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# The Production of Immunity against *Cl. welchii*, Type B, Wilsdon (The "Lamb Dysentery Bacillus").*

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THE investigation of the disease, lamb dysentery, followed well-established lines—field observation, isolation of the causative germ, reproduction of the disease in normal lambs by the administration of this germ, and, finally, the prevention of the naturally-occurring infection by the use of an antiserum or a prophylactic prepared with this microbe. With a toxin-producing germ, as *Cl. welchii*, Type B, one could aim to produce an antitoxic immunity, an antibacterial immunity or both. In actual field work, no purposeful attempt was ever made to produce an antibacterial immunity in sheep. Doubtless, antibacterial bodies were formed, due to the presence, in the material injected (toxin-antitoxin mixtures, formol-toxoids and anacultures) of autolysed bacteria or of the bacteria, themselves. Whether or not their presence played a useful rôle in the prevention of the disease is not known. As Type B is a toxin-producer, every endeavour was made to devise methods for the obtaining of high antitoxic immunity.

In this communication, the following aspects of the production of immunity will be discussed.

1. The effect on immunity production of:—
   (*a*) One and two injections of formol-toxoid (primary and secondary stimulus) and the interval between the injection of the antigen and the test.
   (*b*) The amount of formol-toxoid injected.
   (*c*) The value (total-antitoxin-binding-value) of the toxoid injected.
2. The immunizing power of small amounts of free toxin.
3. The production of immunity to Type B toxin, with Type A toxoid.
4. Methods of increasing immunity.
5. The production of immunity with Type B bacilli, freed from toxin.

* The nomenclature suggested by Wilsdon (1931) for the welch group of anaerobes will be used throughout this article.

Dalling and his colleagues (1928) have shown that the immunization of ewes with formol-toxoids or anacultures (formolised whole cultures) of *Cl. welchii*, Type B has reduced the incidence of lamb dysentery in the lambs issue of such ewes. For a number of years, anaculture only has been used for this purpose, chiefly because of the relative ease of production. In brief, its production is as follows:—The meat particles are removed from an 18 hours' meat broth culture of the germ, enough formalin (40 per cent. formaldehyde) to make a 0·4-0·5 per cent. concentration added, the pH adjusted to 7·4 and the material incubated at 37° C. until more than 0·25 c.c. (on intravenous injection) is required to kill a mouse. From 3-10 days at 37° C. are usually required. The value of the toxoid (the word " toxoid " will be used throughout to denote either formol-toxoid or anaculture) is now determined. Varying amounts of toxoid are mixed with 1 unit of a (laboratory) standard antitoxin, and after standing at room temperature for 1 hour, one-half of a test dose of toxin (i.e. one-half of that amount of toxin which is just neutralized by one unit of antitoxin) is added to each mixture. After a further half to one hour at room temperature, the mixtures are injected intravenously into mice. If, for example, the mouse receiving the mixture containing 1/20 c.c. of toxoid, 1 unit of antitoxin and ½ test dose of toxin dies, and that receiving the mixture containing 1/30 c.c. of toxoid lives, 1.0 c.c. of toxoid is said to be equivalent to 15 units of antitoxin, or briefly is of 15 units value.

In testing the immunity produced in animals by toxoids, two methods have been employed:—

(1) *The Cumulative Minimal Lethal Dose (Cumulative M.L.D.) Test* (Mason, Ross, and Dalling, 1931).—This method has given satisfactory results over a period of 7-8 years when applied to guinea-pigs immunized with Type B antigens. Briefly, it is based on the fact that Type B toxin, administered intravenously (i.v.) kills with great rapidity, so that, in the course of 6 hours, 32 or more minimal lethal doses (M.L.D.) may be given in 5 injections (e.g. M.L.D. 1, 3, 4, 8, 16). The result is recorded as " guinea-pig withstood 4-8, etc., M.L.D.", which means that it survived the injection of 4 M.L.D., but not that of a further 4 M.L.D.

(2) *The Titration of the Serum of the Immunized Animal or of the Pooled Sera of a Group of Animals.*—The latter procedure has proved most satisfactory in the writer's hands. In the results to be presented, it will be observed that, by the cumulative M.L.D. test, the individual variation of immunity response is greatly accentuated, some guinea-pigs dying after the injection of 1 M.L.D. and others of the same group withstanding 8 or 16 M.L.D. By testing the pooled sera (i.v. in mice), these individual variations are masked and a valuation figure can be given to the group. Further, the serum can be titrated to about 15-20 per cent. accuracy; such a difference would be difficult to show up by the cumulative M.L.D. method.

Experience over a number of years has shown that a toxoid of 20 or more units usually produces a satisfactory degree of immunity in guinea-pigs.

## EXPERIMENTAL.

### The Effect of a Single Injection of Toxoid.

The time elapsing between the injection of antigen and the appearance of circulating antitoxin depends, to a large extent, on the value of the antigen. After the injection of 2·0 c.c. of toxoid, value 80 units, into guinea-pigs, antitoxin is demonstrable in 10 days, but whether present before that time has not been ascertained. A low value toxoid of 5-10 units will not stimulate the production of demonstrable antitoxin in 2 up to 4 weeks after injection. However, that it does act as a primary stimulus is shown when a second injection is given 2-4 weeks later; antitoxin is then easily demonstrable 7-12 days after such a secondary stimulus.

In table 1 are recorded the results of testing, by the cumulative M.L.D. method, the immunity produced in guinea-pigs by one subcutaneous injection of a good value toxoid (valued 40 units).

The experiment shows that the maximum immunity is present between the 14th and 21st day after the injection of the antigen, is decreasing at the 28th day and is not demonstrable at the 90th day.

Table 1.

*The Effect of a Single Injection of Toxoid.*

| G.P. | Cum. M.L.D. test after | | | | |
|---|---|---|---|---|---|
| | 14 days. | 21 days. | 28 days. | 60 days. | 90 days. |
| 1..... | <1 | <1 | <1 | <1 | <1 |
| 2..... | 1–2 | <1 | <1 | <1 | <1 |
| 3..... | 3 | 1–2 | <1 | <1 | <1 |
| 4..... | 6–10 | 1–2 | <1 | <1 | <1 |
| 5..... | 6–10 | 2–4 | 1–2 | 1–2 | <1 |
| 6..... | >10 | 4–8 | 1–2 | 4–8 | <1 |

(The guinea-pigs received 2·0 c.c. of the same toxoid s.c., and 6 were removed and tested at the above stated periods.)

*The Effect of Two Injections of Toxoid* (the 2nd injection = " the secondary stimulus ").—Again, the value of the toxoid injected determines, to a large extent, the immunity response of the animals. Experiments, planned to show up differences in response of guinea-pigs, which received the secondary stimulus 14, 21, and 28 days respectively after the primary stimulus, did not give a decisive answer, i.e. no significant differences were got. The writer hesitates to stress this point because of the importance that is placed upon a relatively long interval in immunization with diphtheria and tetanus toxoids. Further, in the immunization of sheep, in the field, with Type B toxoid, a long interval, 4-5 months, is allowed, with excellent results, as judged by the immunity of the lambs to lamb dysentery and by the presence of antitoxin in the ewes' sera and colostra.

*The Effect of the Dose of Antigen.*—As a routine measure, 2·0 c.c. Type B toxoid is given s.c. to guinea-pigs and 5·0 c.c. to sheep. In one experiment, using 2·0 and 0·1 c.c. respectively of toxoid, value 15 units, as primary stimuli and testing, 3 weeks later, the pooled sera of 8 guinea-pigs from each group for its antitoxic power, the following results were got:—

2·0 c.c. guinea-pigs: 0·1 c.c. of serum neutralized 1 M.L.D. of toxin.

0·1 c.c. guinea-pigs: 0·3 c.c. of serum did not neutralize 1 M.L.D. of toxin.

The results of an experiment, planned to test the effect of the size of the secondary stimulus, are noted in table 2. The toxoid was of 25 units value, the primary stimulus was 2·0 c.c. s.c., and the interval between it and the secondary stimulus was 14 days. The immunity test (cumulative M.L.D.) was carried out 10 days after the second injection.

TABLE 2.

