

STUDIES ON  
ANTHRAX  
IMMUNITY



BY  
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Thesis.

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Studies on anthrax immunity.

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STUDIES ON ANTHRAX IMMUNITY.

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INTRODUCTION.

Although in the past the term Anthrax was used rather loosely for different diseases, there seems to be little doubt that the condition as we know it today, has been in existence since the earliest times. In fact several of the ancient authors (Homer, Ovid and Pliny) wrote about a disease most probably identical with Anthrax. That Anthrax is a disease of primary significance may be gauged from the fact that it has meant the death of millions of animals as well as many thousands of human beings. The aetiology of the disease was completely unknown until Robert Koch in 1876 proved the significance of the rod shaped organisms in the blood, subsequently termed the *Bacillus anthracis*. Shortly afterwards Louis Pasteur succeeded to produce a vaccine against the malady. Since the discovery of the causal organism, the disease has received the attention of hundreds of investigators from all parts of the world, and an enormous amount of literature has been published. Even at the present moment research work in connection with Anthrax is being conducted on a large scale for, although we possess valuable weapons for combating the disease, some cardinal points are as yet unexplained, to mention only - the route of infection in the different classes of animals, the pathogenesis of the disease and the biology of the organism in nature outside the animal body.

With reference to the position of Anthrax in South Africa up to the end of 1924, Viljoen, Curson and Fourie have recently published a detailed report in which the

occurrence, diagnosis and preventive measures are fully discussed. Previous to that Kehoe and others have written on the disease in South Africa.

In this report is embodied the greater part of the experimental work carried out, and the observations made by the writer during the years 1925 to 1928, while working in the Anthrax Section of the Veterinary Research Laboratory, Onderstepoort.

It is a well known fact that, apart from the actual destruction of all Anthrax infected material, the active immunization of animals against the disease, is by far the most effective means we possess in eradicating it. For this purpose <sup>three</sup> ~~two~~ main types of vaccine are used more or less exclusively all over the world.

(a) Growth cultures containing the living but much weakened or attenuated vegetative forms of the *Bacillus anthracis*. This vaccine was first introduced by Louis Pasteur, and is still used extensively in certain countries. It is generally prepared as a double vaccine, the first inoculation being made with a weaker vaccine which produces the ground immunity, while the second, which is stronger, is considered to enhance the immunity.

(b) Anthrax Spore Vaccine. This vaccine, which is now favoured in many countries, is of comparatively recent introduction, although based on the same principle as that employed for the Pasteur Vaccine, i.e. the utilisation of attenuated living organisms. It differs, however, in that the more resistant spores of the attenuated vegetative forms, are used rather than the bacilli themselves. Vegetation of the spores in the vaccine is prevented by suspending these in various preservatives, e.g. glycerinised saline. By this procedure the keeping qualities as well as the uniformity of the vaccine is markedly improved, while the immunity conferred is in no way inferior to that produced by the *Pasteur vaccine*.

(c) Sero-vaccination as advocated by Söbernheim:

This method which is extensively used in certain European countries as well as in South America, consists in a simultaneous injection of anthrax immune serum, together with attenuated anthrax cultures. The immune serum produces an immediate passive immunity, while the effect of the living *bact* attenuated organisms is considered to be a rapid replacement of the passive by a solid active immunity against anthrax.

*Follow on page 3.*

~~anthrax vaccine.~~

Further, it may be briefly mentioned that a <sup>fourth</sup> ~~third~~ method of preventive and curative inoculation exists, viz. by means of hyperimmune anthrax sera, <sup>alone</sup> the use of which, however, is markedly limited in comparison with the above-mentioned vaccines, and the immunity conferred of a purely passive and transitory nature.

Although the <sup>three</sup> ~~two~~ types of vaccine mentioned are extensively used, and generally with good results, several investigators, to mention Bait, have from time to time been engaged in an endeavour to demonstrate the presence and value as active immunizing agents, of non-living metabolic products of the Anthrax organism elaborated either artificially in vitro or in the animal body. It may be stated that up to the present the results of these researches have been most conflicting, e.g. some authors claim to have demonstrated definite toxic action of these products while others have failed to do so. Similarly in some cases active immunity seemed to have been provoked while in other cases the results were clearly disappointing. Should it, however, be clearly demonstrated that the Anthrax bacillus is capable of elaborating a potent immunizing substance comparable to the so-called "aggressin" of Black quarter, it would indeed be of inestimable value in fighting the disease.

However efficient our present Anthrax vaccines may be, it should always be borne in mind that the wholesale inoculation with live organisms, although attenuated, is far from the ideal, since they are directly derived from the dangerously virulent organism the biology of which is not completely known. Further it may be noted that some difficulty is experienced in immunizing certain classes of animals, e.g. goats, which are very susceptible to anthrax and may die as the result of vaccination, while horses

are peculiarly susceptible to the development of subcutaneous swellings following on vaccination.

During the course of the last few years, what is described as "true Anthrax aggressin" has appeared on the market, and complete safety and strong immunizing properties are claimed for it.

The object of the experimental work carried out by the author was as follows: -

- (a) To attempt the production of a non-living yet efficient immunizing substance against Anthrax.
- (b) To compare the relative value of different methods of immunization with Anthrax Spore vaccine. The latter experiments were conducted mainly with the view of ascertaining the value of cuti-vaccination as advocated by Besredka and others in comparison with the methods usually employed in South Africa.

The work will be divided into several sections and discussed under the following titles. Tables and protocols of experiments will be included in the text rather than all together in a special appendix.

- 1. Species susceptibility and immunity.  
The selection of experimental animals.
- 2. (a) Attenuation of virulent strains.  
(b) Experiments with disintegrated cultures.  
(c) Experiments with washed cultures.
- 3. Production of immunizing or toxic substances:  
(a) Artificial in vitro experiments.  
(b) Natural, in the animal body.
- 4. Comparative safety and immunity experiments with Anthrax Spore Vaccine.
- 5. Summary and general discussion.
- 6. Bibliography.

1. Species susceptibility and immunity.The Selection of Experimental Animals.

Numerous investigators to mention Sobernheim, Baumgarten, Löffler, Metchnikoff, K. Müller, have pointed out the fact that the different species of animals exhibit marked variations in the degree of susceptibility to anthrax both under natural and artificial conditions. Even in the same class of animal these differences are frequently observed, e.g. whereas poultry show marked resistance, Robertson found the ostrich peculiarly susceptible to the disease. According to K. Müller, the different species of rats exhibit marked variations in susceptibility to anthrax. Carnivorous animals are as a general rule far more resistant than herbivora. Furthermore, differences may be demonstrated in one and the same species of animal kept under different conditions. It has frequently been observed that, although such animals as cattle and horses are markedly susceptible and may die in large numbers from anthrax naturally contracted, it is with great difficulty that a fatal artificial infection can be brought about either by enteral or parenteral administration of virulent anthrax material. As a result of our incomplete knowledge as to the natural routes of infection, this peculiar phenomenon is still a mystery. On the other hand may be mentioned the ease with which sheep, goats and most laboratory animals can be fatally infected.

With reference to immunity, as has been mentioned before, certain species of animals exhibit a marked inherent resistance to the disease, while most of the susceptible species can be successfully immunized by the use of reliable vaccines.



d'Herelle expressed the opinion that the study of immunity in artificially produced infectious diseases does not necessarily mean that the same conditions will prevail under natural conditions. This point is of great significance and should not be lost sight of.

Among the various susceptible species however, notable exceptions are encountered. Before any immunity work on Anthrax is carried out, it is essential that these points be clearly realised, and it is for this reason that this aspect is particularly stressed. Thus it may happen that experiments carried out on certain animal species which are markedly susceptible but difficult to immunize, may result in totally wrong conclusions being drawn. Many of the conflicting results obtained by different workers may probably be ascribed to the different types of animals used.

The ideal species of animal to be used in immunity experiments would obviously be one showing a practically constant and well marked susceptibility to artificial infection combined with the quality of developing a solid immunity after vaccination. It is intended to discuss briefly the more common domesticated animals from this point of view.

(a) The common Laboratory animals. Of these may be mentioned the white rat, white mouse, the guineapig and the rabbit. Among these animals, it is exceedingly rare that a spontaneous outbreak of anthrax should occur, although excepting for the white rat, which exhibits a strong natural immunity, artificial infection of the others is extremely easy. White mice may be killed even by very weak vaccines. Should they survive vaccination, the degree of immunity, if any be developed, is however, negligible. Similarly guineapigs which are extremely susceptible even to weak strains, are particularly difficult, perhaps

impossible, to immunize and hence great care should be exercised in drawing conclusions from any immunity experiments carried out on these animals. Rabbits which are markedly susceptible to virulent but less so to vaccine strains, may develop a certain degree of immunity which, however, is weak and always unreliable. Although these points are vaguely known, they are not fully appreciated with the result that erroneous conclusions may be arrived at.

(b) Goats. The animals here referred to comprise the domesticated species indigenous to South Africa. These animals exhibit a well marked susceptibility both to natural and artificial infection with anthrax. Deaths may occur at times even from the use of weak vaccines. The immunity developed, although as a rule fairly strong, is inclined to be inconsistent.

(c) Sheep. Although under natural conditions outbreaks of anthrax appear to be less frequent than among cattle, merino sheep exhibit a well marked and practically uniform susceptibility to artificial infection with virulent material. At the same time the losses even from strong vaccine are very small, while a solid and reliable immunity is developed.

(d) Cattle. Under natural conditions cattle are perhaps more subject to anthrax than any other species of animal, although great difficulty may be experienced in attempting to kill them by means of artificial infection. That a strong immunity has been developed after vaccination can only be ascertained by the effective stoppage of an active outbreak.

(e) Horses. These animals show a peculiar susceptibility to anthrax under natural conditions. Definite symptoms generally of a subacute or even chronic nature, may be noted, the chief of which are the large oedematous swellings in the

subcutis of certain regions of the body.

