·5	133·0	76·0
8·0	427·5	133·0	38·0

In general the deterioration of beta toxin at 37°C is very rapid but after two weeks it was obvious that pH 6·0 had the least detrimental influence. On the strength of this observation an experiment was arranged in which the pH of a clarified toxic filtrate was adjusted to 6·0, and 100 ml. samples were kept at 4°C, 20°C, 30°C, and 40°C, respectively, after preservation with 1 per cent toluol. The Lf value of the original filtrate was determined and the individual samples were flocculated at the intervals indicated in Table 12.

TABLE 12.—*The keeping qualities of Beta Toxin at different temperatures.*

Storage temperature	Original value Lf/ml.	Value after 1 week	Value after 2 months	Value after 3 months
4°C	304	304	266	247
20°C	304	266	247	247
30°C	304	209	209	209
40°C	304	152	38	38

These results point to the existence of a roughly inverse relationship between the stability of beta toxin and its storage temperature.

The opinion of Weinberg, Nativelle and Prévot (1937) that beta toxin is labile is confirmed by the above two experiments.

The Toxoiding of Beta Toxin

In view of the labile nature of liquid beta toxin, procedures for detoxification were investigated with the object of finding one which would result in minimal destruction. The point at which toxoiding was regarded as complete was reached as soon as 0·2 ml. of the filtrate failed to kill 18 to 20 gm. mice injected intravenously. Commencing the day after a particular method of toxoiding was applied to a solution the test was carried out daily, and since the lethal action of beta toxin is rapid the result could be known within two hours.

Eaton (1937) working with purified diphtheria toxin used hexamethylene tetramine to effect an extremely slow liberation of formaldehyde in the presence of the toxin, thus eliminating the destructive influence of free formaldehyde. An alkaline pH accelerated the process which was carried out at 35°C.

Since beta toxin is unstable at alkaline pH levels (Table 11) a solution of toxin containing hexamethylene tetramine was adjusted to pH 7·0. Both a concentration of 0·5 and 1·0 per cent of this compound was used at 30°C. and 37°C. As soon as the solutions were atoxic their antigen content was determined by flocculation and the results recorded in Table 13.

TABLE 13.—*Toxoiding of Beta Toxin by the action of Hexamethylene Tetramine*

Percentage	Temperature °C	Original value	Time taken for toxoiding	Final value
		Lf/ml.	days	Lf/ml.
1·0.....	37	228	8	38
0·5.....	37	228	8	28
1·0.....	30	228	19	76
0·5.....	30	228	19	76

From Table 13 can be concluded that increasing the percentage of hexamethylene tetramine from 0·5 to 1·0 per cent has no deleterious effect on beta toxin but that an increase in temperature from 30 to 37°C reduces the units of antigen considerably in spite of the longer incubation at the lower temperature.

Hexamethylene tetramine was not investigated further because, as Table 13 shows, it destroyed antigen considerably.

Goldie (1937) investigated the action of ketene on *Corynebacterium diphtheriae* toxin. Working with crude toxic filtrates and filtrates purified by dialysis, he reported that exposure to ketene destroyed a large proportion of their toxicity without affecting the Lf value. The loss of toxicity was proportional to the percentage of primary amino-groups blocked by the ketene. When, however, the treatment was continued until all toxicity was lost the Lf value was reduced to nil.

Employing the methods described by A. M. Pappenheimer Jnr. (1938) a clarified beta toxic filtrate was exposed to the action of ketene for varying periods. One litre of filtrate in a thin cellophane sausage casing was suspended in ten litres of 2M sodium acetate solution which acted as buffer. Ketene was bubbled through a sparger placed in the bottom of the cellophane bag and the buffer outside the bag was stirred continuously by an electric stirrer. During the reaction period the pH inside the bag never fell below pH 6·0. After bubbling for 10, 20 and 30 minutes, respectively, samples were withdrawn and dialyzed against distilled water at 4°C. The samples were tested for the presence of acetate by neutralizing a small volume with excess $CaCO_3$ powder, filtering and then adding a $FeCl_3$ solution to the filtrate. When positive a reddish brown colour developed due to the formation of a complex iron acetate. When free from acetate the Lf and toxicity of each sample were determined. The results are given in Table 14.

TABLE 14.—*The Toxoiding of Beta Toxin by exposure to Ketene*

Period of exposure to ketene	Lf/ml.	Toxicity
0	304	
10 minutes	218·5	
20 minutes	76	
30 minutes	28·5	

Meaning lethal for mouse in dose of 0·2 ml. intravenously

The considerable reduction in antigenic value following an insufficient exposure to effect complete detoxification excludes this method as a practical means of toxoiding toxic filtrates.

Because the above methods offered no distinct advantages over the use of formalin, it was decided to determine the conditions under which formolizing was least destructive to beta toxin. For this purpose a clarified toxic filtrate was used containing—

non-protein-nitrogen	7·28 mg./ml.
protein-nitrogen	0·64 mg./ml.
amino-nitrogen	0·78 mg./ml.
and	399 Lf/ml.

The amino-nitrogen content was determined by the copper method of Pope & Stevens (1939).

Volumes of 500 ml. were measured into separate Erlenmeyer flasks. The contents of groups of five flasks were adjusted to pH 5·0, 6·0, 7·0 and 8·0, respectively. Enough 37·0 per cent formaldehyde solution was added so that in each group the flasks of filtrate contained a final concentration of 0·5, 0·6, 0·7, 0·8 and 0·9 per cent formaldehyde, respectively. All flasks were placed in an incubator at 37°C and the detoxification of the filtrates tested daily. When 0·2 ml. (mouse i.v.) was not lethal, the Lf was ascertained. The results are recorded in Table 15.

TABLE 15.—*The effect of different concentrations of Formaldehyde on Beta Toxin at various pH levels*

Percentage Formaldehyde	pH level			
	5·0	6·0	7·0	8·0
0·5	114 (33 days)	Not detoxified after 2 months	190 (35 days)	209 (11 days)
0·6	152 (19 days)	Not detoxified after 2 months	228 (23 days)	209 (8 days)
0·7	228 (9 days)	228 (35 days)	190 (17 days)	171 (5 days)
0·8	228 (6 days)	266 (8 days)	190 (9 days)	171 (4 days)
0·9	266 (5 days)	266 (6 days)	152 (4 days)	133 (4 days)

(Unbracketed figures =Lf/ml.; 19 days, 9 days, etc. = day on which sample was non-toxic)

By repeating the experiment with three different toxic filtrates similar results were obtained allowing the following generalizations to be made:—

(1) Detoxification takes place more slowly at pH 6·0 than at the other levels tested, but less destruction of antigen ensues.

(2) An increase in the percentage formalin up to 0·9 per cent results in increasingly rapid detoxification at pH 5·0 and 6·0 with less destruction of antigen at the higher concentrations.

(3) At pH 7·0 and 8·0 a higher percentage of formalin causes more rapid detoxification but increased destruction of antigen takes place at the higher concentrations. This effect is more pronounced at pH 8·0 than at pH 7·0.

Since the best results were obtained at pH 6·0 the detoxification of beta toxin at this pH level was investigated at different temperatures. For this purpose 500 ml. quantities of the toxic filtrate used in the previous experiment were measured into three flasks. Formalin to give a final concentration of 0·8 per cent formaldehyde was added to each, the pH was adjusted to 6·0 and the flasks incubated at 30°C, 35°C and 40°C, respectively.

The results are recorded in Table 16.

TABLE 16.—*The effect of Formolization at different temperatures on Beta Toxin*

Temperature	Original value (Lf/ml.)	Value after toxoiding (Lf/ml.)	Time taken (days)
30°C	399	290	10
35°C	399	290	5
40°C	399	290	3

From Table 16 can be seen that 75 per cent of the original antigen was retained in the process of toxoiding at each of the three different temperatures but that the time taken at the lower temperatures was considerably longer than at 40°C.

For the formolization of toxic filtrates used in the experiments described below a concentration of 0·8 per cent formaldehyde per 0·78 mg. amino-nitrogen per ml. was employed at pH 6·0. Toxoiding was effected at 37°C.

The Formolization of Purified Toxin

For the toxoiding of purified toxin the conditions found most favourable in the case of a crude toxic filtrate were employed, viz., pH 6·0 and 37°C.

The optimal concentration of formaldehyde had to be found, however, since the animo-nitrogen content of the purified product was considerably lower than that of crude toxin. The results recorded in Table 17 illustrate the effect of increasing percentages of formaldehyde on purified toxin (toxoiding was taken as complete when 0·2 ml. injected intravenously failed to kill mice).

TABLE 17.—*The influence of increasing percentages of Formaldehyde on Purified Beta Toxin*

Flocculation Value: 910 Lf per ml.
Purity: 5,200 Lf per mg. P.N.
Amino-nitrogen content: 0·224 mg. per ml.

Percentage formaldehyde added	Period required for toxoiding	Floc. value of toxoid	Purity of toxoid Lf/mg. P.N.
	days	Lf/ml.	
0·1	10	Less than 10	Not determined
0·2	10	Less than 10	Not determined
0·3	10	Less than 10	Not determined
0·4	6	754	4,715
0·5	6	754	4,024
0·6	2	251	1,572
0·7	2	251	1,257

With concentrations of 0·1, 0·2 and 0·3 per cent formaldehyde incubation had to be continued for 10 days before the toxin lost its toxicity. In the process, however, the antigen was completely destroyed.

In the range of formaldehyde from 0·4 to 0·7 per cent the higher concentrations effected toxoiding in a shorter period but were responsible for an increased destruction of antigen and a decrease in purity of the final toxoid. A concentration of 0·4 per cent formaldehyde was optimal.

The keeping qualities of Beta Toxoid

Since the rate of deterioration of a toxoid determines the useful life of a prophylactic prepared from it, a study of the stability of beta toxoid was made.

Firstly, volumes of 500 ml. of formolized toxic filtrate were kept in an incubator at 37°C after adjustment of the pH of individual quantities to 5·0, 6·0, 7·0 and 8·0, respectively. The flocculation value of the toxoid was initially determined by blending with toxin and subsequently at intervals as indicated by Table 18.