*The Effect of Varying the Size (Dose) of the Secondary Stimulus.*

*Test by Cum. M.L.D. Method.*

*Secondary Stimulus c.c.*

| G.P. | 2·0 | G.P. | 0·4 | G.P. | 0·1 |
|---|---|---|---|---|---|
| 1..... | 2–4 | 9..... | < 4 | 21..... | <2 |
| 2..... | 8–16 | 10..... | < 6 | 22..... | <3 |
| 3..... | 8–16 | 11..... | <14 | 23..... | <3 |
| 4..... | 16–20 | 12..... | <14 | 24..... | <4 |
| 5..... | 20–30 | 13..... | <14 | 25..... | <6 |
| 6..... | 20–30 | 14..... | <14 | 26..... | <6 |
| 7..... | >40 | 15..... | 4–8 | 27..... | 4–10 |
| 8..... | >64 | 16..... | 4–8 | 28..... | 8–20 |
| | | 17..... | 4–8 | | |
| | | 18..... | 8–16 | | |
| | | 19..... | 16–32 | | |
| | | 20..... | 16–32 | | |

One can say, from the above results, that a 20 times reduction in size of the secondary stimulus results in a decreased immunity response. One would hesitate to say that a 5 times reduction has a definite effect; the indication is that of lessened response, but the experiment would have to be repeated on a large number of animals and the results examined statistically before a final answer could be given.

*The Effect of the Value of the Antigen.*—In table 3 is recorded the immunity produced by 2 injections of toxoids of different value. A similar table could be presented, showing that 1 injection of toxoid (5), value 80 units, so immunized 6 guinea-pigs that they withstood 1-4, 4-8, 4-8, 8, 8-16, and 16-24 M.L.D. of toxin respectively, whereas toxoid (2), value 10 units was incapable of immunizing any one of 6 guinea-pigs to resist 1 M.L.D. of the same toxin.

TABLE 3.

*The Effect of the Value of the Toxoid Injected.*

| *Toxoid.* | | | | |
|---|---|---|---|---|
| (1) | (2) | (3) | (4) | (5) |
| Value (units). 15 | Value (units). 10 | Value (units). 5 | Value (units). $2\frac{1}{2}$ | Value (units). 80 |
| | | *Cum. M.L.D. test.* | | |
| G.P. | G.P. | G.P. | G.P. | G.P. |
| 1..... <1 | 1..... <1 | 1..... <1 | 1..... <1 | 1..... 16–32 |
| 2..... 4–8 | 2..... 1–2 | 2..... <1 | 2..... <1 | 1..... >32 |
| 3..... 4–8 | 3..... 2–4 | 3..... <1 | 3..... <1 | 3..... >32 |
| 4..... 16 | 4..... 2–4 | 4..... <1 | 4..... <1 | 4..... >32 |
| 5..... >32 | 5..... 4–8 | 5..... 4–8 | 5..... 1–2 | 5..... >32 |
| | | 6..... 4–8 | 6..... 1–2 | 6..... >32 |
| | | 7..... 8–16 | | 7..... >32 |
| | | 8..... 8–16 | | |

[The figures opposite G.P. (guinea pig) 1, 2, etc., are the number of M.L.D. tolerated by the animal.]

Table 3 shows clearly that the antigenic power of a toxoid is in direct relation to its antitoxin binding value. However, one cannot assume, from the above results, that a toxoid of 20 units will be a better antigen than one of 15 units, although one could be almost certain that it would be better than one of 5 units and poorer than one of 80 units. Glenny (1931) discusses the effect, on immunity production, of non-specific material in diphtheria toxoid and suggests that toxoids contain 99 per cent. non-specific and only 1·0 per cent. specific substance. It will readily be appreciated that one lot of toxoid may contain more non-specific material than another and that much of this material may, itself, be antigenic. Again, there is the possibility that the antigen, itself, may vary qualitatively from batch to batch.

The effect of the solubility of the antigen and the route of injection will be discussed under "Methods of Increasing Immunity".

*Unresponsive Animals.*—It will have been noticed, in the results recorded, that the immunity response to the injection of the same toxoid varies enormously in individual guinea-pigs. When this happening was first encountered, it was feared that some guinea-pigs had not been injected and that others had received more than the desired number of inoculations. However, when the phenomenon occurred with every lot of toxoid tested, it was realized that the cause of the apparent discrepancy was the animal itself. To ascertain if the immunity response of the individual was of the same order when two antigens were injected at the same time, the following experiment was carried through. Two groups of guinea-pigs received two injections of Type B toxoid (2·0 c.c.) at 21 days' interval and were tested by the cumulative M.L.D. method 14 days after the secondary stimulus. At the time of the first inoculation, 1·0 c.c. of diphtheria toxoid was injected into one group and 5·0 c.c. into another. Three weeks after this inoculation, each guinea-pig was

bled and its serum titrated for the presence of diphtheria antitoxin. (The writer is indebted to his former colleague, Mr. A. T. Glenny, for carrying out these tests.) Table 4 records the results.

TABLE 4.

*Comparison of the Immunity Response of Guinea-Pigs Injected at one time with Type B and Diphtheria Toxoids.*

| GROUP 1. | | | GROUP 2. | | |
|---|---|---|---|---|---|
| 2 injections of 2 c.c. Type B toxoid, 1 injection of 1 c.c. dip. toxoid. | | | 2 injections of 2 c.c. Type B toxoid, 1 injection of 5 c.c. dip. toxoid. | | |
| G.P. | Dip. AT. | Type B. | G.P. | Dip. AT. | Type B. |
| 1..... | 1/1000 | 1–4 | 1..... | 1/1000 | 12–32 |
| 2..... | 1/10 | >32 | 2..... | 1/25 | 1–4 |
| 3..... | 1/1000 | 4–12 | 3..... | 1/25 | 12–32 |
| | | | 4..... | 1/1000 | 4–8 |
| | | | 5..... | 1/1000 | 1–4 |
| | | | 6..... | 1/250 | >32 |

(Dip. AT = diphtheria antitoxin; the figures under Dip. AT. = units; the figures under Type B = the result of the cumulative M.L.D. test.)

Whilst there may be some degree of correlation, the results do not indicate that it is very close.

## METHODS OF INCREASING IMMUNITY.

(1) THE EFFECT OF MAKING ATOXIC TOXOID SLIGHTLY TOXIC.

(*a*) To 2·0 c.c. amounts of a rather low value Type B toxoid (11½ units), 10 mouse M.L.D. of Type A, Type B and *Cl. septique* toxins, respectively, were added. Guinea-pigs received 2·0 c.c. s.c. twice (14 days' interval), and were tested (cumulative M.L.D.) 12 days after the secondary stimulus. The results are given in table 5.

TABLE 5.

*The Effect of making Toxoid Slightly Toxic.*

| G.P. | Toxoid only. | Toxoid plus | | | Type B toxin alone 10 M.L.D. |
|---|---|---|---|---|---|
| | | Type A toxin. | Type B toxin. | *Cl. sept.* toxin. | |
| 1..... | <1 | 4–8 | <1 | <1 | <1 |
| 2..... | <1 | 16–24 | 24–40 | <1 | <1 |
| 3..... | <1 | 16–24 | 24–40 | 8–16 | <1 |
| 4..... | 4–8 | 24–40 | 50–60 | 24–40 | <1 |
| 5..... | 4–8 | 24–40 | 50–60 | | 1–2 |
| 6..... | 16–24 | | >60 | | |

(*Cl. sept.* = *Cl. septique*; the Type B toxin alone was diluted in broth and 2·0 c.c. injected.)

*Local reactions in above guinea-pigs* (necrosis or evidence of necrosis): —

| | 14 *days after first inoculation.* |
|---|---|
| Toxoid alone | 0/6 |
| Toxoid plus Type A toxin | 5/5 |
| Toxoid plus Type B toxin | 5/6 |
| Toxoid plus *Cl. septique* toxin | 4/4 |
| Type B toxin (10 mouse M.L.D.) | 5/5 |

(5/5 = 5 of 5 animals injected had local reactions.)

(*b*) An experiment carried out with the same toxoid as in (*a*) above. The number and the spacing of the injections and the test was the same. The difference was that smaller amounts of toxin were added. The results are given in table 6.