The results of artificial infection, however, are most inconsistent. Thus it frequently happens that animals withstand even large injections of virulent material. On the other hand alarming swellings may follow the injection of small doses of weak vaccine. This phenomenon is as yet unexplained.

To substantiate the above views frequent examples will be mentioned in the text.

It will thus be clear, firstly, that although the small laboratory animals are markedly susceptible to artificial infection, the subsequent immunity developed in surviving cases as tested with more virulent material is unreliable; secondly, susceptible cattle and horses withstand virulent artificial infection in a large percentage of cases. It thus becomes exceedingly difficult to ascertain the degree of immunity developed after vaccination as subsequently tested with fully virulent material.

From numerous immunity experiments conducted, it was clearly evident that only in limited few species could reliable information be obtained as to the actual degree of immunity developed. For this purpose merino sheep were found to yield by far the most constant and reliable results, and consequently practically all the immunity work was carried out on this species. To a lesser extent goats were also used. On account of the wholesale inoculation of farm animals in South Africa against anthrax, attention had to be paid to the source of experimental animals. As anthrax was of uncommon occurrence in the Karroo, and hence practically no animals vaccinated against anthrax, most of the susceptible experimental sheep and goats were derived from that region. Such animals generally show a well marked and practically constant susceptibility to artificial infection.

Horses were used only for purposes of noticing the severity of clinical symptoms following different methods of vaccination.

The small laboratory animals were extensively used mainly to ascertain the degree of virulence of anthrax material, both unattenuated and vaccine strains. Further these animals were used for the purpose of testing the toxicity of different non-living anthrax products.

Excepting in a limited few cases, cattle were not included in these experiments, on account of the difficulty experienced in killing non vaccinated controls with virulent material.

## 2. (a) Attenuation of Virulent Strains.

Toussaint was the first to notice a decrease in the virulence of anthrax organisms in infected blood when exposed for short periods to higher temperatures than the optimal. Through the researches of Pasteur, Chamberland and Roux, a method was devised for the gradual attenuation of the anthrax organism in artificial media. By cultivating the *B. anthracis* in sterile bouillon at 42° C for long periods, a progressive decrease in virulence was noted. It was thus found that such cultures firstly become avirulent for rabbits, later for guineapigs and eventually also for white mice. Although avirulent, these organisms were found to confer an immunity against fully virulent cultures. This formed the basis for the famous Pasteur Anthrax vaccine. Although at the present day numerous other anthrax vaccines are also in use, the principle of attenuation by prolonged incubation at higher temperatures is still maintained in practically every instance. Various other methods have been described for the attenuation of the *B. anthracis*, although apparently without any distinct advantage over the Pasteur method. Preisz drew attention to the possibility\*

of an unequal attenuation of different anthrax strains and even of individual organisms.

According to Koch, Gaffky and Wöfler, the passage of attenuated vaccine strains through experimental animals does not enhance the virulence of such strains except perhaps where the original virulent organisms were attenuated very rapidly in a short time, i.e. gradual attenuation leads to the production of vaccine strains showing greater constancy. From recent investigations Schilling claims to have shown that anthrax organisms could be attenuated by growing on 5% Sodium chloride agar for 6 weeks. Rabbits could be successfully and safely immunized and afterwards withstood virulent anthrax cultures which regularly killed susceptible controls. The same, however, could not be established for guineapigs.

Attenuation of several virulent anthrax strains was attempted in different ways. Pure agar cultures were obtained from six strains of virulent anthrax from different parts of South Africa. All these were definitely proved to be lethal to guineapigs and rabbits. Attenuation was commenced by inoculating broth tubes with the pure strains and consistently incubating these at 42° C. Injections into guineapigs 24 to 48 hours afterwards confirmed the virulence noted in the first instance. After incubating for 25 days, subcultures on to nutrient agar were made every 5 days, incubated at 37° C for 3-4 days, sealed and kept at room temperature for further use. This was continued until all the strains had been incubated at 42° C up to a period of 90 days. Following this, so-called "test batches" were prepared from several selected agar tubes representing different periods of attenuation. Young broth cultures with seed material obtained from the agar slants, were sown on to the thin layers of nutrient agar contained in large flat



bottomed Fernbach flasks and incubated at 37° C. Growth and sporulation were noted daily. Generally, after 4 or 5 days sporulation was practically complete. The growth was then removed by pouring small amounts of sterile normal saline on to the surface together with a few sterile glass beads. By gentle tilting of the flasks, the growth became dislodged. This was carefully decanted into sterilised flasks and an equal weight of pure glycerine added. This formed the concentrated glycerinised spore emulsion with which all further tests were conducted. Glycerine was found to preserve the spores and prevent vegetation. By this procedure spore emulsions were found to maintain a uniform strength for very long periods. Subsequently the degree of virulence of these experimental vaccines was tested out on guineapigs and rabbits by subcutaneous injection of various dilutions made in normal saline. The immunizing properties were afterwards ascertained by vaccination of sheep and goats, but only in those cases where rabbits survived the injection. When rabbits died from the vaccine, it was generally found to cause death in a fair percentage of the sheep and goats. The degree of immunity developed in sheep and goats was subsequently tested after 14 to 21 days by the subcutaneous injection of a virulent glycerinised spore emulsion of known strength. This was prepared in the same way as described for the test batches. By previously conducted experiments the minimal lethal dose, referred to as the M.L.D. for sheep was accurately determined.

The testing of virulent spore emulsion was carried out periodically and the M.L.D. fixed. Diagnosis of anthrax was made by the microscopic examination of blood or spleen smears taken from dead animals. Where these proved to be negative, broth cultures made from the spleen, were injected into white mice and the result noted.

The following experiments will serve to demonstrate the variations in safety and immunizing properties of different test batches: -

Experiment 1.

Strain A. attenuated 50 days.

1 rabbit injected .1 c.c. glycerine emulsion - dead in 4 days Anthrax.

1 guineapig injected .1 c.c. emulsion - dead in 3 days Anthrax.

1 guineapig injected .01 c.c. emulsion - dead in 3 days Anthrax.

Strain A. attenuated 70 days.

1 rabbit injected .1 c.c. emulsion - remained alive.

1 guineapig injected .1 c.c. emulsion - dead in 4 days Anthrax.

1 guineapig injected .01 c.c. emulsion - dead in 3 days Anthrax.

1 guineapig injected .001 c.c. emulsion - dead in 4 days Anthrax.

As the rabbit remained alive this batch was tested out on sheep and goats.

| Animal      | Date vaccine | Result                   | Date virulent spores | Result                   |
|-------------|--------------|--------------------------|----------------------|--------------------------|
|             | 18/8/25.     |                          | 8/9/25.              |                          |
| Sheep 11324 | .02 c.c.     | -                        | 500 M.L.D.           | Alive                    |
| " 7349      | .02 c.c.     | -                        | 500 M.L.D.           | Alive                    |
| " 11022     | .01 c.c.     | -                        | 500 M.L.D.           | Dead 15/9/25<br>Anthrax. |
| " 11217     | .01 c.c.     | -                        | 250 M.L.D.           | Alive                    |
| Goat 11850  | .02 c.c.     | -                        | 250 M.L.D.           | Alive                    |
| " 11856     | .01 c.c.     | dead 29/8/25<br>Anthrax. | -                    | -                        |

One goat is noticed to have died from anthrax after vaccination, while in the immunity test one of the sheep succumbed from anthrax.

Strain A. attenuated 80 days - Single Colony.

1 rabbit injected .1 c.c. emulsion - remained alive.

1 guineapig injected .1 c.c. emulsion - dead in 5 days Anthrax.

1 guineapig injected .01 c.c. emulsion - dead in 3 days  
Anthrax.

1 guineapig injected .001 c.c. emulsion - remained alive.

This was again tested on sheep and goats as follows: -

| Animal     | Date Vacc.<br>2/12/25 | Result | Date virulent<br>spores 18/12/25 | Result |
|------------|-----------------------|--------|----------------------------------|--------|
| Sheep 9005 | .02 c.c.              | -      | 500 M.L.D.                       | Lived  |
| " 8865     | .01 c.c.              | -      | 250 M.L.D.                       | Lived  |
| Goat 14031 | .02 c.c.              | -      | 500 M.L.D.                       | Lived  |
| " 14024    | .01 c.c.              | -      | 250 M.L.D.                       | Lived  |

It is seen that this particular batch was both safe for sheep and goats and conferred a good immunity protecting against as much as 500 M.L.D.

Strain A. - attenuated 80 days.

From the same tube that the above batch was made, another was prepared only that a different colony was selected from the agar plate.

1 rabbit injected .1 c.c. emulsion - remained alive.

1 guineapig injected .1 c.c. emulsion - dead in 5 days  
Anthrax.

1 guineapig injected .01 c.c. emulsion - dead in 5 days  
Anthrax.

1 guineapig injected .001 c.c. emulsion - dead in 5 days  
Anthrax.

Note: in this case the .001 c.c. killed while in the previous batch the guineapig remained alive.

| Animal      | Date Vacc.<br>2/12/25 | Result                   | Date virulent<br>spores 18/12/25 | Result. |
|-------------|-----------------------|--------------------------|----------------------------------|---------|
| Sheep 10647 | .02 c.c.              | -                        | 1000 M.L.D.                      | Alive   |
| " 10724     | .01 c.c.              | -                        | 500 M.L.D.                       | Alive   |
| Goat 14032  | .02 c.c.              | dead 5/12/25<br>Anthrax. | -                                | -       |
| " 14020     | .01 c.c.              | dead 6/12/25<br>Anthrax. | -                                | -       |

In this case the vaccine prepared from a different colony is highly virulent for guineapigs and goats, although it is safe for sheep and confers a strong immunity. This clearly demonstrates the unequal attenuation of different organisms although of the same strain and in the same tube.

Experiment 2.