TABLE 18.—*The effect of storage at different pH levels on Beta Toxoid (Values recorded as Lf/ml.)*

pH	Initial value	1 week	3 weeks	6 weeks	12 weeks	16 weeks
5·0	247	247	247	133	133	76
6·0	247	247	247	171	152	114
7·0	247	247	209	152	152	114
8·0	247	247	171	76	76	38

There was little, if any, difference in the stability at pH 6·0 and 7·0, both of which are more favourable than higher or lower values. On this evidence and for reasons discussed under " precipitation of the crude toxoid by potassium alum " (*vide infra*) pH 6·0 was chosen for further studies on the stability of the toxoid at varying temperatures. Before elaborating on this aspect, however, the influence of

neutralizing the excess formaldehyde (by means of sodium-meta-bisulphite) on the stability of the toxoid should be indicated. For the detection of free formaldehyde 0·3, 0·4 and 0·5 per cent, respectively, of sodium-meta-bisulphite (in 10 per cent solution) was added to 10 ml. samples of toxoid. The mixtures were left at room temperature for 30 minutes and then 1 ml. of a 5 per cent alcoholic solution of Dimedone (Hopkin & Williams, Ltd.) was added to each. In the tubes with free formaldehyde a white precipitate formed and from the reactions in the series of three tubes the quantity of sodium-meta-bisulphite required for addition to the original toxoid could be calculated. After neutralization of the excess formaldehyde 1 per cent toluol was added as preservative.

From an experiment run concurrently with the one last described the results at pH 6·0 are given in Table 19 for comparison and they leave no doubt that the keeping qualities of the toxoid are improved by neutralization of excess formaldehyde.

TABLE 19.—*The effect of neutralizing excess Formaldehyde on the stability of Beta Toxoid*

Interval	Excess formaldehyde neutralized	Excess formaldehyde not neutralized
	Lf/ml.	Lf/ml.
0	247	247
1 week	247	247
3 weeks	247	247
6 weeks	209	171
12 weeks	190	152
16 weeks	152	114

Secondly, the influence of prolonged storage of liquid toxoid at pH. 6·0 at temperatures of 4°C, 30°C and 37°C on its stability was investigated. After neutralization of excess formaldehyde in the bulk liquid toxoid toluol was added to a final concentration of 1 per cent and 200 ml. quantities put into sterile bottles for storage at the different temperatures. The bottles were closed with rubber stoppers to prevent loss of preservative and concentration of the toxoid by evaporation.

The flocculation value was determined initially and again at intervals as indicated by Table 20.

TABLE 20.—*The result of storing Beta Toxoid at different Temperatures at pH* 6·0
(The numbers in the columns indicate Lf per ml.)

Temperature	Original Value	3 mth.	6 mth.	9 mth.	12 mth.	15 mth.	18 mth.	21 mth.
4°C	171	142	133	133	133	133	95	76
30°C	171	142	133	114	76	57	0	0
37°C	171	142	95	76	38	38	0	0

The results show that after one year 23 per cent of the antigen at 4°C is lost, 56 per cent at 30°C and 78 per cent at 37°C. These data have their practical application in the determination of the expiry period of a prophylactic in relation to its beta toxoid content. The 30°C level was chosen intentionally as representing a temperature slightly higher than that to which a prophylactic would be exposed under South African field conditions.

Alum Precipitation of Beta Toxoid

An alum precipitate of a diphtheria toxoid has been shown to be a better antigen than liquid toxoid (Glenny, Buttle & Stevens, 1931; Glenny & Barr, 1931; Glenny, 1930; Holt, 1950). Similar conclusions were arrived at in the case of tetanus toxoid (Glenny, 1930; Pillemer, Grossberg & Wittler, 1946), *Cl. welchii* epsilon toxoid (Thomson & Batty, 1953; Muth & Morrill, 1946) and *Cl. chauvoei* anaculture (J. R. Scheuber: personal communication). Levine, Stone & Wyman (1955) working with diphtheria and tetanus toxoids stated that the effect of an aluminium adjuvant depended on the absorption of toxoid on the surface of the precipitate. Since there appeared to be no reason why the position should be different in the case of beta toxoid, an experiment was planned to determine the conditions under which the largest quantity of a toxoid is removed from solution by alum precipitation.

The flocculation value of a toxoid prepared from a clarified toxic filtrate was found and 500 ml. quantities were measured into separate flasks. To groups of three flasks each a sterile 7·5 per cent potassium-alum solution was added with sterile precautions to give final concentrations of 0·5, 1·0, 1·5, 2·0, 2·5 and 3·0 per cent alum, respectively, per group. The pH of the material in the first flask of each group was adjusted to 5·0, the second to 6·0 and the third to 7·0. After the mixtures had been left at room temperature overnight (following the method of Barr, Pope, Glenny & Linggood, 1941) a sample from each flask was centrifuged until the supernatant was clear. The flocculation value of the supernatant from every sample was then found and the results recorded graphically in Fig. 4. Corrections were made for the dilution effected by the addition of the concentrated alum solution.

From Fig. 4 it can be seen that the precipitation of toxoid at pH 5·0 is more complete than at pH 7·0. At all pH levels the amount of toxoid precipitated increases with the increase in concentration of alum.

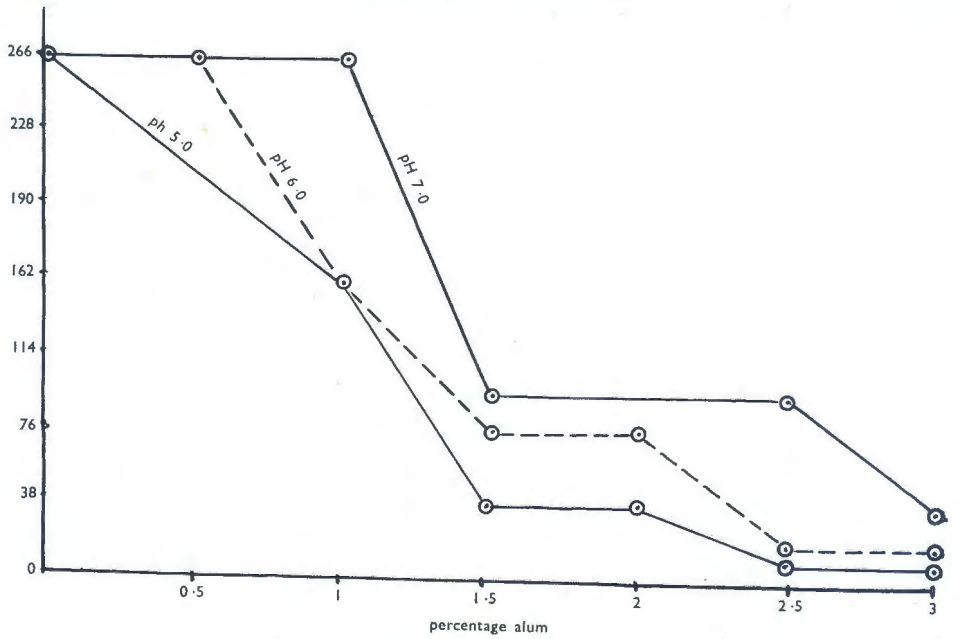


FIG. 4.—Graph showing the Lf values of the Supernatant of a Toxoid after the addition of Increasing Concentrations of Potassium Alum at pH 5·0, 6·0 and 7·0 respectively

For the preparation of an alum-precipitated toxoid to be used in the experiments about to be recorded a concentration of 1·5 per cent alum at pH 6·0 was selected. This concentration is as effective as 2·0 per cent, while mixtures containing more alum cause severe lameness when they are injected into animals. A pH value of 6·0 favours the storage properties of beta toxoid while an alum precipitate at a level below pH 6·0 causes pain when injected subcutaneously or intramuscularly (Levine *et al.*, 1955).

The production of an Immunity against Beta Toxin

Experimental Animals

Sheep were chosen as experimental animals so that the results could be applied directly to antigen production for large scale immunization in the field. The animals were obtained from a lamb dysentery-free area and had not been immunized against the disease. However, so that none be included that had developed a natural immunity, only those were used whose sera, in a dose of 0·2 ml., did not neutralize 1 minimal reacting dose of beta toxin tested intracutaneously in rabbits.

Preparation of the Antigen

The toxoid was prepared from a toxic filtrate and was diluted with broth so that 2·5 ml., the dose chosen for immunisation purposes, would contain the desired number of Lf. Potassium alum, in the form of a 7·5 per cent solution was added to make a 1·5 per cent concentration and the pH adjusted to 6·0. When toxoid without alum was used it was diluted in broth so that 2·5 ml. contained the required number of Lf and the pH was adjusted to 6·0.

The sheep were bled five times at weekly intervals. The first sample was taken seven days after the injection where one injection only was given and seven days after the second injection where two injections were given.

It is generally accepted that the logarithm of the individual antitoxin titres, rather than the titres themselves, are normally distributed in a group of animals responding to a dose of toxoid; and in the experiments reported below the logarithms of the titres recorded in the tables were used for graphical representation and statistical analysis.

A comparison of the mean log titre and the log median titre for the groups of animals for which the former could be calculated showed, as could be expected, a reasonably close agreement. The log median titre was thus accepted for graphical recording in the case of groups for which no mean log titre could be calculated because one or more of the individual titres was recorded as O.

Immunity produced by a primary stimulus of alum-precipitated toxoid and of liquid toxoid

For the comparison between the immunity produced by alum-precipitated beta toxoid (APT) and liquid toxoid six groups of ten sheep were used. Three groups received injections of 50, 100 and 200 Lf respectively of APT and the remaining groups received injections of 50, 100 and 200 Lf respectively of liquid toxoid.

The results are recorded in Table 21.