TABLE 6.

*The Effect of making Toxoid Slightly Toxic.*

| G.P. | Toxoid only. | Toxoid plus | | | | |
|---|---|---|---|---|---|---|
| | | Type A toxin 7 M.L.D. | Type A toxin 3 M.L.D. | Type B toxin 5 M.L.D. | Type B toxin 2 M.L.D. | *Cl. sept.* toxin 10 M.L.D. |
| 1... | <1 | 4–8 | <1 | <1 | <1 | <1 |
| 2... | <1 | 8–16 | <1 | 8–16 | 1–4 | <1 |
| 3... | <1 | 8–16 | <1 | 16 | 4–8 | 1–4 |
| 4... | <1 | 8–16 | 1–4 | 16–32 | 4–8 | 4–8 |
| 5... | <1 | 8–16 | 4–8 | >32 | 16–32 | 4–8 |
| 6... | 1–4 | >32 | 8–16 | >32 | 16–32 | 4–8 |
| 7... | 8–16 | >32 | 8–16 | >32 | >32 | 8–16 |
| 8... | 8–16 | >32 | >32 | | >32 | |
| 9... | >32 | >32 | >32 | | | |

*Local Reactions in above Guinea-pigs.*

| | 14 days after *first inoculation.* |
|---|---|
| Toxoid alone | 0/9 |
| Toxoid plus 7 M.L.D. Type A toxin | 7/9 + 2/9 ? Compare. |
| Toxoid plus 3 M.L.D. Type A toxin | 1/9 + 2/9 ? |
| Toxoid plus 5 M.L.D. Type B toxin | 1/7 + 1/7 ? |
| Toxoid plus 2 M.L.D. Type B toxin | 0/8 |
| Toxoid plus 10 M.L.D. *Cl. septique* toxin | 6/7 + 1/7 ? |

(7/9, etc. = 7 of 9 guinea-pigs injected showed definite reactions; 2/9?, etc. = 2 of 9 guinea-pigs injected showed very mild reactions; guinea-pigs not accounted for showed no reactions.)

(*c*) The effect of the addition of toxin to very low value toxoid was investigated. The toxoid used was of 2½ unit value. The procedure was exactly as for (*a*). Table 7 records the results.

TABLE 7.

*The Effect of making a Low Value Toxoid Slightly Toxic.*

| G.P. | Toxoid ($2\frac{1}{2}$ units) plus | | |
|---|---|---|---|
| | Nil. | Type B toxin 10 M.L.D. | Type A toxin 10 M.L.D. |
| 1 | <1 | <1 | <1 |
| 2 | <1 | <1 | <1 |
| 3 | <1 | 1–4 | <1 |
| 4 | <1 | 1–4 | <1 |
| 5 | <1 | 8–16 | <1 |
| 6 | 1–4 | | <1 |
| 7 | 1–4 | | 1–4 |
| 8 | | | 1–4 |

(*d*) The effect of a single injection of slightly toxic toxoid was investigated. To 2·0 c.c. amounts of a toxoid, value 25 units, no toxin, 10 mouse i.v. M.L.D. of Type B and 8 mouse i.v. M.L.D. of Type A toxin were added, and injected s.c. into guinea-pigs. Fourteen days later, the cumulative M.L.D. test was applied. The results are given in table 8.

TABLE 8.

*The Effect of a Single Injection of Toxoid made slightly Toxic.*

| G.P. | A single injection of toxoid (25 units) plus | | |
|---|---|---|---|
| | Nil. | Type B toxin 10 M.L.D. | Type A toxin 8 M.L.D. |
| 1 | <1 | <1 | <1 |
| 2 | <1 | <1 | 1–2 |
| 3 | <1 | <1 | 3 |
| 4 | <1 | <1 | 6–10 |
| 5 | <1 | 1–2 | 6–10 |
| 6 | <1 | 1–2 | >10 |
| 7 | 1–2 | 1–2 | |
| 8 | | 3–6 | |
| 9 | | 6–10 | |

These four experiments show clearly that amounts of free toxin insufficient, of themselves, to produce demonstrable immunity, are capable, when mixed with toxoid of fair or good value, of improving greatly the antigenic power of the toxoid. However, when added to a very poor toxoid, no significant difference is got. Further, the tests show that only the homologous toxin, Type B, or one having a toxic fraction in common with Type B, viz. Type A, is capable of producing the effect; *Cl. septique* toxin was not able to enhance

the value of the antigen. This also shows that the production of a local reaction does not, *per se*, increase the antigenic power of a toxoid.

(2) The Effect of Adding Non-specific Material to Toxoid.—The problem of increasing the immunity, produced by diphtheria formol-toxoid, has occupied the attention of many workers, in particular, that of Ramon and his co-workers and Glenny and his colleagues. Ramon (1926) noted the stimulating effect on antitoxin production in the horse when finely ground tapioca was injected with the antigen. The formation of an inflammatory reaction was an accompaniment of the increased immunity. Glenny *et al.* (1926) and Glenny and Waddington (1928) reported upon the increased immunity resulting from the addition of potash-alum to toxoid. Mazzucchi (1929) has recorded the increased protection against anthrax, afforded to animals, when the vaccine is injected along with the irritant glucoside, saponin. To detail all the publications dealing with the non-specific stimulation of immunity would be tedious, but among the substances used are the following: calcium chloride, turpentine, toluol, oil, lanoline, killed germs and cholesterin.

There are two aspects of the problem (1) the purely scientific, to discover if immunity can be increased with any substance and (2) the practical, to discover those substances, not, in themselves, unduly irritating or toxic which have an activating effect. The second aspect may be further subdivided into (*a*) the production of hyperimmune sera in horses, where the formation of swellings and abscesses does not cause undue anxiety, (*b*) the production of immunity in man, and particularly in children, where abscess formation and induration is of serious moment, and (*c*) the production of immunity in the domestic animals, where reactions are allowable, so long as they are of mild degree. It was with this last aspect that the writer busied himself. Another point, of definite practical importance, had to be kept in mind. The non-specific activator had to be cheap, the preparation of the final product had to be simple, and finally, this product had to be of such consistency that it could be easily injected with a syringe. From the inception of the work, the writer favoured a method which would render the antigen, itself, relatively insoluble. The work of Glenny, Buttle and Stevens (1931) showed that diphtheria toxoid is rapidly eliminated from the body and that the increased immunity resulting from the injection of an alum-precipitated toxoid is due to the slow absorption of the product. For this reason, a number of experiments were carried out to determine the immunizing effect on guinea-pigs of alum precipitates of Type B toxoids.

*Experiments with Alum.*

(*a*) *Yield.*—The most copious yields were obtained when enough alum was added to toxoids to make a 0·5–3·0 per cent. concentration; at 10·0 per cent. it was small and at 0·1 per cent., very small. (Note.—In the following discussion the expression "1·0, etc., per cent. alum was added" will mean that enough alum was added to make a 1·0 per cent. concentration.)

(*b*) *Value and Nitrogen Content.*—The alum was added to 50 c.c. amounts of toxoid, pH 7·4, in the stated percentages, the precipitate washed twice in distilled water, and the determinations made on the precipitate, dissolved in 2·0 per cent. Rochelle salt. (The writer is indebted to his former colleague, Miss M. Barr, for the nitrogen results.)

| Toxoid B2. | *Per cent. alum.* | | | | | |
|---|---|---|---|---|---|---|
| | 0·0 | 1·0 | 1·5 | 2·0 | 2·5 | 3·0 |
| Value (units)..... | 12·5 | 2·5 | 2·5 | 5·0 | 6·0 | 2·5 |
| Nitrogen (per cent.)..... | 0·36 | 0·014 | 0·046 | 0·05 | 0·058 | 0·036 |

| Toxoid 309. | Per cent. alum. | | | | | |
|---|---|---|---|---|---|---|
| | 0·0 | 1·0 | 1·5 | 2·0 | 2·5 | 3·0 |
| Value (units)..... | 10·0 | 2·5 | 2·5 | ? 2·5 | 2·5 | 2·5 |
| Nitrogen (per cent.)..... | 0·38 | 0·051 | 0·052 | 0·046 | 0·046 | 0·038 |

*Immunity Experiment with Toxoid* 309.—The twice washed 1·5 per cent. alum precipitate, resuspended in saline back to the original volume, and the toxoid, itself, were injected, in 2·0 c.c. amounts, s.c. into guinea-pigs. After 21 days, some animals were tested for immunity (cumulative M.L.D.) and the remainder reinoculated with the antigens. These were tested after a further 10 days. Table 9 records the results.