Strain B attenuated 50 days.

1 rabbit injected .1 c.c. emulsion - remained alive.  
 1 guineapig injected .1 c.c. emulsion - remained alive.  
 1 guineapig injected .01 c.c. emulsion - dead in 7 days  
 Anthrax.  
 1 guineapig injected .001 c.c. emulsion - remained alive.  
 This is obviously a very weak vaccine. Tested out on sheep and goats, it gave the following peculiar results:

| Animal      | Date Vacc.<br>26/10/25 | Result | Date virulent<br>spores 9/11/25 | Result                   |
|-------------|------------------------|--------|---------------------------------|--------------------------|
| Sheep 12249 | .02 c.c.               | -      | 500 M.L.D.                      | Dead 14/11/25<br>Anthrax |
| " 9524      | .01 c.c.               | -      | 250 M.L.D.                      | Dead 12/11/25<br>Anthrax |
| Goat 11859  | .02 c.c.               | -      | 250 M.L.D.                      | Alive                    |
| " 11854     | .01 c.c.               | -      | 100 "                           | Alive                    |

In this case, the vaccine besides being safe for sheep and goats, immunized the two goats while the two sheep readily succumbed to the immunity test.

Strain B. attenuated 75 days.

1 rabbit injected .1 c.c. emulsion - dead in 7 days Anthrax.  
 1 guineapig " .1 " " - " " 3 " "  
 1 " " .01 " " - " " 3 " "  
 1 " " .001" " - " " 4 " "

This batch is markedly virulent although the strain was attenuated for 75 days. It would appear that the colony from which it was prepared was formed from an organism in which no attenuation had taken place. It was not tested on

sheep since it killed the rabbit.

In this way no less than 65 test batches were prepared from different attenuations, selecting different colonies of the 6 strains. These were all tested out on rabbits and guineapigs and when promising, also on sheep and goats. Of these not more than four could be considered of any value as reliable vaccine strains. In numerous cases either the vaccine killed or in the surviving animals immunity was absent or weak.

It was clearly evident that the production of a reliable vaccine strain i.e. one in which safety and immunity were combined, depended on at least two factors, viz. (a) the strain itself, (b) individual organisms comprising the strain. Some strains proved to be altogether useless, while in other strains certain colonies yielded excellent test batches.

In some cases it was found necessary to attenuate up to 90 days before the requisite attenuation was reached, while with other strains attenuation for 45 days proved sufficient. It seemed definite that no fixed period could be prescribed for attenuation, and that constant testing out on animals was the only reliable way of obtaining a suitable vaccine strain.

It was then decided to attempt the attenuation of a virulent anthrax strain by prolonged cultivation in a number of different liquid media. This strain which was known to be markedly virulent in the original state had been attenuated previously by other means yielding an excellent vaccine strain. These media were prepared the same as ordinary nutrient broth except that various other materials were substituted for the minced beef. The sterile medium contained in a litre flask was inoculated with 5 c.c. of a 24 hour broth culture of the virulent strain and incubated for 30 days at a temperature of 37° C. Guineapigs and rabbits were



then inoculated subcutaneously with varying amounts of the culture and the results noted.

Experiment 3.

Controls. Virulent Anthrax 24 hour broth culture.

1 rabbit injected .5 c.c. - dead in 3 days Anthrax.  
 1 guineapig " .5 " - " " 2 " "  
 1 " " .25 " - " " 2 " "

Controls. Virulent Anthrax 30 day broth culture.

1 rabbit injected .5 c.c. - dead in 4 days Anthrax.  
 1 guineapig " .5 " - " " 3 " "  
 1 " " .25 " - " " 2 " "

A. Virulent Anthrax in Pea broth for 30 days.

(ordinary dry peas were used).

1 rabbit injected .5 c.c. - dead in 4 days Anthrax.  
 1 guineapig " .5 " - " " 2 " "  
 1 " " .25 " - " " 2 " "

B. Virulent Anthrax in Bean broth for 30 days.

(ordinary dry beans were used).

1 rabbit injected .5 c.c. - dead in 5 days Anthrax.  
 1 guineapig " .5 " - " " 2 " "  
 1 " " .25 " - " " 2 " "

C. Virulent Anthrax in ordinary broth + whole egg for 30 days.

1 rabbit injected .5 c.c. - dead in 5 days Anthrax  
 1 guineapig " .5 c.c. - " " 2 " "  
 1 " " .25 " - " " 2 " "

D. Virulent Anthrax in Chicken muscle broth for 30 days.

1 rabbit injected .5 c.c. - dead in 5 days Anthrax.  
 1 guineapig " .5 " - " " 2 " "  
 1 " " .25 " - " " 2 " "

E. Virulent Anthrax in Dog muscle broth for 30 days.

1 rabbit injected .5 c.c. - dead in 3 days Anthrax.  
 1 guineapig " .5 " - " " 3 " "  
 1 " " .25 " - " " 4 " "

F. Virulent Anthrax in Potato broth for 30 days.

1 rabbit injected .5 c.c. - dead in 4 days Anthrax.  
 1 guineapig " .5 " - " " 4 " "  
 1 " " .25 " - " " 2 " "

G. Virulent Anthrax in ordinary broth + 10%Anthrax hyperimmune Donkey serum for 30 days.

1 rabbit injected .5 c.c. - dead in 5 days Anthrax.  
 1 guineapig " .5 " - " " 2 " "  
 1 guineapig " .25 " - " " 3 " "

It is thus seen that out of a total of 27 animals injected with the same amounts of different anthrax cultures not a single one survived showing that practically no attenuation had taken place in any of the flasks. The incubation was stopped at 30 days since after this period attenuation might have been expected even in the plain broth control cultures. From these experiments it was evident that by cultivating virulent anthrax organisms in these various media, attenuation was brought about no sooner than in ordinary broth.

Attempted attenuation by freezing and thawing.

As a third method of attenuating virulent anthrax cultures it was decided to attempt this by repeatedly freezing and thawing of the cultures. For this purpose a small apparatus was constructed, consisting of two tubes closed at one end made out of galvanised iron and suspended one within the other. A small inlet in the outer tube was connected with a cylinder containing Carbon dioxide under high pressure. A saline emulsion consisting of a 12 hour agar growth of virulent anthrax organisms was placed in the inner tube. Opening of the cylinder caused the rapid escape of  $\text{CO}_2$  from between the two tubes. This resulted in almost immediate freezing of the emulsion. After being frozen for 10 minutes the inner tube was removed and quickly steeped into water at  $90^\circ \text{C}$ . This caused rapid thawing inside the tube.

As soon as liquefaction was complete the freezing was repeated. Subcultures on nutrient agar were made after every alternate freezing and incubated at 37° C. This freezing and thawing was continued 24 times in succession. After incubating the agar subcultures for 24 hours, approximately equal amounts of growth (one platinum loopful) were removed and suspended in definite amounts of saline. This was subsequently injected into guineapigs and rabbits with the following results: -

Experiment 4.

Virulent Control. 24 hour old virulent agar culture, one loopful suspended in 10 c.c. saline.

1 rabbit injected 1 c.c. emulsion - dead in 3 days Anthrax.  
 1 guineapig " 1 " " - " " 3 " "  
 1 " " .1 " " - " " 3 " "

Frozen 1.

One loopful 24 hour agar culture made from material frozen once and suspended in 10 c.c. saline.

1 Rabbit injected 1 c.c. emulsion - dead in 7 days Anthrax.  
 1 guineapig " 1 " " - " " 2 " "  
 1 " " .1 " " - " " 5 " "

Frozen 21.

This was made from material frozen 21 times.

1 rabbit injected 1 c.c. emulsion - dead in 3 days Anthrax.  
 1 guineapig " 1 " " - " " 3 " "  
 1 " " .1 " " - " " 3 " "

Frozen 24.

As the frozen 21 showed no signs of attenuation the frozen 23, culture was taken and frozen for 2 days on end and termed frozen 24. Saline emulsions were made as above and guinea-pigs and rabbits injected only with much smaller amounts.

|                   |                               |                  |          |
|-------------------|-------------------------------|------------------|----------|
| 1 rabbit injected | .001 <sup>c.c.</sup> emulsion | - dead in 3 days | Anthrax. |
| 1 guinea pig      | " .001 c.c.                   | " - " "          | 2 " "    |
| 1 "               | " .0001 "                     | " - " "          | 5 " "    |
| 1 "               | " .00001 "                    | " - " "          | 3 " "    |

From the above experiment it was clear that the young 12 hour old vegetative forms of virulent anthrax could withstand with impunity persistent rapid alternate freezing and thawing. Microscopic examination of smears made from the frozen material revealed not the slightest signs of disintegration, while the animal tests proved the absence of any attenuation having taken place.

Of the different methods of attenuation attempted, it was decided that the Pasteur method, although tedious and frequently disappointing, was the only one in which attenuation definitely took place.

## 2. (b) Experiments with disintegrated Anthrax Cultures.

The object of this series of experiments was to devise ways and means for the actual disintegration of anthrax organisms and setting free of the protoplasm. Once this had been achieved the sterile germ free product was to be tested out on small laboratory animals in order to ascertain the presence or absence of endocellular toxins, and further to note the degree of immunity developed, if any, in sheep and goats. For these experiments the growth from young 12-24 hour old agar slopes were used, the seed material consisting of a highly virulent anthrax strain. The young growth consisting mostly of vegetative forms was carefully removed from the agar surface by means of a platinum needle and suspended in normal saline. In this way a thick saline emulsion was formed.

As mentioned before the alternate freezing and thawing of such emulsions did not cause disintegration and hence the method was abandoned.

Several other methods employed gave completely satisfactory results.

Experiment 5.

Disintegration by continued shaking.

A saline emulsion prepared as described above was placed in a strong flask together with a number of sterile glass beads and securely corked. It was then shaken continuously in a shaking apparatus for 4 days.