TABLE 21.—*Immunity produced by Primary Stimulus of APT and of Liquid Toxoid*

Dose.	Alum-precipitated						Without alum					
	Sheep No.	Sampling times in weeks					Sheep No.	Sampling times in weeks				
		1	2	3	4	5		1	2	3	4	5
50 Lf...	1	0	5·7	2·6	1·8	1·2	1	0	0	0	0	0
	2	0	40·0	12·3	10·0	2·6	2	0	0	0	0	0
	3	0	4·0	1·6	1·0	0·8	3	0	0	0	0	0
	4	0	0·2	0·4	0·6	0·4	4	0	0	0	0	0
	5	0	1·3	2·0	2·0	1·3	5	0	0	0	0	0
	6	0	0·4	0·5	0·8	0·4	6	0	0	0	0	0
	7	0	4·0	1·6	1·0	0·6	7	0	0	0	0	0
	8	0	4·0	4·0	4·0	2·6	8	0	0	0	0	0
	9	0	5·7	4·4	10·0	2·0	9	0	0	0	0	0
	10	0	4·0	4·0	8·0	2·0	10	0	0	0	0	0
		1	2	3	4	5		1	2	3	4	5
100 Lf...	1	0	20·0	20·0	13·3	8·0	1	0	0	0	0·4	0
	2	0	1·6	1·1	1·0	1·0	2	0	0	0·2	0·2	0·1
	3	0	40·0	40·0	20·0	20·0	3	0	0	0·1	0	0
	4	0	0·8	4·0	1·3	0·8	4	0	0·2	0·1	0	0
	5	0	4·0	3·3	2·6	2·0	5	0	0	0	0	0
	6	0	4·0	3·3	2·6	2·6	6	0	0	0	0	0
	7	4	40·0	20·0	13·3	10·0	7	0	1·0	1·0	1·3	0·6
	8	0	4·0	4·0	2·0	1·0	8	0	0	0	0	0
	9	0	1·6	0·8	0·8	0·4	9	0	0·4	0·2	0	0
	10	0	0·2	0·8	0·8	0·4	10	0	0·4	0·1	0	0
		1	2	3	4	5		1	2	3	4	5
200 Lf...	1	0	133·0	40·0	40·0	20·0	1	0	0·1	0·4	0·2	0·1
	2	0	2·6	1·3	1·3	0·8	2	0	20·0	8·0	5·7	4·0
	3	0	20·0	13·3	8·0	6·6	3	0	0·4	0·4	0·4	0·2
	4	0	13·3	13·3	8·0	5·7	4	0	0·4	0	0	0
	5	0	5·0	4·0	5·0	5·0	5	0	0	0	0	0
	6	0	10·0	5·7	3·3	2·6	6	0	0·1	0	0	0
	7	0	5·0	5·0	5·7	4·0	7	0	0	0	0	0
	8	0	20·0	20·0	20·0	13·3	8	0	0·4	2·0	0·6	0·4
	9	0	40·0	20·0	20·0	13·3	9	0	0·2	0·2	0·1	0·5
	10	0	6·6	4·0	3·3	2·6	10	0	0·2	0·2	0·2	0·2

The conclusion to be drawn from the results given in Table 21 is that APT is a much better antigen, Lf per Lf, than liquid toxoid. This holds for the three doses, 50 Lf, 100 Lf and 200 Lf, given.

Immunity response to a primary stimulus of graded doses of APT

Having shown that APT is a better antigen than liquid toxoid experiments were carried out with APT in an attempt to obtain a graded response. Sheep, in groups of 20, received 6·25, 25, 50, 100 and 200 Lf APT, respectively, and were bled and their sera assayed for antitoxin at the times shown in Tables 22 to 26.

TABLE 22.—6·25 *Lf*

Sheep No.	Sampling times in weeks				
	1	2	3	4	5
1	0	6·6	8·0	6·6	4·0
2	0	0·4	0·4	0·3	0·2
3	0	1·3	2·0	2·0	0·6
4	0	0·4	1·3	1·0	0·4
5	0	5·0	8·0	5·0	4·0
6	0	0·2	0·1	0	0
7	0	0·4	0·6	1·0	1·3
8	0	0·1	0·6	0·6	0·4
9	0	0·1	0·6	0·4	0·4
10	0	1·0	0·3	0·5	0·5
11	0	2·0	5·0	4·0	2·6
12	0	0·1	0·2	0·1	0·2
13	0	2·0	4·0	2·6	1·3
14	0	1·0	4·0	8·0	4·4
15	0	2·0	4·0	2·6	2·0
16	0	0·4	0·1	0	0
17	0	5·0	2·0	2·0	1·3
18	0	5·0	13·3	5·7	4·0
19	0	2·6	1·0	0·6	1·0
20	0	0·9	0·2	0	0
Median	—	1·0	1·1	1·0	0·95
Log median	—	0	0·0414	0	—0·0223

TABLE 23.—25 *Lf*

Sheep No.	Sampling times in weeks				
	1	2	3	4	5
1	0	0·1	1·3	1·3	0·5
2	0	0·2	0·8	0·6	0·6
3	0	0·4	2·0	1·0	0·5
4	0	0·1	0·8	0·2	0
5	0	5·0	5·0	2·6	2·0
6	0	0·8	0·8	1·3	1·0
7	0	0	0	0·2	0·4
8	0	0	0	0·1	0
9	0	6·6	5·7	6·6	2·0
10	0	13·3	4·0	1·3	2·0
11	0	5·0	3·3	2·0	1·3
12	0	1·4	4·0	2·0	1·0
13	0	13·3	2·0	2·0	1·1
14	0	13·3	10·0	4·4	4·0
15	0	6·6	8·0	3·3	2·4
16	0	5·0	4·0	2·0	1·3
17	0	1·1	0·5	0·2	0
18	0	0·2	0	0·2	0
19	0	0	0	0·1	0
20	0	0	0	0	0
Median	—	0·9	1·7	1·3	0·8
Log median	—	—0·0458	0·2304	0·1139	—0·0969

TABLE 24.—50 *Lf*

Sheep No.	Sampling times in weeks				
	1	2	3	4	5
1	0	5·7	2·6	1·8	1·2
2	0	40·0	12·3	10·0	2·6
3	0	4·0	1·6	1·0	0·8
4	0	0·2	0·4	0·6	0·4
5	0	1·3	2·0	2·0	1·3
6	0	0·4	0·5	0·8	0·4
7	0	4·0	1·6	1·0	0·6
8	0	4·0	4·0	4·0	2·6
9	0	5·7	4·4	10·0	2·0
10	0	4·0	4·0	8·0	2·0
11	0	0·1	0·4	0	0
12	0	2·0	1·6	1·2	0·6
13	0	2·0	2·0	4·0	1·0
14	0	0·1	0·1	0	0
15	0	4·0	8·0	1·0	0·6
16	0	2·0	1·6	0·8	0·3
17	0	0	0·4	0	0
18	0	0·4	1·3	4·0	1·3
19	0	40·0	20·0	4·0	2·0
20	0	4·0	4·0	4·0	1·6
Median	—	3·0	2·0	1·6	0·9
Log median	—	0·4771	0·3010	0·2041	—0·0458

818504—3

TABLE 25.—100 *Lf*

Sheep No.	Sampling times in weeks				
	1	2	3	4	5
1	0	20·0	20·0	13·3	8·0
2	0	1·6	1·1	1·0	1·0
3	0	40·0	40·0	20·0	20·0
4	0	0·8	4·0	1·3	0·8
5	0	4·0	3·3	2·6	2·0
6	0	4·0	3·3	2·6	2·6
7	4	40·0	20·0	13·3	10·0
8	0	4·0	4·0	2·0	1·0
9	0	1·6	0·8	0·8	0·4
10	0	0·2	0·8	0·8	0·4
11	0	4·0	2·6	1·6	0·8
12	0	5·0	1·1	0·1	0
13	0	6·6	5·7	5·0	5·0
14	0	0	4·0	2·6	1·6
15	0	1·0	5·7	6·6	2·6
16	0	4·0	1·3	1·0	2·0
17	0	4·0	5·0	20·0	6·6
18	0	1·0	0·5	0·8	0·4
19	0	6·6	4·4	10·0	6·6
20	0	1·3	1·6	2·0	2·0
Median	—	4·0	3·3	2·6	1·8
Log median	—	0·6021	0·5185	0·4150	0·2553

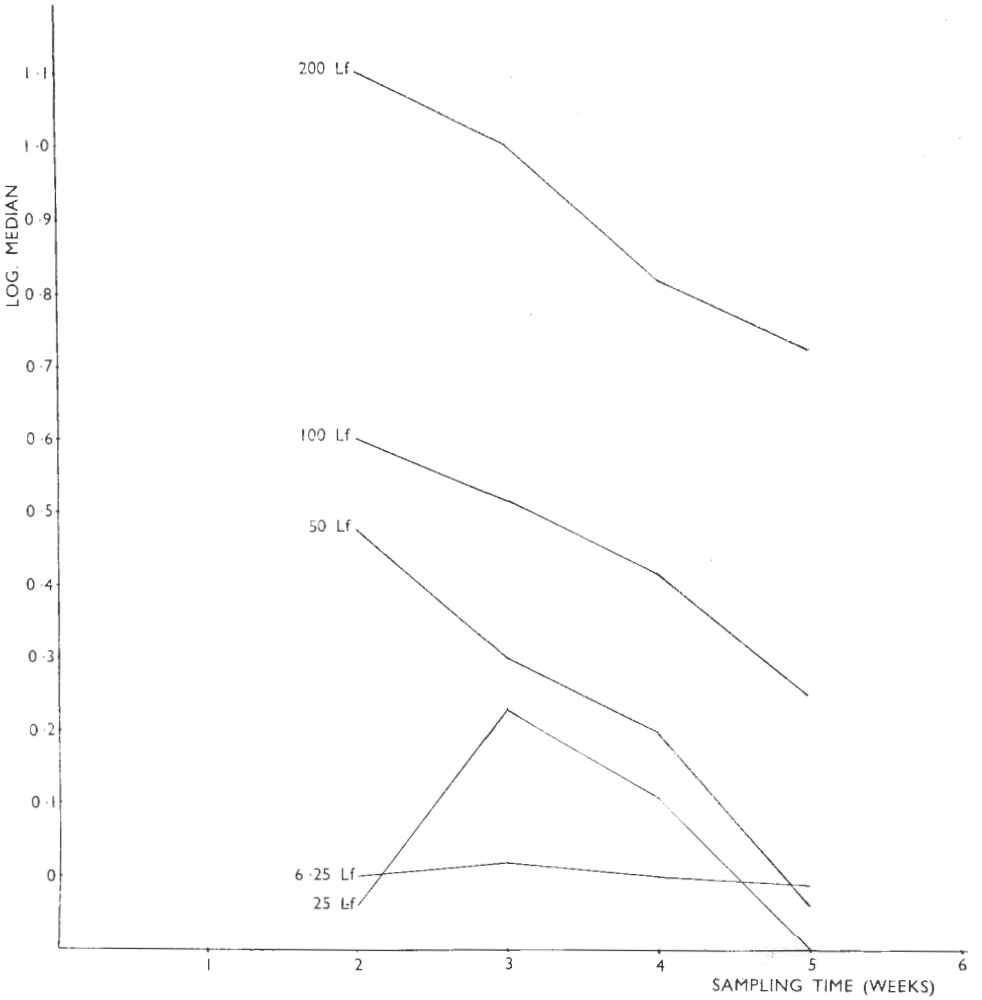
TABLE 26.—200 *Lf*

Sheep No.	Sampling times in weeks				
	1	2	3	4	5
1	0	133·0	40·0	40·0	20·0
2	0	2·6	1·3	1·3	0·8
3	0	20·0	13·3	8·0	6·6
4	0	13·3	13·3	8·0	5·7
5	0	5·0	4·0	5·0	5·0
6	0	10·0	5·7	3·3	2·6
7	0	5·0	5·0	5·7	4·0
8	0	20·0	20·0	20·0	13·3
9	0	40·0	20·0	20·0	13·3
10	0	6·6	4·0	3·3	2·6
11	0	10·0	8·0	6·6	6·6
12	0	13·3	10·0	6·6	2·6
13	0	20·0	13·3	10·0	8·0
14	0	13·3	10·0	6·6	4·4
15	0	5·0	4·0	2·6	2·0
16	0	10·0	8·0	5·0	3·3
17	0	13·3	40·0	20·0	20·0
18	0	20·0	13·3	10·0	6·6
19	0	5·0	2·0	1·6	0·8
20	0	20·0	20·0	8·0	5·7
Median	—	13·3	10·0	6·6	5·35
Log median	—	1·1239	1·0	0·8195	0·7284

An examination of the data in these tables reveals a marked difference in individual response to the same antigenic stimulus.