TABLE 9.

*Immunizing Power of Alum-Toxoid.*

| Toxoid. Immunity after injection. | | | | Alum precipitate. Immunity after injection. | | | |
|---|---|---|---|---|---|---|---|
| G.P. | 1st | G.P. | 2nd | G.P. | 1st | G.P. | 2nd |
| 1.... | <1 | 13.... | <1 | 5.... | <1 | 19.... | < 1 |
| 2.... | <1 | 14.... | <1 | 6.... | <1 | 20.... | 8–16 |
| 3.... | <1 | 15.... | <1 | 7.... | 1–3 | 21.... | 8–16 |
| 4.... | <1 | 16.... | 1–4 | 8.... | 1–3 | 22.... | 8–16 |
| | | 17.... | 8–16 | 9.... | 1–3 | 23.... | >32 |
| | | 18.... | >32 | 10.... | 1–3 | 24.... | >32 |
| | | | | 11.... | 3–6 | 25.... | >32 |
| | | | | 12.... | 3–6 | | |

(Reactions—none of the toxoid guinea-pigs had reactions; all the alum-toxoid animals had small sterile abscesses.)

The indication is strong that, although the alum precipitate was only a quarter of the value of the original, it produced a much higher immunity.

(*c*) Toxoid, value 15 units, pH 7·4; 0·001 per cent., 0·01 per cent., 0·1 per cent., and 1·0 per cent. alum *added;* the precipitate (unwashed) *plus* the supernatant, injected s.c. into guinea-pigs (2·0 c.c.). Test (cumulative M.L.D.) 14 days after one inoculation and eleven days after two inoculations (21 days' interval). The results are given in Table 10.

(*d*) As (*c*), using a toxoid, value 12½ units, and testing the effect of *adding* 1·0 per cent. alum to it. (The precipitate *plus* the supernatant injected.) Table 11 records the results.

TABLE 10.

*Immunizing Power of Toxoid Plus Alum.*

| G.P. | Alum, per cent. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0·0 | | 1·0 | | 0·1 | | 0·01 | | 0·001 | |
| | Immunity after injection. | | | | | | | | | |
| | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) |
| 1..... | <1 | <1 | 1–3 | <25 | <1 | < 5 | <1 | <20 | <1 | <15 |
| 2..... | <1 | 1–3 | <3 | >10 | <1 | <10 | <1 | <20 | <2 | <25 |
| 3..... | 1–2 | 3–7 | <3 | >15 | <1 | <10 | <1 | <10 | <3 | <25 |
| 4..... | | 3–7 | <3 | >20 | | | | >15 | 2–3 | >10 |
| 5..... | | 7–9 | >6 | | | | | 15 | >7 | >15 |
| 6..... | | 10–17 | | | | | | | | >15 |
| 7..... | | >14 | | | | | | | | >20 |

[The (1) and (2) = first and second inoculation.]

TABLE 11.

*Immunizing Power of Toxoid Plus Alum.*

| *G.P.* | *Immunity after one injection of* | |
|---|---|---|
| | *Toxoid.* | *Toxoid plus 1 p.c. alum.* |
| 1..... | <1 | <1 |
| 2..... | 1–2 | <1 |
| 3..... | 2–4 | <1 |
| 4..... | 2–4 | <1 |
| 5..... | 4–8 | 1–2 |
| 6..... | | 4–8 |

(*e*) A toxoid, value 12½ units, pH 7·4 was treated as follows:—

1. 1·0 per cent. alum added, and the precipitate *plus* the supernatant injected.
2. 1·0 per cent. alum added, the precipitate washed twice in distilled water, resuspended in saline to the original volume and injected.

3. A 1·0 per cent. alum precipitate washed twice in distilled water, dialysed against distilled water for 4 days at 5° C., suspended in distilled water to the original volume and injected.
4. 1·0 per cent. finely ground tapioca added, and injected.
5. The toxoid, without treatment, injected.

Guinea-pigs received 2·0 c.c. of the above products and, after three weeks, were bled and 0·1 c.c. of their sera titrated against two mouse i.v. M.L.D. of Type B toxin. No serum neutralized this amount. On testing by the cumulative M.L.D. method, the following results were got (Table 12):—

TABLE 12.

*Immunizing Power of Alum Toxoids and of Toxoid Plus Tapioca.*

| G.P. | Immunity after one injection of modification. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Toxoid (no treatment). |
| 1..... | <1 | <1 | <1 | <1 | <1 |
| 2..... | <1 | 1–2 | <1 | <1 | 1–2 |
| 3..... | <1 | 1–2 | <1 | 1–2 | 1–2 |
| 4..... | <1 | 1–2 | <1 | 1–2 | 1–2 |
| 5..... | <1 | 1–2 | <1 | 1–2 | 1–2 |
| 6..... | <1 | 1–2 | 1–2 | 1–2 | 1–2 |
| 7..... | <1 | | 1–2 | 1–2 | 1–2 |
| 8..... | 1–2 | | 2–4 | 1–2 | |

(*f*) The 1·0 and 10·0 per cent. alum precipitates of a toxoid, value 80 units, were washed twice in saline. Their value was less than $2\frac{1}{2}$ units. The immunity test (cumulative M.L.D.) in guinea-pigs, 21 days after a single injection of 2·0 c.c., is recorded in Table 13.

TABLE 13.

*Immunizing Power of 1·0 per cent. and 10·0 per cent. Alum Precipitates.*

| G.P. | Immunity after one injection of | | |
|---|---|---|---|
| | Toxoid. | 1·0 p.c. Alum precipitate. | 10·0 p.c. alum precipitate. |
| 1..... | 1–4 | <1 | <1 |
| 2..... | 4–8 | <1 | <1 |
| 3..... | 4–8 | <1 | <1 |
| 4..... | 8 | 1–2 | 1–2 |
| 5..... | 16–24 | 1–2 | 1–2 |
| 6..... | 24–40 | 1–2 | 1–2 |
| 7..... | | 1–2 | 4–8 |

These immunity experiments, planned to show the stimulating effect of an alum precipitate (a precipitate which is only very slightly soluble in water) are very disappointing. The reason would appear

to be the inability of alum to precipitate out more than a fraction of the antigenic material. With toxoid 309 [see under (*b*)], 1·5 per cent. alum precipitated 50 per cent. of the active material, whereas with the toxoid used in (*f*) less than 1/30 was obtained in a 1·0 per cent. precipitate. It would appear that with *each* batch of toxoid, a preliminary experiment should be carried out, in which samples are precipitated with 1·0–10·0 per cent. alum and the value and nitrogen content determined. That amount of alum which gives a precipitate, containing the most units per mgm. N should be used, i.e. where the units: mgm. N ratio is high. However, even granting that such a procedure would lead to the obtaining of a better antigen, the results recorded do not indicate that the method has much to recommend it as concerns the increasing of the value of Type B toxoids. In passing, it may be mentioned that the method has proved valuable with toxoids of *Cl. septique* (writer's unpublished observations).

It should also be noted that the alum precipitates produced local reactions in many of the guinea-pigs injected (abscesses containing a thick pus, usually sterile). No indication was got that animals with such reactions were more immune than those without them.

*Experiments with Agar, Saponin and Colloidal Iron.*

These experiments will not be detailed, as no definite indication was got that the addition of the substance to toxoid increased its immunizing value.

(*a*) *Agar.*—Added, whilst liquid, to toxoid to make 0·1, 0·25 and 0·5 per cent. concentrations. The immunizing power tested on sheep and guinea-pigs; no definite advantage over toxoid.

(*b*) *Saponin.*—Added to toxoid to make a 0·025–0·05 per cent. concentration. The guinea-pigs injected all developed necrosis (sometimes severe). Results as for agar. These experiments again show that the production of a local reaction did not, *per se*, increase the immunizing value of the toxoid.