Microscopic examination revealed the presence of a large percentage of disintegrated bacilli in the form of debris. Subcultivation, however, resulted in an abundant growth. The emulsion was, therefore, diluted and carefully passed through a Seitz bacterial filter. The filtrate was completely sterile as shown by subcultivation and smears. The material was then injected into small animals as follows:

1 guinea pig 2 c.c. filtrate - remained alive.  
 1 " 2 " " - " "  
 1 white mouse 1 c.c." - " "

Similarly a young broth culture shaken up in the same way, filtered and injected into small animals produced no effect.

The addition of 1 in 1000 solution of anilin gentian violet to the saline emulsion, was also found to cause rapid disintegration of the bacilli, and sterilisation of the emulsion.

2 guinea pigs injected with 1 c.c. each of the emulsion - both remained alive. Similarly two white mice remained alive.

This material was also tested out on two sheep:

| Animal     | Date emul-<br>sion 16/9/26 | Result | Date virulent<br>scores 29/9/26 | Result                   |
|------------|----------------------------|--------|---------------------------------|--------------------------|
| Sheep 7716 | 5 c.c.                     | -      | 100 M.L.D.                      | Dead 3/10/26<br>Anthrax. |
| " 8722     | 3 c.c.                     | -      | 100 M.L.D.                      | Dead 2/10/26<br>Anthrax. |

Disintegration of virulent material was also carried out by grinding it up in a mortar, after adding about half the



volume of clean sterile sand. In this way practically all the bacilli were disintegrated. After passing the diluted saline emulsion through a bacterial filter, and confirming sterility, the filtrate was again tested out on animals. Guinea pigs and white mice injected with amounts up to 3 c.c. all remained alive. Sheep were injected as follows:

| Animal      | Date fil-<br>trate 21/10/26 | Result | Date virulent<br>spores 1/1/26 | Result.                  |
|-------------|-----------------------------|--------|--------------------------------|--------------------------|
| Sheep 14677 | 5 c.c. filtrate             | -      | 100 M.L.D.                     | Dead 5/11/26<br>Anthrax. |
| " 14680     | 10 " "                      | -      | 100 M.L.D.                     | Remained<br>alive.       |

As one sheep remained alive the experiment was repeated. On two more sheep, injecting the same dose of filtrate and the same amount of virulent material with the result that both died.

From these experiments it was clear that artificial-ly cultivated young bacilli may be regarded as containing no endocellular toxic substance sufficient to kill a mouse. Similarly it was devoid of any substance capable of conferring immunity on sheep.

## 2. (c) Experiments with washed cultures.

The object of these experiments was to determine the virulence and also the immunizing powers of anthrax organisms after complete removal of all traces of their extracellular products or metabolism. This was carried out by the repeated washing in normal saline and centrifugalising of the organisms. It has been shown that certain bacteria e.g. the *Bacillus chauveaui* of Black quarter, when completely separated from all extracellular products both the vegetative forms and the spores are incapable of setting up infection and causing death.

Experiments on anthrax were carried out as follows:

### Experiment 6 - spores only.

For this the glycerinised spore emulsion of a fairly

strong and very reliable vaccine batch was used. Microscopic examination of smears revealed the presence of spores only. A small amount of glycerinised emulsion was diluted in saline and thoroughly centrifuged. The supernatant fluid was siphoned off and the precipitate consisting of spores again shaken up with saline and the centrifuging repeated. This was done 6 times until probably all traces of extracellular substances had been removed. Various saline dilutions of the spores were then made up and injected into small animals as follows: -

|  |   |
|--|---|
| 1 guineapig - 1 c.c. washed spores         | } All animals<br>died within 4<br>days from Anthrax |
| 1 guineapig - 1 c.c. of .1 dilution spores |   |
| 1 " - 1 c.c. " .01 " "                     |   |
| 1 " - 1 c.c. " .001 " "                    |   |

Four white mice injected with the same amounts also died within 4 days. The material was then tested on sheep as follows:

| Animal.     | Date washed<br>vaccine spores<br>25/11/26 | Result | Date virulent<br>spores<br>6/12/26 | Result             |
|-------------|---|--------|------------------------------------|--------------------|
| Sheep 15623 | 1 c.c. subcut.                            | -      | 500 M.L.D.                         | Remained<br>alive. |
| " 14338     | .001 c.c. "                               | -      | 250 M.L.D.                         | Remained<br>alive. |

This experiment therefore clearly demonstrated that the *Bacillus anthracis* (in the spore stage) unlike the *Bacillus chauveaui* is capable of causing death as also of immunizing sheep without the presence of any extracellular products i.e. toxins or aggressins.

#### Experiment 7 - Vegetative forms only.

The spore emulsion from the same vaccine batch was inoculated into broth tubes and incubated for 24 hours. Microscopic examination revealed numerous bacilli but no spores. The broth culture was then centrifuged and the precipitate of bacilli repeatedly washed as in the previous experiment. Various dilutions of the bacilli were again injected into small animals, guineapigs and white

mice. These all died from anthrax following injections of dilutions up to .01 of the original suspension of bacilli. Sheep were also tested as follows: -

| ANIMAL      | Date washed<br>vaccine spores<br>25/11/26 | Result | Date virulent<br>spores<br>6/12/26 | Result                      |
|-------------|---|--------|------------------------------------|-----------------------------|
| Sheep 15621 | 1 c.c. subcut.                            | -      | 500 M.L.D.                         | Remained<br>alive.          |
| " 15592     | .0001 c.c. "                              | -      | 250 M.L.D.                         | Dead<br>9/12/26<br>Anthrax. |

The amount used on the second sheep was obviously too small and on retesting another animal with a larger amount it withstood the immunity test.

From this experiment, as from the previous one, it was clear that the washed bacilli were capable of killing small animals and of immunizing sheep.

### 3. (a) Production of immunizing or toxic substances.

#### Artificial in vitro experiments.

Numerous investigations have been conducted with the object to ascertain the actual cause of death following a fatal anthrax infection. The view is frequently held that the tremendous accumulation of anthrax bacilli in the general blood circulation towards the end of an infection is in itself sufficient to cause death, i.e. by purely mechanical means through a blockage of numerous capillaries supplying vital organs. According to this view death could result from internal asphyxia. Other authors again maintain that it may be due to pulmonary embolism. Although apparently of great significance, this mechanical factor alone does not explain the cause of death in every instance, since it may happen that death takes place without the presence of many organisms in the circulation. This is especially noticeable in equines where only very few organisms may be found in the blood after death. The possibility of toxic substances

elaborated by the *B. anthracis* has, therefore, to be considered. From this point the results of the investigations of the various authors are most conflicting.

According to Pasteur and Joubert, Levy and Beckman, Sanarelli, Hruska and others, no toxins are elaborated by the *B. anthracis*. Conradi claims to have shown that neither endo- nor extracellular toxins are formed by the *B. anthracis* whether cultivated in vitro or in the animal body. Against this many workers claim to have demonstrated definitely toxic substances formed by the anthrax organism. Hoffa, Hankin and Westbrook, Martin, Brieger and Fraenkel, Karmier and more recently Markoff, Bierbaum and Boehmke, all maintain that toxins, capable of causing definite symptoms and even death, are formed by anthrax organisms. Further, the immunizing property of these metabolic products of the *B. anthracis* has formed the subject for close investigation. Here again the evidence brought forward is very conflicting, some authors claiming reliable immunizing properties for certain germ free anthrax products, while others report completely negative results.

Bail was the first to apply his conception of aggrassin immunity to anthrax. By the use of sterile material collected from the oedematous infiltrations of animals dead from anthrax, he was able to cause a strong immunity in sheep, rabbits and guineapigs. That this was not of the nature of a passive immunity was shown by the fact that it only developed 8 to 10 days after vaccination of the animals. Markoff also found shaken extracts prepared from anthrax cultures protecting rabbits against a subsequent virulent infection. Working on Bail's aggrassin theory Okuda described the collection of subcutaneous oedema fluids from anthrax infected rabbits and the strong immunizing powers of such fluids for other rabbits. Likewise Matsumoto found the sterile oedema fluid of infected

rabbits of definitely immunizing value. He concludes that the metabolic products of the *B. anthracis*, present in such fluids are responsible for the immunity produced, and further that such aggrassin are in some way comparable to bacterial toxins. Guinea-pigs, however, were found to be far more difficult to immunize with the aggrassin. According to Salsbery recent investigations have resulted in the production of a true anthrax aggrassin capable of conferring a solid immunity in different classes of domesticated animals. The aggrassin is constituted of the sterilised and filtered body juices collected from the carcasses of animals which have succumbed to an infection of virulent anthrax. Experiments conducted on laboratory animals as well as on sheep showed that the so-called aggrassin had well marked antigenic properties, while outbreaks of anthrax amongst cattle under natural conditions could be stopped by aggrassin injections.

Since the results obtained by different workers were so conflicting, it was decided to conduct some experiments with the *B. anthracis* both in vitro and in vivo in order to demonstrate the presence or absence of any such active metabolic products.

For this purpose a number of different liquid media was prepared. The sterile medium was then inoculated either with a fairly strong vaccine strain of anthrax, or with fully virulent cultures, and incubated at 37° C for varying periods. After this the culture was filtered through a Seitz bacterial filter and the filtrate tested for sterility by subcultivation and injection of small animals. Should these animals succumb to anthrax, it was clear proof that filtration had been incomplete, while if smears were negative and subcultures of both the filtrate and the spleen of the dead animal sterile, death was regarded as being due to toxic action of the filtrate. The immunizing power of the



filtrate was tested out on sheep and goats in the manner already described.

Experiment 8.

Six flasks each containing one litre of sterile nutrient broth of F# 7.6 were inoculated with 15 c.c. each of 24 hour broth culture made from a fairly strong vaccine strain, and incubated at 37° C.

Flask 1. inoculated and kept as a control.

Flask 2. 15 c.c. 24 hour broth culture of the same strain added every 14 days.