These results are graphically represented in Fig. 5.

FIG. 5.—Graph showing the Immunity Response to a Primary Stimulus of Graded Doses of APT.



For reasons already stated the log median titre was used for the graphical representation shown in Fig. 5. From this graph it can be seen that an increase in the dose results in an increased response.

For doses of 50 Lf, 100 Lf and 200 Lf, the observed maximum response occurred at the second week while for doses of 6·25 Lf and 25 Lf at the third week.

The immunity response after two injections of APT at one week's interval

In this experiment groups of ten sheep were given injections of 6·25, 25, 50, 100 and 200 Lf, respectively, and again seven days later.

The results are recorded in Tables 27 to 31.

TABLE 27.—6·25 *Lf*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	0·2	1·3	1·3	1·0	0·5
2	2·6	2·6	2·0	1·0	0·8
3	0·1	0·1	2·0	0·5	0·4
4	1·0	4·0	2·6	1·6	2·0
5	0·1	0·1	0·4	0·4	0·2
6	4·0	4·0	2·0	1·0	0·4
7	2·0	2·0	3·3	4·0	2·0
8	5·0	2·0	2·6	1·6	1·6
9	1·3	6·6	6·6	4·4	3·3
10	4·0	6·6	4·0	2·6	2·0
Mean log titre	0·0033	0·1974	0·3391	0·1370	—0·0269

TABLE 28.—25 *Lf.*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	13·3	13·3	8·0	5·0	5·7
2	4·0	4·0	4·0	1·0	0·8
3	40·0	20·0	10·0	6·6	5·7
4	8·0	13·3	10·0	5·7	4·0
5	4·0	5·7	40·0	4·0	3·3
6	0·2	1·3	0·8	0·5	0·6
7	5·7	5·7	4·0	2·0	3·3
8	5·0	2·0	2·0	2·4	1·3
9	2·0	1·0	2·0	0·6	0·6
10	40·0	10·0	10·0	6·6	4·0
Mean log titre	0·7492	0·7078	0·7215	0·3855	0·3326

TABLE 29.—50 *Lf.*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	0·8	2·0	2·0	2·0	1·0
2	4·0	4·0	4·0	4·0	2·0
3	4·0	2·0	2·0	0·6	0·6
4	4·0	4·0	4·0	4·0	4·0
5	1·3	2·6	10·0	1·2	0·8
6	40·0	57·0	26·6	20·0	20·0
7	40·0	80·0	40·0	20·0	8·0
8	400·0	400·0	40·0	40·0	13·3
9	13·3	13·3	13·3	4·0	4·0
10	100·0	400·0	80·0	40·0	40·0
Mean log titre	1·0754	1·2208	1·0462	0·771	0·6117

TABLE 30.—100 *Lf.*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	0·6	4·0	5·0	3·3	3·3
2	40·0	8·0	5·7	4·4	4·0
3	1·3	2·6	2·0	2·0	1·0
4	80·0	40·0	40·0	13·3	6·6
5	80·0	80·0	50·0	20·0	13·3
6	133·0	66·0	66·0	40·0	40·0
7	20·0	20·0	8·0	4·4	3·3
8	40·0	8·0	3·3	0·8	0·6
9	4·4	10·0	10·0	6·6	5·0
10	4·0	3·3	3·3	1·6	1·0
Mean log titre.	1·1573	1·0968	0·9817	0·7060	0·5662

TABLE 31.—200 *Lf*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	80·0	200·0	50·0	50·0	40·0
2	40·0	20·0	20·0	13·3	10·0
3	80·0	100·0	100·0	40·0	20·0
4	4·0	6·6	3·3	2·0	2·0
5	5·7	8·0	13·3	8·0	8·0
6	10·0	40·0	8·0	8·0	4·4
7	133·0	200·0	200·0	50·0	50·0
8	13·3	13·3	5·7	4·4	2·6
9	2·6	3·3	2·6	2·0	1·3
10	80·0	50·0	40·0	20·0	40·0
Mean log titre.	1·3332	1·4569	1·2620	1·0477	0·9581

From Fig. 6 in which the mean log titres of the data in Tables 27 to 31 are plotted against the sampling times for each dosage level, it can be seen that a definite graded effect was obtained, the higher doses showing a more marked response. Furthermore, an analysis of variance of the means at any of the five sampling times revealed—

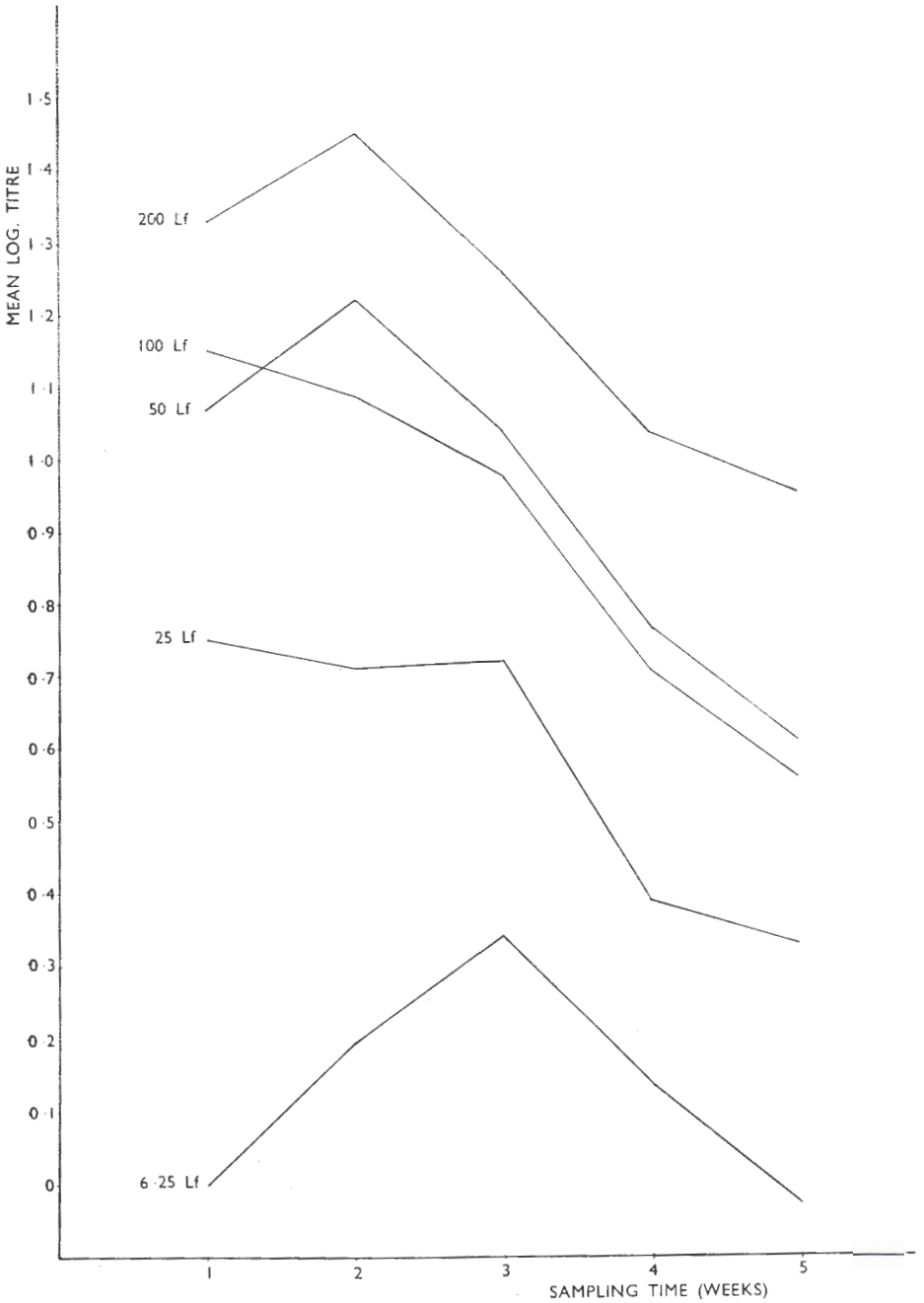
(*a*) a significant difference between the 6·25 Lf group and any one of the rest;

(*b*) a significant difference between the 25 Lf group and the 50 Lf and 200 Lf groups; and

(*c*) no significant difference between the 50 Lf and 100 Lf groups, between the 50 Lf and 200 Lf groups and between the 100 Lf and 200 Lf groups.

It can therefore be concluded that when the primary and secondary stimuli were given at a week's interval, the results obtained with 6·25 Lf and 25 Lf were inferior to those obtained with 50 Lf, 100 Lf and 200 Lf.

FIG. 6.—Graph showing the Immunity Response to Primary and Secondary Stimuli given at One Week's Interval at Different Dosage Levels



The immunity response after two injections of APT at two weeks' interval

The third experiment was conducted similarly to the second except that the interval between the injections was increased to 14 days. The results given in Tables 32 to 36 and in Fig. 7 were obtained.

For the sake of brevity in graphical interpretation and statistical analysis, letters were assigned to the experimental groups of sheep.