(*c*) *Colloidal Iron.*—Equal parts of toxoid and negatively charged iron (8 mgm. Fe/cc) and positively charged iron (2·4 mgm. Fe/cc.) respectively tested on guinea-pigs. All animals developed large swellings. Results as for agar. (The writer's colleague, Dr. A. I. Malan, kindly prepared the colloidal iron.)

*Experiments with Zinc Chloride and the Intraperitoneal Route.*

Alum-precipitates having proved disappointing, the writer looked around for other protein precipitants, the precipitates of which were relatively insoluble in water. Zinc chloride at once suggested itself. (The writer is aware of the use of this substance for the purification of toxoids and toxins, but recalls no published work, in which zinc chloride precipitates have been used for immunization purposes. However, that they have been used for such purposes, he can hardly doubt.) The procedure adopted was similar to that mentioned under " alum precipitation ". The $ZnCl_2$ was added to the toxoid, the precipitate washed twice in water, and resuspended in saline. At the same time, the effect of injecting toxoid intraperitoneally was established.

*Details of Experiments.*

(*a*) *Toxoid* 7.—Value 80 units; *$ZnCl_2$ precipitate*, value 50 units (the precipitate dissolved in 2·0 per cent. sod. citrate and the T.C.P. established; the value is that in terms of the original volume of toxoid precipitated).

*Dose:* 2·0 c.c. *Route:* s.c. and i.p. for toxoid and s.c. for $ZnCl_2$ precipitate. *Test*: the titration of the pooled sera of each group of guinea-pigs against toxin (mouse i.v.) and the cumulative M.L.D. test.

(*b*) *Toxoid* 8.—Value 80 units; *$ZnCl_2$ precipitate*, value 50–60 units. Dose and injections as toxoid 7. Cum. M.L.D. test not done.

(*c*) *Toxoid* 9.—Value 20 units; *$ZnCl_2$ precipitate*, value 20 units (suspended in saline to half the original volume). *Dose:* 2·0 c.c. *Route:* s.c. only. *Test:* pooled sera only tested.

In the case of toxoid 9, a preliminary experiment was carried out to determine, if possible, the optimum conditions for precipitation with $ZnCl_2$. The toxoid was precipitated at pH 6 and at pH 7·4 with 0·1, 0·25, 0·5, 1·0, 2·5 and 5·0 per cent. $ZnCl_2$, and the values of the precipitates determined. About 50 per cent. of the antigen was obtained in the 0·5–5·0 per cent. precipitates, and only traces in the 0·1 and 0·25 per cent. precipitates. Further, it seemed as if the 2·5 per cent. yield of the toxoid, pH 7·4, was the least soluble in 2·0 per cent. sod. citrate, but the difference in the solubilities of the various products was very slight.

The tests of the immunity produced were carried out 21 days after the injection of the antigens. Table 14 records the results.

TABLE 14.

*No. Mouse i.v. M.L.D. Neutralized by 0·1 c.c. Pooled Serum.*

| | S.C. | I.P. | Zn. |
|---|---|---|---|
| *Toxoid* 7..... | 4–5 | 40 | 40 |
| *Toxoid* 8..... | 5 | 50 | 12 |
| *Toxoid* 9..... | ? 1 | | 3–4 |

TOXOID 7.

*Cumulative M.L.D. Test.*

| G.P. | S.C. | I.P. | Zn. |
|---|---|---|---|
| 1..... | 1–4 | 16–40 | 16–24 |
| 2..... | 4–8 | >40 | 24–40 |
| 3..... | 4–8 | >40 | 24–40 |
| 4..... | 8 | | >40 |
| 5..... | 16–24 | | >40 |
| 6..... | 16–24 | | >40 |
| 7..... | 24–40 | | |

(S.C. = immunization by subcutaneous route; I.P. = immunization by intraperitoneal route; Zn. = immunization by $ZnCl_2$ percipitate.)

In all three experiments, the $ZnCl_2$ precipitates (toxoid 7, 1·0 per cent., toxoid 8, 1·0 per cent., toxoid 9, 2·5 per cent.) proved superior as antigens to the toxoids, themselves. A possible and even probable explanation of the failure of the precipitates of toxoids 8 and 9 to produce the same degree of immunity as that of toxoid 7 is as follows. That of toxoid 7 settled out quickly in saline, was with difficulty dissolved in 2·0 per cent. sod. citrate and produced mild local reactions in guinea-pigs. Those of toxoids 8 and 9 formed colloidal-like suspensions in saline, were easily dissolved in citrate and produced no local reactions. Thus, it would appear that the question is that of solubility; the precipitate of toxoid 7 was very slowly absorbed, high immunity resulting, whilst those of toxoids 8 and 9 were more easily dissolved in the animal body and thus more easily absorbed, producing, as consequence, a lower degree of immunity.

One cannot invoke slow absorption as an explanation for the very high degree of immunity produced by the intraperitoneal injection of toxoids 7 and 8. As Table 14 shows, the immunity was about 10 times greater by this route than by the subcutaneous. Rather would the opposite hold—that the very rapid absorption and "shocking" of the system was the cause. It is well known that this "shock" method answers well in the production of antivirucidal sera and in the later stages of the production of hyperimmune antitoxic sera. However, the writer prefers to present the facts without offering a dogmatic opinion.

*Testing of Immunity with Activated Spores.*

The method of preparing the spore suspension is as follows. By culturing *Cl. welchii*, Type B on solid serum for a few days, a suspension, rich in spores, may be obtained. By heating such material at 65° C. for half an hour, most or all of the vegetative elements are killed; the suspension of spores, so produced, has proved stable, in the writer's hands, for at least three months. These spores may or may not, depending upon the number injected, kill a guinea-pig, but 1/100 of a border-line lethal dose will, when "activated", produce death. The activating agent may be calcium chloride, lactic acid, or glycerine, but, in the author's experience, adrenalin has proved eminently satisfactory. In practice, a dose of spores is added to 0·02 c.c. of 1/1000 adrenalin (as received from the manufacturers) and enough saline added to bring the total volume of fluid to 2·0 c.c. Such, injected i.m. or s.c. into guinea-pigs produces typical gas gangrene. One must exercise the greatest care in working with adrenalin; 0·1 c.c. diluted to 1·0 c.c. with saline will, on injection, produce death with a post mortem picture, simulating that of gas gangrene.

Great use has not been made of this method of testing immunity, but, as will be seen later, guinea-pigs, immune to toxin, resist the injection of activated spore suspension. To dilutions of the stock suspension (held at 5° C.) 0·02 c.c. of adrenalin was added and the total volume of fluid made up to 2·0 c.c. with saline. The dilutions were injected intramuscularly into guinea-pigs. The animals chosen

for the test were those immunized with toxoid 8 (s.c. injection), toxoid 8 (i.p. injection) and the $ZnCl_2$ precipitate of toxoid 8 (s.c. injection). The test was carried out 28 days after the immunizing dose. Table 15 records the results.

TABLE 15.

*Test of Immunity with Spore Suspension.*

| G.P. | Antigen. | | | |
|---|---|---|---|---|
| | Toxoid i.p. | Toxoid s.c. | $ZnCl_2$ ppt. | Dose spores c.c. |
| 1..... | L | L | + 72 hr. | 0·5 |
| 2..... | L | L | L | 0·5 |
| 3..... | L | + 72 hr. | L | 0·25 |
| 4..... | L | + 72 hr. | L | 0·25 |
| 5..... | L | + 72 hr. | L | 0·1 |
| 6..... | L | L | L | 0·1 |
| 7..... | L | L | | 0·1 |

| Control G.Ps. | Dose spores c.c. | Result. |
|---|---|---|
| 1..... | 0·01 | + o/n |
| 2..... | 0·03 | + 36 hr. |
| 3..... | 0·1 | + o/n |
| 4..... | 0·25 | + 24 hr. |

(L = alive at the end of a week; + = died; hr. = hours; o/n = overnight. Activating dose of adrenalin = 0·02 c.c.; total volume injected = 2·0 c.c.)