Flask 3. 200 c.c. of a 2% peptone solution added every 14 days.

Flask 4. 200 c.c. fresh broth added every 14 days.

Flask 5. 15 c.c. fresh culture - 200 c.c. broth added every 14 days.

Flask 6. 15 c.c. fresh culture - 200 c.c. 2% peptone added every 14 days.

These materials were added in an attempt to promote and continue the growth. All flasks after being incubated for five weeks, were filtered through a Seitz bacterial filter and tested for sterility.

Subsequently small animals were injected with the filtrate from each flask as follows: -

1 rabbit - 5 c.c. filtrate subcutaneously.

1 guineapigs - 4 c.c. " "

1 " - 2 " " "

1 white mouse - 1 " " "

1 " " - .5 " " "

Of the 30 animals thus injected every one remained in good health. It was then decided after 14 days to test the immunity and the same animals now injected with virulent spore emulsion rabbits 100 M.L.D. guineapigs 50 M.L.D. and white mice with one needle prick from the virulent material. The result was that all the animals, excepting two mice,

died from anthrax within 5 days after the virulent inoculation.

The material was then tested out on sheep and goats, using two sheep and two goats for each of the six filtrates, as follows: -

|         |           |          |         |                   |           |
|---------|-----------|----------|---------|-------------------|-----------|
| 1 sheep | - 10 c.c. | filtrate | subcut. | later tested with | 500 M.L.D |
| 1 "     | 5 "       | "        | "       | "                 | 250 "     |
| 1 goat  | 10 "      | "        | "       | "                 | 250 "     |
| 1 "     | 5 "       | "        | "       | "                 | 100 "     |

Of the 24 animals injected with filtrate not one showed any ill effect. The immunity was tested after 20 days with virulent spores ranging from 500 to 100 M.L.D.

The result was that every animal, except one sheep from flask 4, died from anthrax within 5 days.

From this experiment it was clear that the broth filtrates were neither toxic to small animals nor did they possess any marked immunizing power for larger animals.

#### Experiment 9.

In this case a flask containing one litre liver broth, as prepared for the growth of *B. chauveaui* in the production of Blackquarter filtrate, was inoculated with a young broth culture made from a reliable vaccine anthrax strain and incubated at 37° C for 23 days, and then filtered. The sterile filtrate was injected into guineapigs and mice and also into sheep.

Two guineapigs each injected with 5 and 3 c.c. respectively, and two mice with one c.c. each, all remained alive and healthy.

| Animal      | Date filtrate  | Date virulent  | Result     | Result                   |
|-------------|----------------|----------------|------------|--------------------------|
|             | 10/6/26        | spores 22/6/26 |            |                          |
| Sheep 13887 | 5 c.c. subcut. | -              | 100 M.L.D. | Dead 25/6/26<br>Anthrax. |
| " 12711     | 20 " "         | -              | 100 M.L.D. | Dead 26/6/26<br>Anthrax. |

This filtrate therefore exhibited neither toxic action nor

immunizing properties.

Experiment 10.

It was then decided to grow fully virulent anthrax organisms (a) in ordinary broth, (b) broth + 10% anthrax immune serum. This was done for a period of 30 days and the cultures filtered, and the filtrate injected into animals.

Two guineapigs were injected subcutaneously 5 and 5 c.c. respectively from each of the two filtrates. They all remained alive and were injected 14 days later with 35 M.L.D. virulent spore emulsion. They all died from anthrax within 4 days.

Sheep were then injected as follows:

(a) Filtrate from plain broth culture.

| Animal.     | Date Filtrate<br>10/6/26 | Result | Date virulent<br>spores<br>22/6/26 | Result                   |
|-------------|--------------------------|--------|------------------------------------|--------------------------|
| Sheep 13587 | 5 c.c. subcut.           | -      | 100 M.L.D.                         | Dead 24/6/26<br>Anthrax. |
| " 13884     | 20 " "                   | -      | 100 M.L.D.                         | Dead 25/6/26<br>Anthrax. |

(b) Filtrate from broth + anthrax immune serum culture.

| Animal      | Date Filtrate<br>10/6/26 | Result | Date virulent<br>spores<br>22/6/26 | Result                   |
|-------------|--------------------------|--------|------------------------------------|--------------------------|
| Sheep 13954 | 5 c.c. subc.             | -      | 100 M.L.D.                         | Dead 24/6/26<br>Anthrax. |
| " 13639     | 20 " "                   | -      | 100 M.L.D.                         | Dead 26/6/26<br>Anthrax. |

As in Experiment 9 these filtrates were neither toxic nor did they produce immunity in sheep.

Two horses were injected subcutaneously on the left side of the neck with 10 c.c. and 5 c.c. respectively of the plain broth culture filtrate. At the same time 10 c.c. of ordinary sterile broth was injected into a third horse acting as control. The object of this experiment was to note the development of oedematous swellings frequently seen in horses after vaccination. In all three animals the temperature remained normal and not the sign of a swelling

Noted.

Experiment 11 (see also Expt. 3).

For this purpose the same cultures as used in experiment 3 were filtered, and the sterile filtrates tested out on animals. This consisted of virulent anthrax grown for 30 days in pea broth, bean broth, dog broth, chicken broth, egg broth, potato broth, broth + 10% hyperimmune serum and ordinary broth. Whereas all the small animals died from anthrax following the injection of the live cultures not a single one died from the filtrate, two guinea-pigs each being injected with 5 c.c. The filtrate was also tested out on 2 sheep in each case receiving 15 c.c. and 5 c.c. respectively. After 14 days the immunity was tested by injection of 100 M.L.D. and 50 M.L.D. respectively. The result was that with three exceptions from different filtrates, all the sheep died from anthrax within 5 days. This once more proved that by growing virulent anthrax in different media neither toxic nor immunizing substances were elaborated to any appreciable extent.

Experiment 12.

In this experiment different body fluids were collected and used either as such or diluted with nutrient broth as media in which fully virulent anthrax organisms were grown for several weeks.

(a) A culture consisting of ordinary sterile sheep's blood in a flask inoculated with a 24 hour broth culture of virulent organisms was incubated for three weeks at 37°C. It was then diluted with two volumes of normal saline to facilitate filtration and passed through a Seitz bacterial filter. The sterile filtrate was injected into guinea-pigs in 5 c.c. amounts, without however producing any ill effect. It was then tested out on sheep:

| Animal         | Date filtrate<br>16/9/26 | Result | Date virulent<br>spores 29/9/26 | Result                   |
|----------------|--------------------------|--------|---------------------------------|--------------------------|
| Sheep<br>13042 | 10 c.c.                  | -      | 250 M.L.D.                      | Dead 2/10/26<br>Anthrax. |
| 6572           | 5 c.c.                   | -      | 100 M.L.D.                      | Dead 1/10/26<br>Anthrax. |

This filtrate therefore again showed neither toxic action nor immunizing properties.

(b) The fluid present in the abdominal cavity of a dog suffering from ascites was carefully drawn off and diluted with an equal volume of nutrient broth. In this medium virulent organisms were grown for 3 weeks and then filtered. The filtrate was injected into guineapigs without causing any symptoms or death. It was then injected subcutaneously into sheep:

| Animal         | Date filtrate<br>21/10/26 | Result | Date virulent<br>spores 1/11/26 | Result                    |
|----------------|---------------------------|--------|---------------------------------|---------------------------|
| Sheep<br>14260 | 5 c.c. filtrate           | -      | 100 M.L.D.                      | Dead 11/11/26<br>Anthrax. |
| 12376          | 10 " "                    | -      | 100 M.L.D.                      | Remained alive.           |

As the sheep injected with the smaller 5 c.c. dose of filtrate only died from anthrax eleven days after the injection of the virulent test dose, while the one injected with the 10 c.c. filtrate withstood the virulent test, it was decided to repeat the experiment as follows: -

| Animal         | Date filtrate<br>1/12/26 | Result | Date virulent<br>spores 13/12/26 | Result                    |
|----------------|--------------------------|--------|----------------------------------|---------------------------|
| Sheep<br>15171 | 10 c.c. filtrate         | -      | 100 M.L.D.                       | Remained alive            |
| 15178          | 10 " "                   | -      | 100 M.L.D.                       | Dead 20/12/26<br>Anthrax. |

It is thus seen that in a retest one sheep again remained alive after the virulent dose whereas the other died from anthrax 7 days after the second injection. From the latter experiment it would appear that by cultivating virulent anthrax organisms in a medium consisting of ascites fluid mixed with ordinary broth some metabolic substance is elaborated which is capable of producing an immunity in a

certain percentage of sheep. Except perhaps in the last mentioned experiment no definitely toxic or inert but immunizing substance could be demonstrated in any of the various liquid media in which vaccine or even virulent anthrax organisms had been cultivated for long periods. In this respect the *Bacillus anthracis* differs materially from such organisms as the *Bacillus tetani* or *Bacillus chauveaui*.

3. (b) Production of Immunizing or Toxic Substances  
in the <sup>a</sup>Animal body.

What is termed a "true anthrax aggressin" has within recent years been placed on the market. It is stated to consist of the sterilised and filtered body juices collected from the carcasses of animals which have died as the result of artificial infection with anthrax. For this product is claimed the property of conferring a solid active immunity against anthrax and besides this, complete safety to vaccinated animals.

As a supply of this material had been introduced into South Africa it was decided to ascertain experimentally its degree of safety and immunizing properties on different animals.

Experiment 13.