TABLE 32.—*Group A.* 6·25 *Lf*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	100·0	50·0	40·0	20·0	20·0
2	66·0	50·0	40·0	20·0	13·3
3	40·0	20·0	20·0	6·6	4·0
4	100·0	50·0	40·0	20·0	20·0
5	50·0	40·0	20·0	8·0	5·0
6	100·0	40·0	13·3	5·7	5·7
7	40·0	20·0	13·3	5·7	5·0
8	400·0	200·0	66·0	40·0	40·0
9	40·0	20·0	10·0	4·4	4·0
10	40·0	20·0	10·0	5·7	4·0
Mean log titre.	1·8529	1·5806	1·3476	1·0140	0·9288

TABLE 33.—*Group B.* 25 *Lf.*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	57·0	40·0	20·0	20·0	20·0
2	400·0	133·0	57·0	50·0	40·0
3	133·0	100·0	50·0	20·0	20·0
4	40·0	10·0	8·0	3·3	2·0
5	57·0	40·0	13·3	8·0	6·6
6	400·0	100·0	57·0	40·0	40·0
7	40·0	40·0	10·0	8·0	6·6
8	40·0	10·0	8·0	6·6	4·0
9	40·0	20·0	10·0	6·6	5·7
10	40·0	40·0	13·3	13·3	10·0
Mean log titre.	1·8850	1·5833	1·2566	1·0992	1·0105

TABLE 34.—*Group C.* 50 *Lf*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	200·0	200·0	40·0	20·0	6·6
2	12·3	6·6	5·7	4·0	2·6
3	200·0	200·0	40·0	40·0	40·0
4	40·0	80·0	20·0	10·0	6·6
5	57·0	57·0	26·6	20·0	10·0
6	80·0	40·0	20·0	10·0	8·0
7	200·0	200·0	80·0	80·0	40·0
8	40·0	13·3	6·6	5·7	3·3
9	200·0	40·0	26·6	20·0	10·0
10	40·0	6·6	2·0	1·6	2·0
Mean log titre	1·8759	1·6530	1·2436	1·0970	0·8982

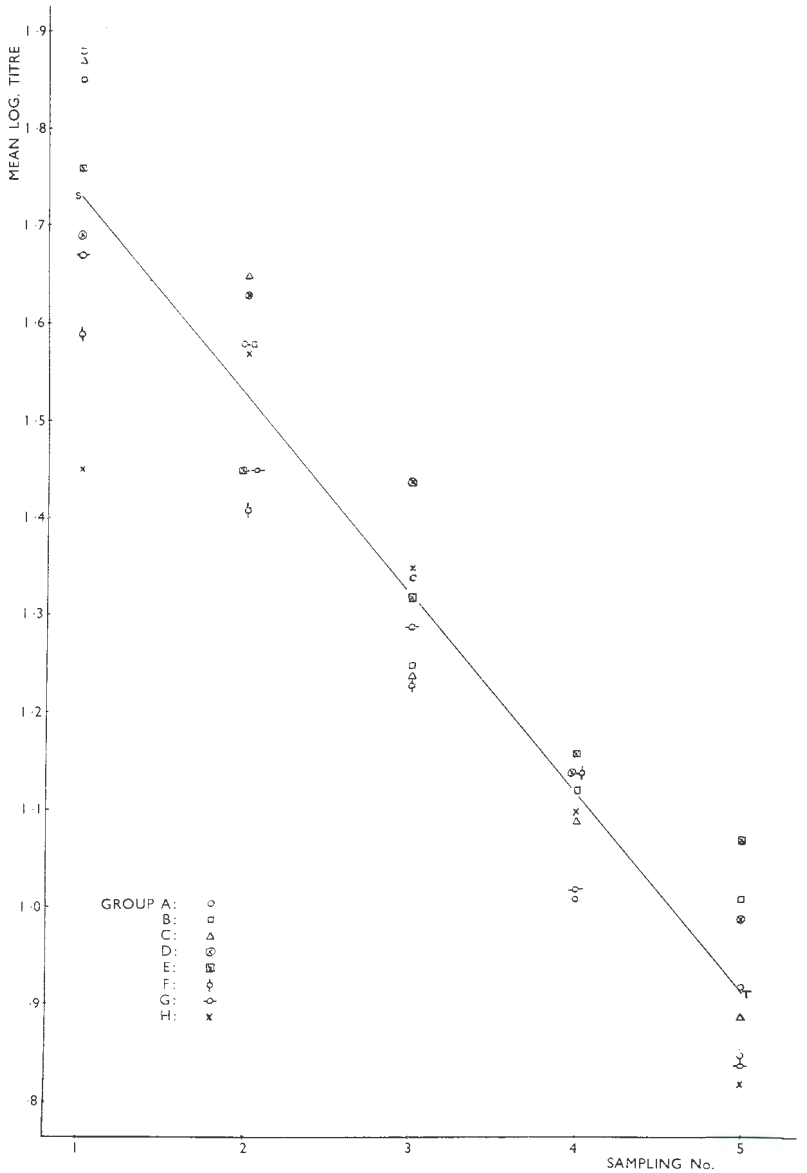
TABLE 35.—*Group D.* 100 *Lf*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	44·0	44·0	20·0	8·0	6·6
2	40·0	13·3	13·3	5·7	5·7
3	66·0	80·0	40·0	20·0	10·0
4	13·3	20·0	13·3	3·3	3·3
5	80·0	80·0	50·0	20·0	20·0
6	40·0	66·0	40·0	40·0	20·0
7	40·0	13·3	10·0	6·6	5·0
8	40·0	13·3	13·3	6·6	4·0
9	57·0	57·0	40·0	40·0	20·0
10	200·0	400·0	200·0	66·0	40·0
Mean log titre	1·6956	1·6301	1·4479	1·1444	0·9901

TABLE 36.—*Group E.* 200 *Lf*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	100·0	40·0	40·0	20·0	20·0
2	400·0	66·0	50·0	40·0	40·0
3	50·0	13·3	20·0	20·0	10·0
4	10·0	10·0	10·0	6·6	5·7
5	13·3	13·3	5·7	5·7	6·6
6	13·3	10·0	5·0	4·0	2·6
7	80·0	40·0	40·0	13·3	8·0
8	40·0	40·0	20·0	13·3	10·0
9	200·0	50·0	50·0	50·0	40·0
10	200·0	100·0	40·0	20·0	20·0
Mean log titre	1·7656	1·4573	1·3261	1·1630	1·0700

FIG 7.—Graph showing the Immunity response Resulting from two injections of 6·25, 25, 50, 100 and 200 Lf APT respectively at an interval of two weeks and two Injections of 6·25 Lf APT at intervals of 3, 4 and 5 weeks respectively



Group A: 2 injections of 6·25 Lf at 2 weeks' interval.
Group B: 2 injections of 25 Lf at 2 weeks' interval.
Group C: 2 injections of 50 Lf. at 2 weeks' interval.
Group D: 2 injections of 100 Lf. at 2 weeks' interval.
Group E: 2 injections of 200 Lf. at 2 weeks' interval.
Group F: 2 injections of 6·25 Lf. at 3 weeks' interval.
Group G: 2 injections of 6·25 Lf. at 4 weeks' interval.
Group H: 2 injections of 6·25 Lf. at 5 weeks' interval.

The above results, given in Tables 32 to 36 and plotted in Fig. 7, indicate that the log titre for each dose group shows a linear regression on sampling times and further that there is no real difference in response to the different doses since the slope and position of the different lines show no obvious disagreement.

An analysis of variance for the data obtained in this last experiment confirms that for any given dose the regression of the mean log titres on sampling times shows no significant deviation from linearity and also that the regression lines obtained for the different doses do not differ significantly with regard to slope or position. It is unnecessary to include details of this analysis at this point as the conclusions drawn are verified by the broader analysis which follows below.

This means that the response obtained after two injections spaced at an interval of 14 days was independent of the dose within the range of doses chosen. On the strength of this finding the effect of longer intervals was investigated and for the purpose the lowest dose, viz. 6·25 Lf was chosen.

The immunity response following on two injections of 6·25 *Lf APT spaced at intervals of* 3, 4 *and* 5 *weeks respectively*

Three groups of ten sheep each received a primary stimulus of 6·25 Lf per sheep. The first group received a secondary stimulus three weeks after the primary; the second group received its secondary stimulus four weeks after the primary and in the third group the two stimuli were separated by five weeks. The results are shown in Tables 37 to 39.

TABLE 37.—*Group F. Two injections of* 6·25 *Lf at an interval of three weeks*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1.....	6·6	6·6	5·7	4·0	2·6
2.....	50·0	40·0	40·0	40·0	10·0
3.....	200·0	100·0	40·0	40·0	20·0
4.....	200·0	200·0	66·0	50·0	20·0
5.....	6·6	4·0	2·0	1·6	1·3
6.....	6·6	4·0	2·6	2·6	1·6
7.....	100·0	50·0	40·0	40·0	20·0
8.....	100·0	40·0	20·0	20·0	8·0
9.....	8·0	8·0	4·4	5·0	5·0
10.....	200·0	100·0	100·0	50·0	20·0
Mean log titre.	1·5965	1·4131	1·2344	1·1425	0·8539

TABLE 38.—*Group G. Two injections of 6·25 Lf at an interval of four weeks*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	100.0	100.0	100.0	40.0	20.0
2	13·3	13·3	8·0	5·7	4·0
3	20.0	20.0	13·3	8·0	5·7
4	100.0	66.0	66.0	40.0	20.0
5	6·6	0·4	0·1	0·2	0·2
6	400.0	400.0	400·0	100.0	100.0
7	50.0	40.0	20.0	10.0	6·6
8	66.0	40.0	40.0	20.0	13·3
9	50.0	20.0	20.0	8·0	4·4
10	50.0	40.0	20.0	8·0	4·4
Mean log titre.	1·6764	1·4556	1·2954	1·0271	0·8493

TABLE 39.—*Group H. Two injections of 6·25 Lf at an interval of five weeks*

Sheep No.	Sampling times in weeks after secondary stimulus				
	1	2	3	4	5
1	200·0	200·0	66·0	50·0	40·0
2	50·0	50·0	20·0	10·0	5·7
3	66·0	100·0	66·0	40·0	20·0
4	4·0	2·6	2·0	1·3	1·3
5	40·0	40·0	40·0	40·0	4·4
6	100·0	200·0	100·0	5·7	5·7
7	1·0	4·0	2·6	2·0	0·8
8	40·0	50·0	40·0	40·0	20·0
9	66·0	66·0	50·0	40·0	20·0
10	13·3	20·0	10·0	5·7	4·0
Mean log titre.	1·4570	1·5740	1·3560	1·1034	0·8280

When these data are plotted (Fig. 7) it can be seen that the responses obtained in Groups F, G and H do not differ obviously from those of Groups A to E. This was verified by a complete analysis of the pooled data for these eight groups. For the purpose of this analysis x represents the sampling time in weeks and y the logarithm of the titre. Before the analysis was carried out it was ascertained that the variance of y seemed to be independent of the mean. The details of the analysis are recorded in Tables 40 and 41.