The results given above confirm in some measure those of the titration of the antitoxin, as given in Table 14. More guinea-pigs survived the spore injection in the groups whose pooled sera contained the most antitoxin (toxoid i.p. and $ZnCl_2$ ppt.). However, it will be noticed that the test is more qualitative than quantitative; three guinea-pigs of the toxoid s.c. group did not witstand 0·25 c.c., 0·25 c.c. and 0·1 c.c. respectively of spores, whereas two others resisted 0·5 c.c. Further, the survivors of all groups had hard, swollen legs, due probably in great measure to the adrenalin.

*Production of Immunity with Heated Toxin and Toxoid.*

*Material Used.*—The toxin from which toxoid 8 was made and the toxoid itself.

*Treatment.*—The toxin boiled half an hour; the toxoid heated at 60° C. for half an hour; the toxoid boiled half an hour.

*Values.*—Toxoid, 80 units; 60° C. toxoid, 40 units; boiled toxoid, $<2\frac{1}{2}$ units; boiled toxin, $<2\frac{1}{2}$ units.

*Injections.*—Toxoid and 60° C. toxoid—1 injection s.c., test 21 days later; boiled toxin and boiled toxoid—2 injections, 21 days' interval, test 10 days after the 2nd injection.

*Results: Toxoid.*—0·1 c.c. of the pooled sera neutralized 5 mouse i.v. M.L.D. toxin.

60° *C. Toxoid.*—0·1 c.c. of the pooled sera neutralized 1 mouse i.v. M.L.D. of toxin. By the cumulative M.L.D. method, 4 guinea-pigs did not withstand 1 M.L.D. and 3 stood 1—3.

*Boiled Toxin and Toxoid.*—The pooled sera of neither group neutralized 1 mouse i.v. M.L.D. of toxin; no animal withstood 1 M.L.D. of toxin i.v.; the three guinea-pigs of the boiled toxoid group tested did not survive the injection of 0·1 c.c. of activated spore suspension (given in the same way was as noted in Table 15).

These results show clearly that the antigen contained in Type B toxin and toxoid is thermo-labile; it is destroyed on boiling and its value halved when heated at 60° C. for half an hour.

### Production of Immunity by the Injection of Small Amounts of Toxin.

(1) *Immunity after One Sub-lethal i.v. Dose of Toxin.*—Into seven guinea-pigs, which had received, i.v., from $\frac{1}{2}$–$\frac{3}{4}$ of one sure M.L.D. of toxin, was injected, also i.v., one certain fatal dose of the same toxin 15 days later. All died.

(2) *Immunity after Several Sub-lethal i.v. Doses of Toxin.*

(*a*) A guinea-pig received $\frac{1}{2}$ M.L.D. of toxin i.v., 5 times within 5 hours. Twenty-four hours later, it withstood 1 sure M.L.D. of toxin i.v.

(*b*) As (*a*), but $\frac{1}{3}$ M.L.D. 6 times. Withstood 1 M.L.D. 24 hours later.

(*c*) As (*a*), but $\frac{1}{2}$ M.L.D. 6 times. Did not withstand 1 M.L.D. 24 hours later.

(*d*) As (*b*); withstood $1\frac{1}{2}$ M.L.D. 24 hours later.

(*e*) Five guinea-pigs received $\frac{1}{2}$ M.L.D. toxin i.v. Tested with 1 M.L.D. after $\frac{1}{2}$, 1, 3, 6 and 24 hours—all died.

(*f*) A guinea-pig received $\frac{1}{2}$ M.L.D. 3 times within 2 hours and another $\frac{1}{2}$ M.L.D. 24 hours later. After a further 24 hours, it survived the injection of 1 sure M.L.D.

(*g*) A guinea-pig received $\frac{1}{3}$ M.L.D. 4 times within 3 hours. It died 15 hours later.

The toxin used in the above experiments was a dry one and the M.L.D. had been established in some dozens of guinea-pigs. Further, the necessary controls were included in each test. One may conclude that a guinea-pig may tolerate a fatal dose of toxin as soon as 24 hours after the i.v. administration of 4–6 sub-lethal doses of the same toxin.

(3) *Immunity after the Intradermic Injection of Small Amounts of Toxin.*

(*a*) Two guinea-pigs received 1, 2 and 3 M.R.D. of toxin i.d.—7 days later tested with 1 M.L.D. of toxin i.v., both died.

(*b*) Six guinea-pigs received 1, 2 and 3 M.R.D. of toxin i.d., repeated in 7 days. Tested 14 days later with 1 M.L.D. of toxin i.v.—4 died and 2 survived the 1 M.L.D. but not a further 2.

(*c*) As (*b*), but the i.v. test 7 days after the 2nd i.d. injection—none survived 1 M.L.D. i.v.

(*d*) As (*b*), but the i.v. test 2 days after the 2nd i.d. injection—none survived 1 M.L.D. i.v.

(*e*) Six guinea-pigs received 1, 2 and 3 M.R.D. toxin i.d., repeated in 14 days. Only one survived 1 M.L.D. of toxin i.v. 14 days later.

(*f*) Guinea-pigs received 2·0 c.c. s.c. of Type B toxoid (20 units). Five were tested 14 days later with toxin i.v. *Result:* G.P. (1) <1, (2) 1-3, (3) 1-3, (4) 1-3, (5) 3-7. The 6 animals remaining received 1, 2 and 3 M.R.D. of toxin i.d., and were tested i.v. in a further 2 days. *Result:* G.P. (1) 1-3, (2) 1-3, (3) 1-3, 4 (7-15), (5) 7-15, (6) >15.

Although the injection of a small amount of toxin i.d. produces only a slight degree of immunity, the same amount i.d. acts as a satisfactory secondary stimulus when the primary stimulus was a toxoid of fair value.

## Immunity Experiments in Guinea-pigs, using Type A and/or Type B Antigens.

*General Scheme.*—Guinea-pigs received 2·0 c.c. of toxoid, repeated in 14 days, and the test (cumulative M.L.D.) applied 14 days after the 2nd inoculation.

*Experiment* 1.—Two injections of Type A toxoid. *Result:* G.Ps. not immune to 1 M.L.D. of either Type A or B toxin.

*Experiment* 2.—Two injections of Type B toxoid. *Result:* G.Ps. highly immune to Type B but not to Type A toxin.

*Experiment* 3.—First inoculation—Type A toxoid, second inoculation—Type B toxoid. *Result:* G.Ps. not immune to either toxin.

*Experiment* 4.—First inoculation—Type B toxoid, second inoculation—Type A toxoid. *Result:* G.Ps. highly immune to Type B toxin but not immune to Type A toxin.

*Experiment* 5.—First inoculation—Type B toxoid plus 1·0 c.c. of a high value Type B antitoxin, second inoculation, toxoid only. *Result:* G.Ps. not immune to Type B toxin.

*Experiment* 6.—As experiment 5, but using a high value Type A antitoxin. *Result:* G.Ps. immune to Type B toxin.

*Experiment* 7.—

(*a*) Three injections of Type A toxoid—G.Ps. not immune to 1 M.L.D. of Type A toxin.

(*b*) Six injections of Type A toxoid—G.Ps. immune to at least 1 M.L.D. of Type A toxin but not to 1 M.L.D. of Type B toxin.

(*c*) Six injections of Type B toxoid—G.Ps. highly immune to Type B toxin and to at least 1 M.L.D. of Type A toxin.

These experiments show that:—

(1) It is difficult to produce immunity to Type A, even with the use of a Type A antigen.

(2) Type A toxoid will act as a secondary stimulus to a primary stimulus of Type B toxoid.

(3) Type A toxoid cannot be used as a primary stimulus (Type B toxoid being the secondary) in the production of immunity to Type B toxin.

(4) When Type B toxoid (used as a primary stimulus) is "flooded" with homologous antitoxin, no immunity is produced to Type B toxin.

(5) The "flooding" of a primary stimulus of Type B toxoid with Type A antitoxin does not influence the degree of anti-Type B immunity produced.

(6) Six injections of Type B toxoid produce immunity to Type A toxin, whereas the same number of injections of Type A toxoid produce no anti-type B immunity.