Subcultures made from the "aggressin" in broth and on agar all remained completely sterile. According to the directions for use guineapigs were injected subcutaneously with the following amounts:

Two guineapigs 1 c.c. each and 2 guineapigs 2 c.c. each. No symptoms were shown, all the animals remaining in good health. It was then decided to test the material on sheep and goats as follows: (The dose injected corresponded with that advised by the manufacturers).

| Animal      | Date aggressin<br>10/8/26 | Result | Date virulent<br>spores 24/8/26     | Result               |
|-------------|---------------------------|--------|-------------------------------------|----------------------|
| Sheep 12068 | 3 c.c. subcut.            | -      | 500 M.L.D.                          | Dead 30/8/26 Anthrax |
| " 10850     | 3 " "                     | -      | 250 "                               | " 27/8/26 "          |
| " 12990     | 3 " "                     | -      | 30/8/26                             | " 2/9/26 "           |
| " 12970     | 3 " "                     | -      | 100 M.L.D.<br>30/8/26<br>100 M.L.D. | " 4/9/26 "           |
| Goat 14487  | 3 " "                     | -      | 250 "                               | " 26/8/26 "          |
| " 14449     | 3 " "                     | -      | 100 "                               | " 27/8/26 "          |
| " 14604     | 3 " "                     | -      | 30/8/26<br>50 M.L.D.                | " 2/9/26 "           |
| " 14595     | 3 " "                     | -      | 30/8/26<br>50 M.L.D.                | " 2/9/26 "           |

Thus of the 4 sheep and 4 goats vaccinated not a single one survived the immunity test, clearly showing that the immunity produced, if any at all, must have been very weak indeed. In none of the animals vaccinated was any temperature reaction noticed so that complete safety of the vaccine could not be disputed.

Seeing that no artificial product with reliable immunizing properties could be obtained, it was decided to ascertain whether in the animal body any such substances were elaborated after artificial infection with anthrax. With this object in view a series of experiments were conducted as follows:-

Susceptible sheep and goats were to be artificially infected with anthrax and blood smears examined at regular intervals. As soon as the smears revealed numerous bacilli and signs of approaching death noticed, blood from the jugular vein was to be drawn off aseptically into sterile flasks. Immediately after death, the carcass was to be carefully opened and whatever other body fluids could be found besides the blood, were to be collected in sterile vessels. Parts of the different organs were also to be collected.

The material thus collected were to be treated in different ways:

(a) Some of the body fluids including the blood were to be passed through Seitz bacterial filters and sterile filtrate obtained. To facilitate filtration the blood was citrated at the moment of collection so as to prevent clotting, and afterwards diluted with two or three volumes of sterile normal saline to decrease the viscosity.

(b) In some flasks containing these fluids the anthrax infection was to be killed off by chemical means. In this connection various preliminary experiments were conducted both on anthrax cultures and anthrax infected blood. Of the various chemical substances tested out, it was found that neosalvarsan in a dilution 1 : 5000 caused the complete sterilisation of both anthrax cultures and infected blood within two hours. This was clearly proved by numerous subcultures and injections into small animals. Smears made from such material revealed marked disintegration of the organisms within 24 hours, while after a few days, only the debris of the disintegrated organisms could be made out.

Similar experiments were conducted with anilin gentian violet in various dilutions. It was found that a dilution of 1 : 2000 killed off the anthrax infection within 24 hours, and that in the course of a few days complete disintegration had resulted. Various dilutions of Yatren were also tested but with less satisfactory results. For this series of experiments it was, therefore, decided to use unfiltered citrated blood treated either with neosalvarsan 1 : 5000 or anilin gentian violet 1 : 2000.

(c) Extracts from different organs were made by macerating these up with sterile normal saline in a mortar and subsequently filtering through a bacterial filter.

All these materials were then tested (1) on small animals for toxic properties, and (2) on larger animals for their



immunizing value.

Experiment 14. Unfiltered Blood.

Goat 14471 was injected subcutaneously with 250 M.L.D. virulent spore emulsion. On the second day the temperature rose to 103.5°F. Twenty minutes before death blood smears revealed numerous bacilli. Blood was collected from the jugular vein (a) in Citrate + neosalvarsan (b) in citrate + anilin gentian violet. Subcultures made on the following day all remained sterile. Guinea pigs injected with 2 c.c. and white mice with .5 c.c. each of the two samples of blood all remained alive. The material was then tested out on sheep as follows:

| Animal      | Date sterile blood<br>6/7/26            | Result | Date virulent<br>spores 15/7/26 | Result          |
|-------------|---|--------|---------------------------------|-----------------|
| Sheep 12898 | 2 c.c. citr. + neosalvarsan blood       | -      | 100 M.L.D.                      | Remained alive. |
| " 7539      | 5 " " " "                               | -      | 250 "                           | Remained alive. |
| " 13821     | 2 " " + an.gent. <sup>viol.</sup> blood | -      | 100 "                           | " "             |
| " 12889     | 5 " " + " " " "                         | -      | 250 "                           | " "             |

As all 4 sheep survived the immunity test, there were definite signs that an immunity had been established. It was then decided to retest the material using a larger number of animals.

| Animal      | Date sterile blood<br>20/7/26     | Result | Date virulent<br>spores | Result                   |
|-------------|-----------------------------------|--------|-------------------------|--------------------------|
| Sheep 13841 | 2 c.c. citr. + neosalvarsan blood | -      | 100 M.L.D. 8/8/26       | Remained alive           |
| " 13891     | 5 " " " "                         | -      | 250 " 16/8/26           | " "                      |
| " 13873     | 2 " " + an.gent.viol. blood       | -      | 100 " 16/8/26           | " "                      |
| " 9213      | 5 " " " " " "                     | -      | 100 " 8/8/26            | " "                      |
| Goat 14493  | 2 " " + neosalvarsan Blood        | -      | 100 " 16/8/26           | " "                      |
| " 14491     | 5 " " " " " "                     | -      | 100 " 8/8/26            | " "                      |
| " 14452     | 2 " " + an.gent.viol. blood       | -      | 100 " "                 | " "                      |
| " 14486     | 5 " " " " " "                     | -      | 250 " 16/8/26           | Dead 21/8/26<br>Anthrax. |

Thus from the 4 sheep and 4 goats used for the retest, in which some were injected with 5 c.c. and others with 2 c.c. of the two samples of blood, only one goat succumbed from anthrax 5 days after the immunity test.

In some cases the immunity was tested 18 days after vaccination while in the rest 27 days after vaccination. The goat that died was injected with 250 M.L.D. 27 days after vaccination.

Thus the preliminary evidence that an active immunity had been established by this material was proved beyond any doubt. The fact that in some cases the immunity was tested as long as 27 days after vaccination, would tend to exclude the possibility of a purely passive immunity, as e.g. shown by anthrax sera, and which generally passes off within the first week after injection.

In order to establish the effect that the neosalvarsan in the blood might have produced on the immunity test several experiments were conducted with this drug: -

| Animal      | First injection                                 | Second injection                  | Result                   |
|-------------|---|-----------------------------------|--------------------------|
| Sheep 11785 | 100 M.L.D. subcut.                              | 1 gram neosalvarsan 48 hrs. later | Dead on 3rd day Anthrax. |
| " 13618     | 50 " "  | 1 " "                             | Dead on 3rd day Anthrax. |
| " 13893     | 100 " "   | 1 " " 24 hrs. later               | Dead on 6th day Anthrax. |
| " 13774     | 50 " "  | 1 " "                             | Dead on 4th day Anthrax. |
| " 13065     | Simultaneously 100 M.L.D. + 1 gram neosalvarsan |                                   | Remained alive           |
| " 10456     | 1.5 gram "                                      | 100 M.L.D. 12 hours later         | Dead on 3rd day Anthrax. |

Of the 6 sheep tested in different ways only one remained alive viz. in the case where the virulent material and the neosalvarsan injections were made simultaneously, the first subcutaneously and the latter intravenously. As was shown in the case of sheep 10456, where 1.5 gram neosalvarsan was injected 12 hours before the virulent material, the bactericidal effect of the drug was so reduced within this short

period that the animal succumbed to anthrax within 3 days. From these experiments it was clear that the survival of the animals treated with the blood samples and afterwards with virulent material could not have been due to any salvarsan still present in the body after 27 days.

After a period of 7 weeks this same material was again tested both for its keeping qualities as also for its thermostability. For this purpose some of the blood was pasteurised in a water bath at 60° C for 30 minutes while some was pasteurised at 80° C for 30 minutes. It was then injected into sheep:

| Animal      | Date sterile blood injected 20/8/26                              | Result | Date virulent spores 3/9/26 | Result         |
|-------------|--|--------|-----------------------------|----------------|
| Sheep 12038 | 5 c.c. citr. + neosalvarsan past. 60° C for 30 min.              | -      | 100 M.L.D.                  | Remained alive |
| " 13846     | 5 c.c. citr. + An. gent. viol. blood past. at 60° C. for 30 min. | -      | 100 "                       | " "            |
| " 10414     | 5 c.c. citr. + neosalvarsan past. at 80° C for 30 min.           | -      | 100 "                       | " "            |
| " 10588     | 5 c.c. citr. + An. gent. viol. blood past. at 80° C for 30 min.  | -      | 100 "                       | " "            |

After a period of 5 months this material was again tested and some pasteurised at 92° C for 30 minutes.

| Animal      | Date sterile blood injected 23/11/26                               | Result | Date virulent spores 6/12/26 | Result                 |
|-------------|--|--------|------------------------------|------------------------|
| Sheep 15536 | 5 c.c. citr. + neosalvarsan past. at 80° C for 30 minutes          | -      | 100 M.L.D.                   | Remained alive         |
| " 15648     | 5 c.c. citr. + neosalvarsan bld. past. at 92° C for 30 minutes.    | -      | 100 "                        | " "                    |
| " 15114     | 5 c.c. citr. + An. gent. viol. bld. past. at 80° C for 30 minutes. | -      | 100 "                        | " "                    |
| " 15522     | 5 c.c. citr. + An. gent. viol. bld. past. at 92° C for 30 minutes. | -      | 100 "                        | Dead 10/12/26 Anthrax. |

In this test one of the sheep died from anthrax 4 days after the virulent injection. Thus out of the 20 animals vaccinated with this material 18 were definitely immunized, even after keeping the blood for 5 months and then pasteurising it up to 92° C for 30 minutes.