TABLE 40.—*Analysis of Covariance of* x *and* y

Nature of variation	Deg. Freedom	S*xx*	S*xy*	S*yy*	$\frac{(Sxy)^2}{Sxx}$
Between groups	7	0	0	1·0442	
Within groups, between sampling times	32	800	— 163·086	35·5126	33·2463
Between sampling times	39	800	— 163·086	36·5568	
Within sampling times	360	0	0	96·1438	
TOTAL	399	800	— 163·086	132·7006	
Between sampling times of Groups—					
A	4	100	— 24·148	5·9628	5·8313
B	4	100	— 22·331	5·2611	4·9867
C	4	100	— 25·114	6·4777	6·3071
D	4	100	— 18·967	3·7428	3·5975
E	4	100	— 16·855	2·9797	2·8409
F	4	100	— 17·558	3·1534	3·0828
G	4	100	— 20·827	4·3581	4·3376
H	4	100	— 17·286	3·5770	2·9881
TOTAL		800	— 163·086	35·5126	33·9720

TABLE 41.—*Analysis of Variance for* y

Nature of variation	Degree of freedom	Sum of squares	Mean square
Between groups	7	1·0442	0·1492
Regression of *y* on *x*	1	33·2463	33·2463
Difference of regression co-efficients within groups	7	0·7257	0·1037
Within groups between sampling times	24	1·5406	0·0642
Between sampling times	39	36·5568	
Within sampling times	360	96·1438	0·2671
TOTAL	399	132·7006	

A comparison of the appropriate mean squares recorded in Table 41 with the error mean square (variation with sampling times) indicates that the regression of *y* on *x* for individual groups shows no deviation from linearity and no evidence of non-parallelism between the individual regression lines. These eight regression lines are so similar in position that they can be represented by the average regression line ST in Fig. 7 representing the equation.

$$y = 1{\cdot}9340 - 0{\cdot}204x$$

The results and their statistical analysis show that sheep, immunized with two doses of APT, each containing from 6·25 Lf to 200 Lf and separated by an interval of two weeks, would have a mean circulating antitoxin titre of about 50 units per ml. one week after the second stimulus. This titre will drop at a constant rate given by the regression co-efficient of ST, viz. — 0·204, during the following five weeks so that at any time it will be about two-thirds of the titre of the previous week. This, of course, will not hold for much longer than the five-week period when the titres can be expected to remain at a steadier level.

The observation that the same level of immunity can be produced with 6·25 Lf as with 200 Lf beta toxoid has an important bearing on the economy of lamb dysentery vaccine production. The *Cl. welchii* Type B formolized culture filtrates produced in the bacteriological laboratory at Onderstepoort contain on an average 300 Lf beta toxoid. When it is taken into account that lamb dysentery vaccine is injected in a dose of 2 ml. per sheep these filtrates can be diluted considerably without losing their efficacy.

These results raise the question whether two doses of APT containing less than 6·25 Lf beta toxoid would elicit the same response as a product containing 6·25 Lf. This point was investigated by giving two groups of ten sheep two injections of APT. In one group the dose was 6·25 Lf per injection and in the other about half this quantity, viz. 3 Lf. The primary and secondary doses were spaced at an interval of three weeks. A different batch of toxoid to the one used in the previous experiment was used. The sheep were bled one week after the second injection and the antitoxin titres of their sera determined. The results are recorded in Table 42.

TABLE 42.—*Group having received* 6·25 *Lf.*

Sheep No.	*Antitoxin titre (units/ml.)*
1	200·0
2	13·3
3	40·0
4	40·0
5	40·0
6	66·0
7	13·3
8	20·0
9	50·0
10	20·0
Mean log titre	1·5480

Group having received 3·0 *Lf.*

Sheep No.	*Antitoxin titre (units/ml.)*
1	0
2	0·2
3	5·0
4	0·0
5	0·0
6	0·7
7	5·7
8	6·7
9	5·7
10	5·7

When the titres of the group that received 6·25 Lf toxoid are compared with those obtained at the first bleeding of the group that received two injections of 6·25 Lf at three weeks' interval in the previous experiment, no significant difference can be demonstrated. A comparison of the mean log titres gives a value $t = 0·1998$ for 18 degrees of freedom.

The titres of the 3 Lf group, however, are obviously significantly lower than those of the 6·25 Lf group; three of the ten sheep in the former group showed no response at all and two failed to reach one unit per ml., while none developed more than seven units per ml.

A reasonable conclusion would therefore be that, for practical purposes, 6·25 Lf beta toxoid per dose of APT is the lowest limit at which a response similar to that obtained by the injection of doses up to 200 Lf, can be produced.

The response to a booster injection of beta toxoid

The effect of a booster injection of beta toxoid was investigated in sheep immunized twelve months previously. The dose in Lf of the booster was varied but the volume injected was kept constant at 2·5 ml. The same type of APT but not the same batch as in the previous experiments was used. One week after the injection bleeding was commenced and continued at varying intervals until 28 weeks after the start of the experiment. The level of immunity was determined over 28 weeks, representing the gestation period of a sheep, to provide information on the longest time interval before parturition a booster injection could be given and yet allow for adequate immunity at lambing.

Nine groups of sheep were immunized in different ways as shown in Table 43. Five of these groups were strictly comparable in that the sheep were immunized and sampled on identical dates, and these form a single experiment, Experiment 1. Similarly, the other four groups make up Experiment 2.

TABLE 43.—*Scheme of Injections received by the groups of Sheep used in Experiment*

Exp. No.	Group	Primary stimulus	Secondary stimulus	Booster
1	I	50 Lf liquid toxoid	Nil	6·25 Lf APT
	II	50 Lf APT	Nil	6·25 Lf APT
	III	100 Lf APT	100 Lf APT	6·25 Lf APT
	IV	50 Lf APT	Nil	6·25 Lf liquid toxoid
	V	100 Lf APT	100 Lf APT	6·25Lf liquid toxoid
2	VI	100 Lf APT	Nil	100 Lf APT
	VII	100 Lf APT	Nil	6·25 Lf APT
	VIII	50 Lf APT	50 Lf APT	100 Lf liquid toxoid
	IX	50 Lf APT	50 Lf APT	6·25 Lf liquid toxoid

The detailed results are recorded in Tables 44 to 52 and summarized graphically in Fig. 8 (Experiment 1) and Fig. 9 (Experiment 2) in which the mean log titre is plotted against sampling time in weeks for all groups except Group I. For reasons already stated, the log median titre was employed for this group.

TABLE 44.—*Group I*

Sheep No.	Sampling times in weeks after booster injection					
	1	2	4	6	8	10
1	0·2	1·6	1·3	0·5	0·2	0
2	0·2	1·1	0·8	0·42	0·2	0·1
3	2·7	1·0	1·0	0·1	0·13	0
4	0	4·0	2·0	0·53	0·4	0·2
5	6·7	4·0	13·3	4·0	1·3	0·63
6	2·7	1·0	1·3	0·9	0	0
7	6·7	5·0	1·6	0·8	0·4	0·2
8	0	0	0·2	0·2	0·13	0
Log median	0·1761	0·1461	0·1614	—0·284	—0·5229	—1·301

TABLE 45.—*Group II.*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	28
1.....	100·0	200·0	200·0	50·0	20·0	10·0	6·7	4·4	2·7	2·7	2·7
2.....	20·0	40·0	10·0	4·0	2·0	1·6	1·6	0·88	0·72	0·72	0·7
3.....	10·0	10·0	5·0	2·7	2·0	0·7	0·57	0·5	0·4	0·4	0·4
4.....	2·7	2·7	2·0	0·9	0·57	0·4	0·4	0·4	0·4	0·2	0·13
5.....	10·0	20·0	10·0	5·7	4·0	2·0	2·0	1·3	1·3	1·0	1·0
6.....	10·0	10·0	5·7	4·4	2·7	2·0	1·6	1·0	1·0	0·72	0·7
7.....	66·0	50·0	20·0	10·0	5·7	5·0	5·0	2·7	2·7	1·6	1·3
8.....	50·0	50·0	20·0	13·3	6·7	4·0	4·0	2·0	2·0	2·0	1·6
9.....	200·0	200·0	200·0	66·0	50·0	20·0	20·0	20·0	13·3	13·3	10·0
10.....	4·0	5·0	5·0	2·0	1·3	0·72	0·72	0·72	0·8	0·53	0·57
Mean log titre..	1·3159	1·4034	1·1659	0·8335	0·6087	0·3713	0·3353	0·1894	0·1366	0·0533	−0·0089

TABLE 46.—*Group III*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	28
1.....	40·0	50·0	20·0	8·0	5·0	2·7	2·7	1·6	1·6	1·6	1·3
2.....	10·0	20·0	8·0	5·7	4·0	2·7	2·7	2·0	1·6	1·6	1·3
3.....	100·0	100·0	50·0	40·0	13·3	8·0	6·7	2·0	2·0	1·9	1·6
4.....	50·0	20·0	13·3	5·7	4·0	2·7	2·7	1·6	2·0	1·6	1·3
5.....	200·0	200·0	100·0	100·0	66·7	20·0	20·0	20·0	20·0	13·3	10·0
6.....	40·0	40·0	40·0	13·3	6·7	4·0	4·0	2·0	2·7	2·0	2·0
7.....	20·0	13·3	1·3	1·3	0·13	0·1	0·1	0·1	0·1	0·1	0·1
8.....	2·0	50·0	6·7	8·0	6·7	6·7	6·7	6·7	5·0	5·0	4·0
9.....	6·7	6·7	6·7	5·7	4·0	2·7	4·0	2·0	2·7	2·7	2·7
10.....	50·0	200·0	100·0	50·0	40·0	20·0	3·05	2·0	1·6	1·9	1·3
Mean log titre..	1·4331	1·6154	1·2395	1·0613	0·7822	0·5659	0·4839	0·3047	0·3077	0·2725	0·1994

TABLE 47.—*Group IV*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	28
1.....	5·0	2·7	2·0	1·3	0·8	0·4	0·4	0·4	0·4	0·4	0·4
2.....	2·7	6·7	1·14	0·7	0·63	0·4	0·4	0·4	0·4	0·4	0·4
3.....	50·0	40·0	40·0	13·3	5·7	4·0	4·0	2·0	2·0	1·3	1·3
4.....	2·7	5·0	1·14	0·7	0·53	0·2	0·13	0·13	0·13	0·13	0·1
5.....	5·0	5·0	2·7	2·0	1·6	0·72	0·72	0·72	0·8	0·6	0·6
6.....	20·0	13·3	13·3	4·0	1·6	1·6	2·0	2·0	2·0	2·0	1·6
7.....	40·0	20·0	20·0	6·7	5·0	4·0	4·0	2·0	2·7	1·6	1·3
8.....	20·0	40·0	20·0	8·0	4·4	4·0	2·7	2·7	2·7	2·7	2·0
Mean log titre..	1·0205	1·0356	0·7718	0·4450	0·2417	0·0466	−0·0236	−0·0613	−0·0393	−0·1069	−0·1606