### The Production of Immunity with Type B Bacilli.

This aspect of the production of immunity has proved the most interesting and most astonishing of all. In comparatively recent times, a considerable amount of work has been carried out to test the immunizing power of the bacilli of the anaerobic group of germs. Green (1929) showed that washed living and washed formolized suspensions of *Cl. chauvœi* and *Cl. septique* were capable, on s.c. injection into sheep, of immunizing them against the i.m. inoculation of virulent culture. Robertson and Felix 1930) were able to produce an immune serum in horses by injecting washed and heated *Cl. septique* bacilli. This antiserum contained no antitoxin, but protected mice against the injection of washed spores, activated with

calcium chloride. Craddock and Parish (1931) in an attempt to repeat this work found that high value *Cl. septique* antitoxin protected mice against 100 M.L.D. of an activated spore suspension. Henderson (1932) proved that laboratory animals could be immunized against the activated spores of *Cl. chauvœi* by injections of the washed, boiled organism. Later, Henderson (1933) showed that an antiserum, active against the germ, could be prepared by injecting goats with boiled suspensions of the microbe.

The writer has carried the investigation further, and has shown (article in preparation for press) that washed, boiled suspensions of *Cl. chauvœi* immunize sheep against the i.m. injection of *culture*. These suspensions proved to be excellent immunizing agents and, in controlled experiments, were often superior to anacultures or formol-toxoids. In this investigation, the writer also demonstrated the presence of a toxin in the filtrates of cultures. Such filtrates (or the dialysed ammonium sulphate precipitates) produced reactions when injected i.d. into guinea-pigs or sheep, killed guinea-pigs, mice and sheep on i.v. injection and were specifically neutralizable by antitoxin. Sheep immunized against the toxin (2 s.c. injections of formol-toxoid) developed circulating antitoxin, were immune to culture injected i.m., showed no reaction when toxin was given i.d. and resisted the i.v. administration of a dose of toxin lethal for normal sheep. Sheep hyperimmunized with boiled bacilli (6-8 s.c. injections) were resistant to culture injected i.m., but had no circulating antitoxin and were not immune to the i.d. or i.v. administration of toxin. Further, the injection of boiled formol-toxoid produced a slight degree of immunity, not so high as that evoked by the unboiled material, but sufficient to allow of the animal resisting 1-2 M.L.D. of culture, injected i.m. Such sheep had no circulating antitoxin. One could reasonably conclude that two antigens were involved in the production of immunity to *Cl. chauvœi*, (1) a thermo-stabile antigen, producing, probably, antibody only against the bacilli and (2) a thermo-labile antigen (the toxin was destroyed on heating to 60° C. for half an hour) capable of stimulating the formation of antitoxin.

Experiments were planned to prove that the same held good for *Cl. welchii*, Type B. Whilst the presence of a thermo-stable antigen, directed only against the bacilli, has not been disproved, the evidence points to the toxin, or a modification of it, being the chief, and probably the only, antigen connected with the production of immunity to this germ.

*Experiment* 1.—Goats received dense, washed saline suspensions of bacilli, i.v. Some suspensions were living, others heated at 60° C. for half an hour and others boiled for two hours. The sera were destined for agglutinative purposes. Two years later, the sera were titrated against toxin (mouse i.v.) merely to prove that they contained no antitoxin. The astonishing result, recorded in Table 16, was obtained. In the table, the type of germ injected, the number of injections, the length of the course of immunization, whether heated or unheated germs were administered and the titre of the antitoxin in the serum are recorded.

TABLE 16.

*Production of Immunity in Goats by i.v. Injection of Living and/or Killed Type B Bacilli.*

| Goat. | Type. | No. injts. | Length of course. | Titre of serum. | |
|---|---|---|---|---|---|
| | | | | Mouse M.L.D. | Units. |
| 1..... | B. (S) | 14 (545 c.c.) | 50 days<br>285 c.c. boiled bs.<br>200 c.c. living bs.<br>60 c.c. 60° C. bs. | 266 | 200 |
| 2..... | C. (RS) | 5 (215 c.c.) | 26 days<br>Living bs. only injected | 2,000 | 1,400 |
| 3..... | B. (R) | 7 (305 c.c.) | 28 days<br>255 boiled bs.<br>50 c.c. living bs. | <1 | |
| 4..... | C. (R) | 7 (170 c.c.) | 28 days<br>155 c.c. boiled bs.<br>15 c.c. living bs. | <1 | |

[(S), (RS) and (R) = smooth, rough-smooth and rough variants; under "length of course" the order of the injections is given; under "Mouse M.L.D." the number of mouse i.v. M.L.D. of toxin neutralized by 0·1 c.c. of serum is given; a unit of Type B antitoxin was fixed at the Wellcome Laboratories several years ago.]

Although all the animals received living bacilli, only two produced demonstrable antitoxin. Goat 1 received 3 injections, goat 2, 5 injections, goat 3, 1 injection, and goat 4, 2 injections, of the livng germ. The period elapsing after the last administration of the live culture was 7 days in the case of goats 3 and 4, and 21 days for goat 1 (goat 2 received living suspension only). A careful examination of the history of these animals showed that they had been in no other experiment whatsoever. Although the antitoxic titre of their sera had not been ascertained prior to immunization, it was hardly conceivable that it would be in the region of those recorded for goats 1 and 2. To eliminate all sources of error and doubt, a new experiment was commenced.

*Experiment* 2.—A dense suspension of Type B bacilli was washed 5 times in saline. A portion of it was boiled for 2 hours. To a quantity of this boiled suspension, 2 mouse i.v. M.L.D. per 0·5 c.c. were added. The toxicity (mouse i.v.) of the 3 suspensions was now established.

(*a*) *Living Suspension.*—Mice showed no symptoms after 5 hours when injected with 0·5, 0·25 and 0·1 c.c. i.v.; however, all were dead over-night. In view of the rapidity with which Type B toxin kills, one is justified in concluding that 0·5 c.c. of the material did not contain 1 M.L.D., and that the death of the mice was due to the growth of the germs *in vivo*. An antitoxin binding power test could not be satisfactorily carried out for this reason.

(*b*) *Boiled Suspension.*—0·5 c.c. was non-toxic, and the value, in terms of antitoxin, was less than 2½ units.

(c) *Boiled Suspension Plus Trace of Toxin.*—0·25 c.c. was toxic and 0·1 c.c. non-toxic. The value was less than $2\frac{1}{2}$ units.

The points that required solution were: (1) will living bacilli, only, stimulate the formation of antitoxin, (2) if so, is this due to the presence of minute amounts of toxin in or adsorbed to the germs, (3) if so, will the presence of small amounts of toxin in a boiled suspension produce the same effect, and (4) will boiled bacilli lead to the production of antioxin? The results of the experiment are recorded in Table 17. The injections were all made i.v. and just prior to the administration of the antigen, a sample of blood was withdrawn.

Reference to the table shows that 14 days after the commencement of the course, goat 7 (living bacilli) had developed circulating antitoxin, and that the maximum titre was reached on the 19th day. Goat 5 (boiled bacilli) showed immunity on the 19th day, the antitoxin reaching maximum titre on the 23rd day. Goat 6 (boiled bacilli plus toxin) had circulating antitoxin on the 21st day (probably before) but was not followed further because of the result obtained with goat 5.

TABLE 17.

*Production of Immunity in Goats by i.v. Injection of Living and Killed Type B Bacilli.*

| Date. | Injection. | No. mouse i.v. M.L.D. neut. by 0·1 c.c. serum. | | |
|---|---|---|---|---|
| | | Goat 5. (boiled bacilli.) | Goat 6. (boiled bacilli + toxin.) | Goat 7. (living bacilli.) |
| 27.2..... | 5, 6, 7 yes | <1 | <1 | <1 |
| 4.3..... | 5, 6, 7 yes | <1 | <1 | <1 |
| 6.3..... | 5, 6, 7 yes | <1 | <1 | <1 |
| 8.3..... | 5, 6, 7 yes | <1 | <1 | <1 |
| 11.3..... | 5, 6, 7 yes | <1 | <1 | <1 |
| 13.3..... | 5, 6, 7 yes | <1 | <1 | 4–10 |
| 15.3..... | 5, 6, yes 7, no | 2 | 2 | 40–100 |
| 18.3..... | 5, 6, yes 7, no | 20 | ND | 200 |
| 22.3..... | 5, 6, yes 7, no | 200 | 20–40* | 200 |
| 26.3..... | 5, 6, yes 7, no | 200 | ND | 200 |

(* Tested on the 20.3; 5, 6, 7, yes and no = goats 5, 6, or 7 did or did not receive injections. ND = not tested.)