Experiment 15 - Filtered Blood.

In this experiment the filtered blood from sheep dead from anthrax, was tested.

Sheep 12927 was injected with 250 M.L.D. and blood collected 15 minutes before its death from anthrax, blood smears showing numerous bacilli. The citrated blood was diluted with an equal volume of saline/and immediately filtered. The sterile filtrate was injected into guineapigs without any ill effect. It was then injected into susceptible sheep:

| Animal      | Date sterile filtrate   | Result | Date virulent spores | Result.               |
|-------------|-------------------------|--------|----------------------|-----------------------|
| Sheep 14324 | 10 c.c. subcut. 16/9/26 | -      | 250 M.L.D. 29/9/26   | Dead 3/10/26 Anthrax. |
| 13771       | 5 " " 16/9/26           | -      | 100 " 29/9/26        | Dead 2/10/26 Anthrax. |

As these two sheep died rather unexpectedly after the immunity test, it was decided to procure a fresh sample of blood from another sheep infected with anthrax.

Sheep 13675 was injected with 250 M.L.D. virulent spores. The animal was bled a few minutes before its death on the third day. Blood smears showed numerous anthrax bacilli. The citrated blood diluted with an equal volume of saline was again immediately filtered and the sterile filtrate tested out on guineapigs without any ill effect. It was then injected into sheep:

| Animal      | Date sterile filtrate   | Result | Date virulent spores | Result                 |
|-------------|-------------------------|--------|----------------------|------------------------|
| Sheep 11671 | 2 c.c. subcut. 30/10/26 | -      | 250 M.L.D. 11/11/26  | Dead 13/11/26 Anthrax. |
| 14279       | 5 " " 30/10/26          | -      | 250 " 11/11/26       | Dead 14/11/26 Anthrax. |

As in the previous test the two sheep rapidly succumbed from anthrax after the immunity test. It was thus clear that the blood from which the anthrax organisms had been separated by filtration showed no immunizing powers as compared with the immunity provoked by whole blood in which disintegration

of the organisms had been brought about by chemical means. All evidence pointed to the bacillary debris in the blood as being the real immunizing substance in these experiments. The same immunizing properties, however, could not be claimed for anthrax bacilli cultivated on artificial media and disintegrated either by Anilin gentian violet or by actual grinding up. (See Experiment 5).

Experiment 16.

In this experiment the sterile filtered body juices of several animals dead from anthrax, as well as filtered organ extracts were tested on sheep.

(a) A sterile saline filtrate of minced spleen obtained from goat 15225 dead from virulent anthrax was injected into sheep:

| Animal      | Date sterile spleen filtrate<br>30/10/26 | Result | Date virulent spores<br>11/11/26 | Result                    |
|-------------|--|--------|----------------------------------|---------------------------|
| Sheep 13951 | 2 c.c. subcut.                           | -      | 250 M.L.D.                       | Dead 3/11/26<br>Anthrax   |
| " 13947     | b " "                                    | -      | 250 "                            | Dead 14/11/26<br>Anthrax. |

(b) Sterile filtered bile obtained from the same goat was also tested on sheep:

| Animal      | Date sterile filtered bile<br>30/10/26 | Result | Date virulent spores<br>11/11/26 | Result                   |
|-------------|--|--------|----------------------------------|--------------------------|
| Sheep 14326 | 2 c.c. bile filtrate                   | -      | 100 M.L.D.                       | Remained alive.          |
| " 13725     | 5 c.c. bile filtrate                   | -      | 100 "                            | Dead 12/11/26<br>Anthrax |

(c) Sterile filtered peritoneal fluid obtained from goat 14391, dead from virulent anthrax, was injected into sheep:

| Animal      | Date sterile fluid<br>18/9/26 | Result | Date virulent spores<br>29/9/26 | Result                   |
|-------------|-------------------------------|--------|---------------------------------|--------------------------|
| Sheep 12201 | 10 c.c. subcut.               | -      | 100 M.L.D.                      | Dead 5/10/26<br>Anthrax. |
| " 13992     | 5 " "                         | -      | 100 "                           | Remained alive.          |

On repeating the latter two experiments both animals died after the virulent test. Thus, as in the case of the

filtered blood the other body fluids and organ extracts showed no definite immunizing powers.

Experiment 17. - Oedema fluid.

A number of horses vaccinated with a certain fairly weak batch of anthrax spore vaccine developed well marked subcutaneous oedematous swellings. It was decided to collect some of this oedema fluid and test it out on animals. Horse 18028 showing a severe oedema of the lower cervical and sternal regions following vaccination was used for supplying the oedema fluid.

The clear fluid was drawn off into a sterile flask. Subcultures made from the fluid all remained sterile. Guinea pigs injected with 5 c.c. each of the pure oedema fluid all remained alive.

| Animal      | Date oedema fluid 30/8/26 | Result | Date virulent spores 13/9/26 | Result                |
|-------------|---------------------------|--------|------------------------------|-----------------------|
| Sheep 13909 | 10 c.c. oed. fluid        | -      | 250 M.L.D.                   | Dead 16/9/26 Anthrax. |
| " 13748     | 10 " "                    | -      | 100 "                        | Remained alive.       |
| " 13069     | 5 " "                     | -      | 250 "                        | Dead 18/9/26 Anthrax. |
| " 12695     | 5 " "                     | -      | 100 "                        | Dead 17/9/26 Anthrax. |

Thus out of the 4 sheep injected with as much as 10 c.c. of the pure fluid three died from anthrax after the immunity test showing that the immunizing power, if any, was decidedly very weak. At the same time this fluid was also injected into two non-vaccinated horses, the object being to note the possible development of similar swellings. Horse 17827 was injected with 5 c.c. and horse 17700 with 10 c.c. both subcutaneously on the side of the neck. The animals were examined daily for 12 days and temperature records kept. During this time neither of the animals showed any temperature disturbances nor was there any swelling noticed. The latter experiment would, therefore, tend to exclude the possibility of a toxic substance being the cause of the subcutaneous oedema in horses.

Experiment 18.

The object of this experiment was to ascertain the effect of various non living anthrax products on the course of an anthrax infection in guineapigs. <sup>were injected subcut. with equal amount.</sup> Guineapigs of a definite dilution of Vaccine Anthrax spores. In some of these left as controls the time of death was carefully noted, while the others were injected simultaneously with the various anthrax products which by themselves showed no harmful effects on these animals. Again the time of death was noted.

All animals injected on the 17/9/26.

(a) Controls.

1 guineapig - 1 c.c. of .01 dilution spores - dead in 4 days  
 Anthrax.  
 1 " - 1 " " .01 " " - dead in 5 days  
 Anthrax.

(b) Vaccine spores + sterile broth filtrate obtained from 30 days old virulent anthrax broth culture.

1 guineapig - 1 c.c. of .01 dilution spores + 2 c.c. filtrate  
 dead in 4 days Anthrax.  
 1 " - 1 " " .01 dilution spores + 2 c.c. filtrate  
 dead in 4 days Anthrax.

(c) Vaccine spores + oedema fluid from horse 18028 showing anthrax swellings after vaccination.

1 guineapig - 1 c.c. of .01 dilution spores + 2 c.c. oedema  
 fluid - dead in 4 days Anthrax.  
 1 " - 1 " " .01 dilution spores + 2 c.c. oedema  
 fluid - dead in 5 days Anthrax.

(d) Vaccine spores + sterile filtrate obtained from 30 day old blood culture of virulent Anthrax.

1 guineapig - 1 c.c. of .01 dilution spores + 2 c.c. filtrate  
 dead in 4 days Anthrax.  
 1 " - 1 " " .01 dilution spores + 2 c.c. filtrate  
 dead in 4 days Anthrax.

(e) Vaccine spores + sterile filtrate obtained from anthrax blood of sheep 12927.

1 guineapig - 1 c.c. of .01 dilution spores + 2 c.c. filtrate  
 dead in 4 days Anthrax.  
 1 " - 1 " " .01 dilution spores + 2 c.c. filtrate  
 dead in 6 days Anthrax.

(f) Vaccine spores + sterile filtered peritoneal fluid from goat 14391 dead from anthrax.

1 guineapig - 1 c.c. of .01 dilution spores + 2 c.c. filtrate  
 dead in 3 days Anthrax.  
 1 " - 1 " " .01 dilution spores + 2 c.c. filtrate  
 dead in 4 days Anthrax.

(g) Vaccine spores + imported sterile Anthrax aggrassin.

1 guineapig - 1 c.c. of .01 dilution spores + 2 c.c. aggrassin  
 dead in 3 days Anthrax.  
 1 " - 1 " " .01 dilution spores + 2 c.c. aggrassin  
 in - dead in 4 days Anthrax.

From these experiments it is clear that the different Anthrax products used, had no effect on the course of an anthrax infection in guineapigs when injected simultaneously with the live spores. In none of the cases was death definitely hastened or delayed since all the animals died practically on the same day as the controls did. It should be noted that the spores used in this experiment were repeatedly washed and centrifuged in order to free them from any adherent exo-products.

From all the experiments recorded in this section the following points were clearly evident:

(a) In every instance the metabolic products elaborated by the B. anthracis when cultivated in various liquid media for long periods and then filtered off, failed to reveal any toxic action in small animals or immunizing effect in sheep and goats.

(b) Various body juices, including blood, collected from anthrax infected animals and passed through bacterial filters were neither toxic nor of immunizing value.

(c) Anthrax blood in which disintegration of the B. anthracis had been brought about by chemical means possessed definite immunizing properties which resisted heating at 92°C for 30 minutes. The same qualities could not be claimed for disintegrated organisms after cultivation on artificial media.



(d) No soluble filterable "aggressin" could be demonstrated in any of the experiments. The immunizing substance present in anthrax blood was not found to be filterable, i.e. it is probably very closely associated with the organism in the blood, although it was not found in the case of organisms cultivated on artificial media.