TABLE 48.—*Group V.*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	28
1.....	20·0	13·3	8·0	5·0	4·0	4·0	4·0	2·7	2·7	2·7	2·7
2.....	20·0	20·0	13·3	10·0	5·7	6·7	4·0	4·0	4·0	4·0	4·0
3.....	50·0	40·0	20·0	13·3	6·7	6·7	5·7	4·0	4·0	4·0	4·0
4.....	10·0	6·7	8·0	4·0	2·7	2·0	2·0	1·6	1·6	1·14	1·14
5.....	50·0	40·0	40·0	20·0	13·3	13·3	13·3	10·0	8·0	8·0	8·0
6.....	50·0	40·0	20·0	20·0	8·0	5·0	5·0	4·4	2·7	2·0	2·0
7.....	5·0	4·0	5·0	4·0	2·7	2·0	2·0	1·6	1·3	1·0	1·3
8.....	6·7	20·0	8·0	5·0	2·7	1·6	2·0	1·14	1·14	0·72	0·7
9.....	50·0	20·0	13·3	6·7	4·0	2·0	2·0	2·0	1·3	1·14	1·14
10.....	50·0	40·0	40·0	10·0	6·7	5·0	4·4	4·4	2·0	2·0	2·0
Mean log titre..	1·3622	1·2864	1·1462	0·9154	0·6934	0·5883	0·5672	0·4689	0·3801	0·2941	0·3214

TABLE 49.—*Group VI*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	23
1.....	66·0	133·0	40·0	20·0	20·0	20·0	20·0	13·3	10·0	10·0	10·0
2.....	20·0	20·0	10·0	11·8	7·6	5·6	5·9	4·1	3·3	3·3	3·0
3.....	50·0	40·0	40·0	20·0	20·0	20·0	20·0	10·0	10·0	10·0	6·7
4.....	50·0	50·0	40·0	13·3	10·0	5·7	5·7	2·7	2·7	2·7	2·0
5.....	66·0	50·0	50·0	40·0	13·3	8·0	10·0	6·7	5·0	4·4	4·0
6.....	20·0	40·0	100·0	40·0	20·0	13·3	20·0	13·3	10·0	10·0	10·0
7.....	10·0	8·0	6·7	4·0	2·0	1·14	1·14	0·9	0·9	0·8	0·6
8.....	50·0	40·0	40·0	10·0	5·7	5·7	5·7	5·0	2·7	2·7	2·7
9.....	2·7	5·0	2·0	1·14	0·9	0·6	0·5	0·5	0·5	0·5	0·5
Mean log titre..	1·4198	1·4703	1·3594	1·0734	0·8799	0·7471	0·7712	0·6078	0·5259	0·5141	0·4572

TABLE 50.—*Group VII*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	28
1.....	10·0	10·0	6·7	4·4	4·0	1·6	1·6	1·0	1·0	0·8	0·7
2.....	20·0	40·0	50·0	40·0	10·0	6·7	5·7	5·7	4·4	4·0	2·7
3.....	66·0	400·0	50·0	20·0	10·0	10·0	6·7	4·0	4·0	4·0	4·0
4.....	50·0	666·0	50·0	40·0	13·3	10·0	10·0	6·7	5·0	4·4	4·4
5.....	20·0	13·3	10·0	5·7	4·0	2·7	2·7	2·0	1·6	1·6	1·6
6.....	10·0	20·0	20·0	8·0	5·7	4·0	4·0	1·6	1·6	1·6	1·3
7.....	50·0	20·0	13·3	5·0	4·0	2·0	1·3	0·8	0·8	0·6	0·53
8.....	66·0	133·0	100·0	50·0	20·0	10·0	10·0	5·7	4·4	4·4	4·4
9.....	20·0	40·0	40·0	20·0	8·0	5·7	5·7	4·4	2·7	2·7	2·7
Mean log titre..	1·4388	1·7201	1·4389	1·1674	0·8767	0·6801	0·6322	0·4435	0·3701	0·3347	0·2933

TABLE 51.—*Group VIII*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	28
1	6·7	5·0	4·4	2·0	1·14	1·0	1·0	0·72	0·6	0·47	0·47
2	6·7	6·7	5·0	2·7	1·6	1·3	1·3	1·3	1·0	1·0	0·72
3	6·7	4·0	2·7	1·6	0·9	0·8	0·53	0·53	0·53	0·5	0·4
4	50·0	50·0	40·0	13·3	10·0	10·0	10·0	5·0	4·0	4·0	4·0
5	6·7	5·7	4·4	2·7	1·6	1·6	1·3	1·14	1·0	0·9	0·8
6	20·0	13·3	10·0	8·0	5·7	5·0	5·7	4·4	4·0	4·0	4·0
7	50·0	40·0	20·0	8·0	4·4	4·4	4·4	2·7	2·7	2·7	2·7
8	50·0	133·3	200·0	100·0	50·0	50·0	50·0	20·0	20·0	20·0	20·0
9	20·0	20·0	20·0	10·0	6·7	5·0	5·7	4·0	4·0	2·7	2·7
10	200·0	100·0	100·0	20·0	20·0	20·0	20·0	20·0	20·0	20·0	20·0
Mean log titre	1·3304	1·1733	1·1923	0·8599	0·6645	0·6263	0·7107	0·4730	0·4342	0·3994	0·3704

TABLE 52.—*Group IX*

Sheep No.	Sampling times in weeks after booster injection										
	1	2	4	6	8	10	12	15	18	21	28
1	20·0	40·0	40·0	13·3	8·0	5·0	5·0	5·0	4·4	4·4	4·0
2	5·0	4·4	4·0	2·7	1·3	1·14	1·3	1·14	1·14	1·0	0·9
3	20·0	13·3	6·7	5·0	2·0	1·6	1·14	1·14	0·9	0·8	0·8
4	10·0	13·3	13·3	6·7	4·4	2·7	4·0	2·0	2·0	2·0	2·0
5	20·0	13·3	8·0	4·0	2·0	1·3	1·14	0·9	0·8	0·8	0·6
6	20·0	20·0	20·0	10·0	5·7	5·7	5·7	5·7	5·0	4·4	4·4
7	4·0	4·0	2·0	2·0	2·0	2·0	0·9	0·7	0·53	0·47	0·2
8	50·0	40·0	20·0	10·0	5·0	4·4	5·0	2·7	1·6	2·0	1·14
9	20·0	20·0	13·3	10·0	5·7	5·0	5·0	5·0	2·7	1·8	1·4
10	66·0	50·0	50·0	13·3	13·3	13·3	13·3	13·3	8·0	8·0	6·7
Mean log titre	1·2329	1·2123	1·0783	0·8107	0·5353	0·5159	0·4818	0·3923	0·2821	0·2526	0·1512

FIG 8.—Graphical Representation of the Results obtained with Groups I, II, III, IV and V.