*Experiment* 3.—The sera used in this experiment were prepared in the same way and at the same time as those noted in experiment 1. However, in this case, 2 rough and 2 smooth variants of 3 strains of *Cl. welchii*, Type A were used. The goats received living, boiled and 60° C. heated suspensions. The course of immunization lasted between 3 and 7 weeks. One goat only, that receiving 190 c.c. of a living suspension of an R variant in 16 days, developed antitoxin.

Its serum, in a dose of 0·05 c.c., neutralized 20 M.L.D. of toxin; 0·1 c.c. of the serum of the animal which got the same amount of suspension in the same time, but in this case using the S variant of the same strain, did not neutralize 2 M.L.D. The sera of the other goats, in a dose of 0·1 c.c., did not neutralize 2 M.L.D.

*Experiment* 4.—The immunizing power of one injection of 2·0 c.c. of a dense suspension of washed, boiled Type B bacilli was ascertained.

(*a*) The bacilli were spun out from an 18 hours' meat broth culture, washed twice in saline and boiled for 2 hours. The value of the suspension was less than 2½ units. A formol-toxoid, prepared from the filtrate of the same culture was of 80 units value. One group of guinea-pigs received, by s.c. injection, 2·0 c.c. of the boiled organisms and another 2·0 c.c. of the toxoid. After 21 days, the pooled sera from each group was tested for the presence of antitoxin. In addition, the tolerance of the animals in the boiled bacilli group to toxin i.v. was ascertained.

*Result:*

*Boiled Bacilli Group.*—0·1 c.c. of serum neutralized 4-6 mouse i.v. M.L.D. of toxin.

*Cumulative M.L.D. Test.*—G.P. (1) <1, (2) <1, (3) <1, (4) 1-3, (5) 1-3, (6) 3-7, (7) >12, (8) >12.

*Toxoid Group.*—0·1 c.c. of serum neutralized 5-6 mouse i.v. M.L.D. of toxin.

(*b*) The procedure was exactly as for (*a*). The bacilli and the toxoid had the same culture as origin. The value of the bacillary suspension was less than 2½ units, and that of the toxoid was 20 units.

*Result:*

*Boiled Bacilli Group.*—0·1 c.c. of serum neutralized 20-30 mouse i.v. M.L.D. of toxin.

*Toxoid Group.*—0·1 c.c. of serum did not neutralize 1 mouse i.v. M.L.D. of toxin.

*Experiment* 5.—In experiment 2, an attempt was made to immunize goats with washed bacilli, heated for half an hour at 60° C. On two occasions, the i.v. injection of 5·0 c.c. of a dense suspension killed the goats within 18 hours. In the region of the site of inoculation (jugular vein), the muscles were oedematous. Attempts to cultivate *Cl. welchii*, Type B from the oedematous fluid failed. The most probable explanation for the oedema was a leakage of suspension on withdrawing the needle from the vein. This would mean that a minute amount of the material was capable of producing the effect because, on each occasion, after injecting the antigen, 5·0 c.c. of blood was drawn into the syringe and this reinjected before the needle was withdrawn. The toxicity of the suspension for mice (i.v. injection) and guinea-pigs (id. injection) was investigated.

*Mice, i.v. Injection.*—0·5 c.c. + at once, 0·25 c.c. + 24 hours, 0·1 c.c. + 24 hours, 0·05 c.c. + 36 hours, 0·025 c.c. + 3 days, 0·01 c.c. L; 2 M.L.D. were not neutralized by 0·1 c.c. of 3 high value Type B antitoxins or by 0·1 c.c. of 3 antisera prepared by injecting goats with living and/or boiled organisms. (+ = died, L = lived.)

*Guinea-pig, i.d. Injection.*—0·025 c.c. produced a small, yellow, circumscribed necrotic area, quite unlike the reaction evoked by the toxin of Type B; 3 M.R.D. were not neutralized by 0·1 c.c. of the sera noted under " mice i.v. injection ".

The supernatant of the spun suspension proved non-toxic in a dose of 0·2 c.c., both i.v. and i.d.

These five experiments are of intense practical and scientific interest. To one, like the writer, who has spent years in the preparation of toxins and toxoids for the immunization of horses and sheep, the method offers a probable solution for many difficulties. The antitoxin produced by goat 2, Table (16) in 26 days is as good as the majority produced by horses after a course of injections of toxoid and toxin, lasting 2-3 months. The antitoxins, produced by goats 1, 5 and 7, are of low titre but it has been the writer's experience that the goat does not yield such high value antitoxin as the horse (immunization with toxoids and toxins of *Cl. welchii*, Types A and B, *Cl. septique*, and *Cl. botulinum*, Types A, B, C and D). In addition to the speed of immunization, it is possible that the bacilli, obtained from cultures, the filtrates of which were either of high or low value, would act equally well as antigens. If such were the case, the production of high value toxin and toxoid would be unnecessary; the organisms could be grown in large quantities of glucose broth, spun out in a Sharples centrifuge, resuspended in a small amount of saline, boiled and injected. Such a procedure could easily be carried through by the average laboratory assistant. There are two disadvantages. About 5-10 times more medium would be required than by the usual method, but, on the assumption that consistent results would be obtained, this would be offset by the saving in time and labour accorded to a senior worker, and the saving of laboratory animals. In the immunization of the goats, swelling and oedema of the muscles of the jugular vein region were common. This could doubtless be overcome by flushing the needle out with saline, prior to its withdrawal.

For the immunization of sheep, the " boiled bacilli " method has definite possibilities. In the guinea-pigs, immunized in experiment 4, swelling and necrosis of the injected region (s.c. on abdomen) was the rule. Such reactions, in sheep, would not be tolerated by sheep farmers. However, in view of the high degree of immunity produced by *one* injection, an investigation of methods, capable of preventing the reaction, would be well repaid.

From the purely academic side, the problem is fascinating. A suspension of killed organisms, containing no demonstrable toxin or toxoid, is capable, on injection into animals, of stimulating the formation of antitoxin. It is probable, in the light of the recent work on the immunizing power of specific soluble substances and

polysaccharides when adsorbed on protein or colloids, that the antigen in the boiled suspensions is in the nature of a haptene, linked to, or adsorbed on, the protein of the bacillus.

The experiment, conducted with a bacillary suspension, heated at 60° C., indicates that, in addition to the known exo-toxin, Type B produces an endo-toxin, serologically distinct from the exo-toxin.

It is proposed to continue this investigation, to discover, *inter alia*, the best method of hyper-immunizing animals with boiled bacillary suspensions, a way of overcoming the reactions, produced by them and the physical and serological properties of the endo-toxin.

## CONCLUSIONS.

1. The total antitoxin binding value of a toxoid of *Cl. welchii*, Type B (the "lamb dysentery bacillus") has a direct bearing on its immunizing capacity. The more antitoxin a toxoid binds, the greater is the degree of immunity produced by it.

2. Atoxic toxoid, rendered slightly toxic by the addition of the toxins of *Cl. welchii*, Types A or B proved superior as an antigen to the toxoid, itself. The addition of the toxin of *Cl. septique* did not increase the immunizing value of toxoids.

3. The intravenous injection of 4-6 sub-lethal doses of Type B toxin, within 4-5 hours may so immunize a guinea-pig that it resists a fatal dose of toxin 24 hours later.

4. Alum-precipitated toxoids proved, on the whole, inferior as antigens to the toxoids, themselves. The addition of agar, colloidal iron and saponin to toxoids did not increase their immunizing power.

5. The washed zinc chloride precipitates of toxoids proved superior as antigens to the toxoids, themselves. The solubility of the precipitate appears to have a direct bearing on the degree of immunity produced.

6. Washed living or washed boiled suspensions of Type B bacilli (free from demonstrable toxin) stimulated the formation of antitoxin when injected into goats and guinea-pigs.

7. Washed suspensions of Type B bacilli, heated at 60° C. for half-an-hour, contain a toxic material, not neutralizable by antitoxin.

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