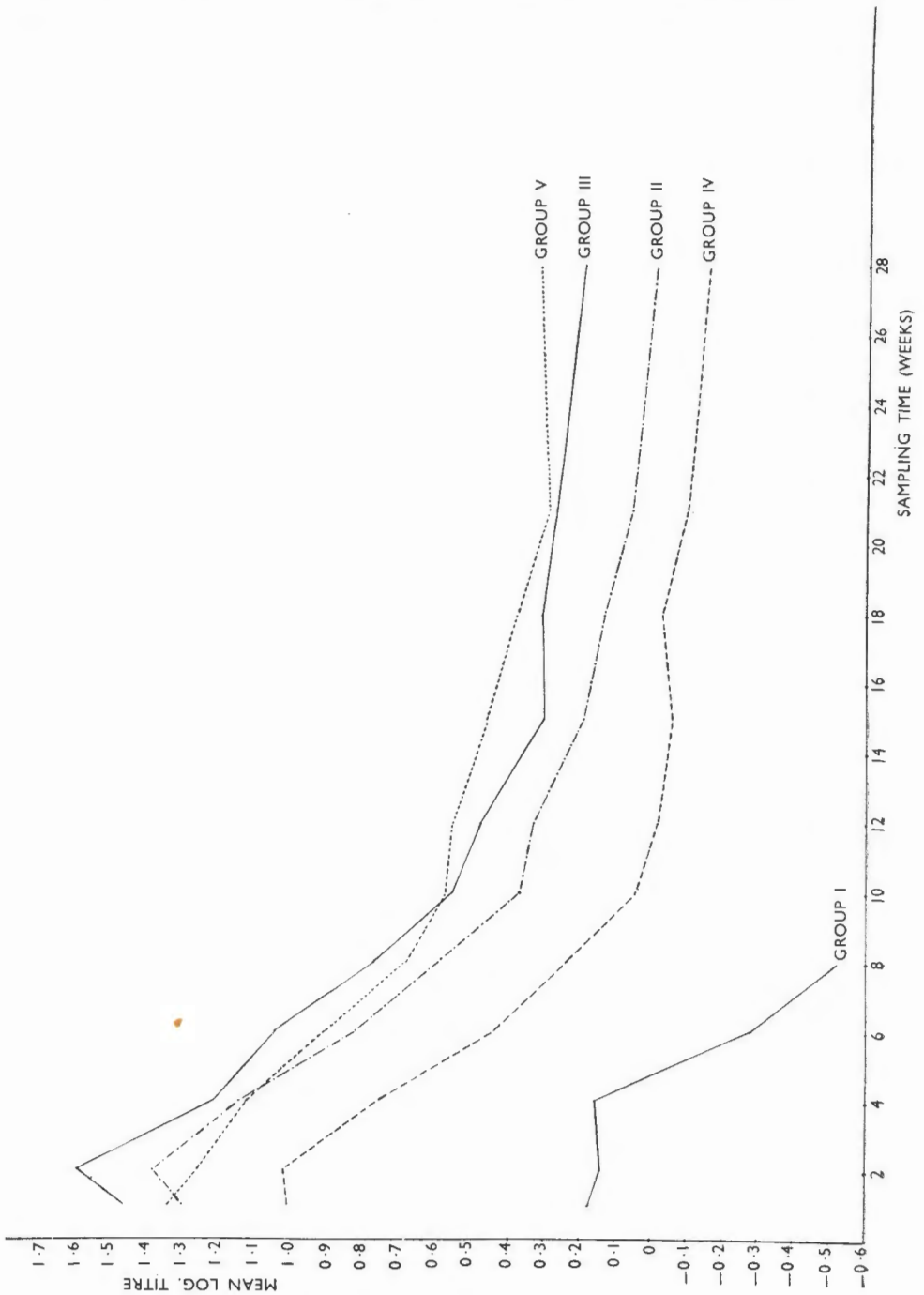
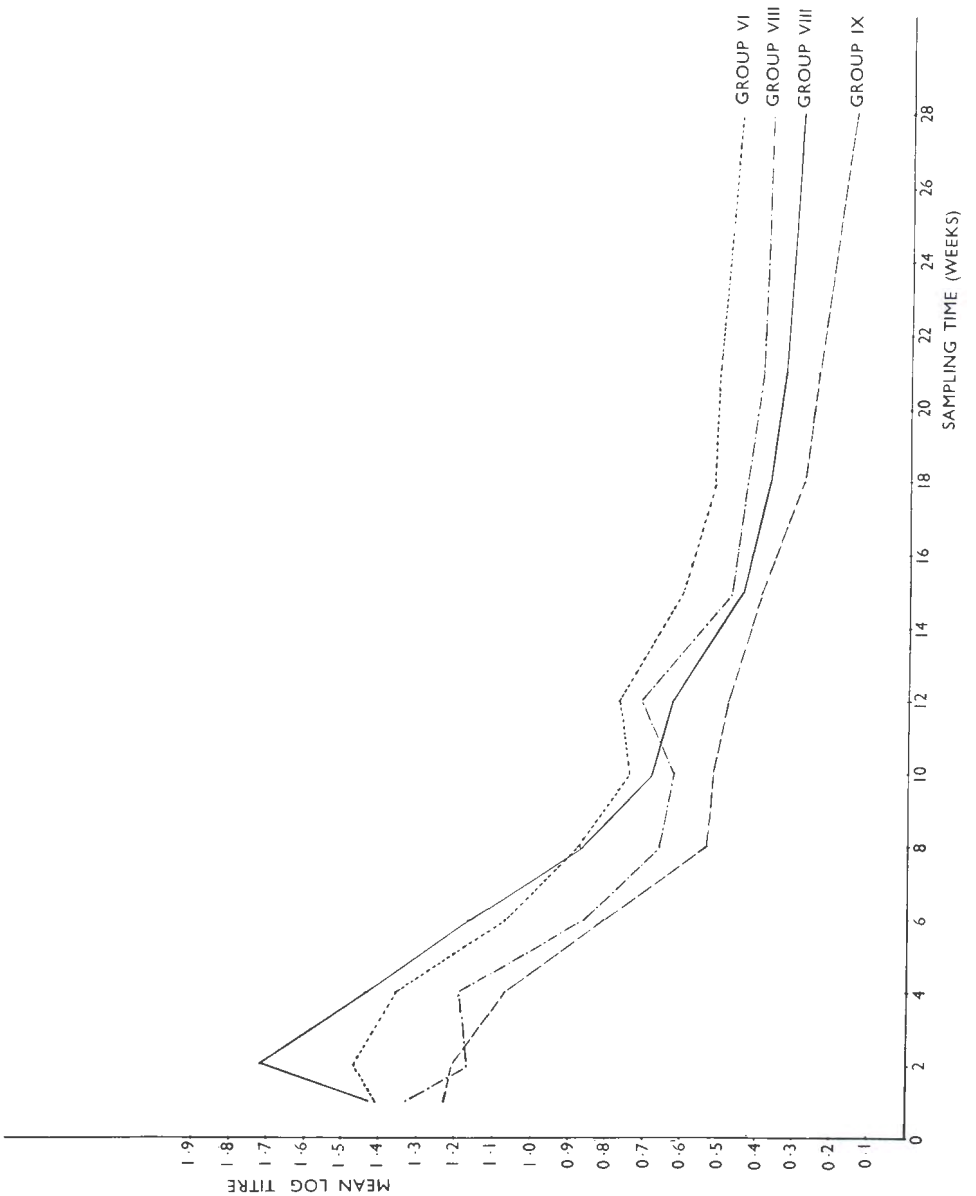


FIG. 9.—Graphical Representation of the Results obtained with Groups VI, VII, VIII and IX.



It is obvious from Fig. 8 that the degree of immunity of the sheep of Group I is inferior to that of any of the other groups, indicating that a single dose of 50 Lf liquid toxoid is a comparatively poor primary stimulus.

The responses in Groups 2, 3, 4 and 5 were very similar. An analysis of variance revealed no significant differences in the four group means at any of the eleven times of sampling. Although the variance ratio in no instance reached the level of significance ($F = 2{\cdot}88$, d.f. = 3 and 34, $P = 0{\cdot}05$), six of the eleven ratios exceeded the value 2. Furthermore, the four curves in question show regularity with regard to their relative positions: Curves III and V are more or less overlapping, Curve II is somewhat lower from the sixth to the 28th week and Curve IV is lowest through the entire sampling period. It is therefore not unreasonable to conclude that one year after a moderate primary stimulus of 50 Lf APT the effect of a small dose (6·25 Lf) of liquid toxoid may not be quite as good as the same dose of APT (Curve IV versus Curve II) but this possible difference disappears in sheep with a more solid basic immunity (Curve III versus Curve V) and such animals respond on the whole better (Curves III and V versus Curves II and IV).

On the basis of the results of Experiment 2, shown in Fig. 9, it might be tempting to conclude that the response to a booster dose of 100 Lf is better than that to a dose of 6·25 Lf, whether given in the form of liquid toxoid (Curve VIII versus Curve IX) or as APT (Curve VI versus Curve VII). However, the differences between the group means were negligible (eight of eleven variance ratios fell between 0·4 and 1·0) and the safest conclusion appears to be that neither the dose nor the nature of the toxoid used to reinforce immunity is of any great moment in sheep with a solid basic immunity, a confirmation of the results of Experiment 1.

From a consideration of the experimental results presented in this thesis a conclusion is justified that an APT containing 6·25 Lf per dose is adequate for a primary, secondary and booster stimulus. This point was further investigated experimentally by giving ten (Group X) fully susceptible sheep two injections of APT containing 6·25 Lf toxoid per dose (prepared to the same specifications as previous injections but using a different batch of toxoid) at an interval of three weeks. Three hundred and sixty-five days after the primary stimulus had been given they were bled (for the determination of the antibody titre of the sera) and given a booster dose of APT containing 6·25 Lf toxoid (prepared to the same specifications as previous injections but from yet another batch of toxoid). They were bled at weekly intervals subsequently and the antibody titre of their sera determined.

The results are given in Table 53.

TABLE 53.—*The antibody levels of the Sera of Sheep prior to a Booster Dose of* 6·25 *Lf APT and at weekly intervals subsequently* (*Units/ml.*)

Group X

Sheep No.	Titre prior to booster injection	Titre after one week	Titre after two weeks	Titre after three weeks
1	1·3	200·0	666·0	500·0
2	0·8	13·3	13·3	6·7
3	0·4	40·0	40·0	20·0
4	0·8	20·0	40·0	20·0
5	0·4	20·0	40·0	40·0
6	0·57	10·0	8·0	5·7
7	0·4	10·0	40·0	20·0
8	0·13	10·0	13·3	13·3
9	0·4	6·7	6·7	4·0
10	0·4	6·7	6·7	5·0

When the antibody levels, attained two weeks after the booster stimulus, are compared with those shown by the sheep in Groups III and VII (in which cases the mean log titres were the highest of the groups in Experiments 1 and 2 respectively, two weeks after the booster stimulus) the following will be found:—

(*a*) A comparison of the mean log titre of Group X with the mean log titre of Group III gives a value $t = 0 \cdot 8642$ for 18 degrees of freedom.

(*b*) A comparison of the mean log titre of Group X with the mean log titre of Group VII gives a value $t = 1 \cdot 5200$ for 17 degrees of freedom.

There is therefore no statistically significant difference in the responses between Groups III and X and between Groups VII and X, implying that an APT containing 6·25 Lf toxoid per dose is adequate for a primary, secondary and booster stimulus.

Conclusion

By a systematic study of the nutritional requirements of a variant strain of *Cl. welchii* Type B a procedure for obtaining considerably increased yields of toxin from the production medium was developed. While previously yields of 50 Lf per ml. were customary, values of 500 Lf per ml. were obtained in routine batches subsequent to the investigation. The deterioration of the active antigen during formolization was minimized by determining the optimal conditions for toxoiding. Furthermore, an increase in efficacy of the antigen effected by alum-precipitation was demonstrated. The routine application of the flocculation test for the quantitative determination of the antigen has been shown to save time, labour and experimental animals. These findings have decided economic significance in a laboratory where prophylactics are produced on a large scale.

Immunity studies in sheep delivered the following results of practical importance:—

(*a*) The primary and secondary injections of lamb dysentery vaccine can be separated by an interval of two, three, four or five weeks with the same measure of success. This allows a farmer considerable latitude in arranging his vaccination programme to suit his farming activities.

(*b*) When a booster injection is given as early as two months before lambing the ewes will have a sufficiently high level of immunity at parturition—thus the handling of heavily pregnant animals is eliminated.

(*c*) The economy of lamb dysentery vaccine production is affected by the fact that the same results can be obtained with APT containing 6·25 Lf per dose as with 200 Lf per dose.

In conclusion, therefore, the object of developing a proved, cheap and practicable method of producing a protective beta antitoxin level in ewes' blood under extensive farming conditions, has been attained.

Summary

Evidence is provided to prove the specificity of the flocculation test used as a tool for assaying the beta fraction of *Cl. welchii* Type B toxin and its corresponding antitoxin.

The separation from *Cl. welchii* Type B (" 1930 strain ") of variants which differ in the production of toxin and mucoid material is described.

A medium and method of cultivation are described by means of which a maximum yield of 560 Lf per ml. of beta toxin was obtained.

By using a sodium acetate-acetic acid buffer system a purified beta toxin containing 7,396 Lf per mg. P.N. was produced.

The keeping qualities of beta toxin under varying conditions were determined.

The toxoiding of beta toxin (both crude and purified) was investigated.

The stability of beta toxoid was determined.

A study was made of alum precipitation of beta toxoid with varying concentrations of potash alum at different pH levels.

APT was proved to be a better antigen than liquid toxoid.

It was proved experimentally that sheep, immunized with two doses of APT, each containing from 6·25 Lf to 200 Lf and separated by an interval of two weeks, would have a mean circulating antitoxin titre of about 50 units per ml. one week after the second stimulus.

As a result of a series of experiments in which the booster effect produced by beta toxoid was investigated the conclusion was reached that the essential requirement for an effective booster response is a solid basic immunity. When the latter is present the dose and nature of the booster injection are of secondary importance.

It was proved that APT containing 6·25 Lf beta toxoid per dose could be used as a primary, secondary and booster stimulus for the prevention of lamb dysentery.

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