#### 4. Comparative Safety and Immunity experiments with Anthrax Spore Vaccine.

Based on the results of various experiments conducted, Besredka has within recent years announced an original theory as to the mechanism of infection and immunity in Anthrax. According to this theory animals which are highly susceptible to anthrax can only become infected through the skin, which is the only organ capable of acting as the portal of entry for the anthrax organism. Where an infection of the skin was definitely excluded, a fatal artificial infection by any other route could not be brought about even in the case of highly susceptible animals such as guineapigs.

Further, successful immunization of susceptible animals is dependent on the primary immunity of the skin. Thus immunization of the skin is essential and sufficient in order to render animals immune to anthrax.

It is for these reasons that Besredka strongly advocates skin vaccination as the rational mode of immunizing animals against anthrax. Laboratory animals such as rabbits and guineapigs which by the usual methods of vaccination were very difficult to immunize could by cuti-vaccination be protected. These findings of Besredka have resulted in numerous investigators focussing attention on cuti-vaccination and cuti-immunity. The results obtained by different workers are, however, again markedly conflicting. While the findings of some are wholly in accordance with

those of Besredka, others were able to substantiate only certain points in the original theory. Some investigators on the other hand, have failed to confirm any of Besredka's findings. Of the various authors who have confirmed the results obtained by Besredka, the following may be mentioned: Velu believes that extensive vaccination of animals in Morocco has shown that a single intradermal injection of vaccine is sufficient to provoke a strong and lasting immunity even where no thermal or local reaction follows vaccination. The method of vaccination is practical and the immunity lasting 6 to 7 months.

According to Nevodov, horses and cattle can be successfully immunized by cuti-vaccination <sup>using</sup> ~~with~~ the vaccine 1 only. No complications are noted while animals may be worked immediately after vaccination.

From numerous experiments Bracq-Rousseau concludes that cuti-vaccination of horses leads to a strong and rapidly forming immunity without, however, any untoward complications.

Similarly Balteano, Plotz, Nicolas, Stoicescu, Vallee, Hruska, Kudwjavzew, Baliberdin, Rossi, Urbain and Rossi<sup>S</sup>, were able to confirm the findings of Besredka.

Several authors although partly in agreement with Besredka's theory, find that the skin is not the only organ susceptible to anthrax.

Thus Combiesco, Popesco, Gratia, Kritschewsky and Brussin, and more recently Matz and Amiraslanow, maintain that animals may be fatally infected by routes other than the skin. Furthermore, numerous experiments have shown that infection through the skin is less certain than by other routes, i.e. the skin is actually less susceptible than other tissues.

From various experiments conducted, Viljoen, Curson and Fourie found the skin no more susceptible than any other

organ and further the immunity following subcutaneous vaccination more reliable than that following cuti-vaccination. According to Gusman, Neri, Pazewicz, Lanfranchi<sup>n</sup> and Casalotti, their experiments did not confirm Besredka's findings, i.e. cuti-vaccination of laboratory animals was not followed by reliable immunity against anthrax.

Seeing that there exists a wide difference of opinion as to the relative susceptibility of animals to different routes of infection as well as the merits of different methods of vaccination, a lengthy series of experiments were conducted with the following objects in view: -

- (a) To attempt the artificial infection of small animals with anthrax in different ways, at the same time excluding as far as possible infection in other ways.
- (b) To attempt the immunisation of small animals by different methods of vaccination.
- (c) To compare the safety and immunity conferred by anthrax spore vaccine on sheep and goats by different methods of vaccination.
- (d) To note the clinical reaction produced in horses following various methods of vaccination.

In all these experiments the vaccine consisted of a glycerinised spore emulsion of a batch of anthrax vaccine, the safety and immunizing properties of which had been carefully ascertained beforehand. The vaccine in each case was prepared by cultivating the attenuated organisms on nutrient agar and collecting the growth as soon as sporulation was practically complete. Subsequently the spore emulsion preserved in an equal weight of glycerine was tested out on guineapigs and rabbits by subcutaneous injection of various fractions of one cubic centimetre, and the lethal effect noticed. Should the rabbits survive, the material was tested out on sheep and goats in amounts ranging from 20 c.c. to .001 c.c. of the emulsion by subcutaneous injection.

After a period of 2 to 3 weeks the immunity was ascertained by the subcutaneous injection of virulent Anthrax Spore emulsion. This constitutes the routine procedure for the testing of different batches of anthrax vaccine before issue.

Experiment 19.

In this experiment only guineapigs were used. Precautions were taken to select animals that had not been in anthrax experiments previously. In order to prevent skin contamination with anthrax material in the course of injections made, where this was to be excluded, long hypodermic needles of very small bore were used. The point of the needle was introduced as far as possible from the skin puncture before the injection was made. On withdrawing the needle the edge of the skin at the puncture was immediately thoroughly disinfected by introducing the points of a very fine pincet rolled in cotton wool and steeped into a 10% solution of tincture of iodine. By twisting the points of the pincet in the skin puncture, the edges were immediately thoroughly disinfected. This procedure was adopted in every case where skin contamination was not desired. Guineapigs were injected with a definite dilution of a fairly weak vaccine known to kill them in that dilution after the usual subcutaneous inoculation. Animals that survived the first injection were afterwards injected subcutaneously with a stronger vaccine and which was regarded as the immunity test.

(a) Intraperitoneally - preventing skin contamination.

1 guineapig - .01 c.c. - dead after 7 days Anthrax.

1 " - .01 " - " " 8 " "

(b) Deep intramuscular - preventing skin contamination.

1 guineapig -.01 c.c. - dead after 6 days Anthrax.

1 " - .01 " - " " 6 " "

(c) Subcutaneous - preventing skin contamination.

1 guineapig - .01 c.c. - dead after 7 days Anthrax.

1 " - .01 " - survived first injection.

Injected 14 days later with .2 c.c. stronger vaccine. Dead 4 days later Anthrax.

(d) Scarification of skin and rubbing on of Vaccine.

1 guineapig - .01 c.c. } Both survived first injection. In-  
 1 " - .01 " } jected 14 days later with .2 c.c.  
 stronger vaccine. One dead 3 days  
 later Anthrax.

(e) Intratracheally - preventing skin contamination.

1 guineapig - .01 c.c. } Both survived first injection. In-  
 1 " - .01 " } jected 14 days later with .2 c.c.  
 stronger vaccine. Both dead 3 days  
 later Anthrax.

(f) Per os. On to the back of the tongue.

1 guineapig - .1 c.c. } Both survived first dose. injected  
 1 " - .1 " } subcutaneously 14 days later with .2  
 c.c. stronger vaccine. Both dead 4  
 days later Anthrax.

Controls: Two guineapigs injected subcutaneously in the ordinary way with .01 c.c. each of the first weaker vaccine died in 4 and 5 days respectively from anthrax.

Two guineapigs injected subcutaneously with .2 c.c. each of the second stronger vaccine both died in 4 days from Anthrax.

Thus in the above experiments guineapigs were readily killed by intraperitoneal, intramuscular, and subcutaneous injections of a weak vaccine, while all the guineapigs survived the intratracheal, oral, and intradermal by scarification, administration of the weak vaccine. These animals, however, all died after the test injection of a stronger vaccine 14 days later.

The above experiment was repeated, using smaller doses of the weak first vaccine, in order to lose fewer animals and to be able to test the immunity developed by means of a second injection of the same stronger vaccine.

(a) Intraperitoneal preventing skin contamination.

1 guineapig - .001 c.c. } Both survived first injection. In-  
 1 " - .001 " } jected 14 days later with .2 c.c.  
 stronger vaccine. Both died 4  
 days later from anthrax.

(b) Intramuscular - preventing skin contamination.

- 1 guineapig - .001 c.c. - dead 6 days later from Anthrax.  
 1 " - .001 " - survived first injection. Injected  
 14 days later with .2 c.c.  
 stronger vaccine. Remained alive.

(c) Subcutaneous - preventing skin contamination.

- 1 guineapig - .001 c.c. - dead after 9 days from Anthrax.  
 1 " - .001 " - Survived first injection. Injected  
 14 days later with .2 c.c. strong-  
 er vaccine. Dead 4 days later  
 from Anthrax.

(d) Scarification of skin and rubbing on of vaccine.

- 1 guineapig - .001 c.c. - dead 7 days afterwards Anthrax.  
 1 " - .001 " - survived injection. Injected 14 days  
 later with .2 c.c. stronger vacci-  
 nation. Dead 3 days later from  
 Anthrax.

(e) Intratracheal - preventing skin contamination.

- 1 guineapig - .001 c.c. } Both survived first injection. In-  
 1 " - .001 " } jected 14 days later with .2 c.c.  
 stronger vaccine. Both dead 4  
 days later Anthrax.

(f) Per os - on to the back of the tongue.

- 1 guineapig - .01 c.c. } Both survived first dose. Injected  
 1 " - .01 " } subcutaneously 14 days later with  
 .2 c.c. stronger vaccine. Both  
 dead 4 days later Anthrax.

A third immunity test was conducted in which case a larger number of guineapigs were vaccinated, with the same first vaccine in a dilution of 1 : 300 as issued to stock-owners. The immunity was subsequently tested with .01 c.c. instead of .2 c.c. of the second stronger vaccine, by subcutaneous injection.

(a) Subcutaneous vaccination - preventing skin contamination

immunity.

| Animal      | Date vaccine<br>3/5/27 | Result                       | Date stronger<br>vaccine<br>16/5/27 | Result                   |
|-------------|------------------------|------------------------------|-------------------------------------|--------------------------|
| 1 guineapig | 1 c.c. (dil.1:300)     | dead 11/5/27<br>not Anthrax. | -                                   | -                        |
| 1 "         | 1 " "                  | alive                        | .01 c.c. subc.                      | dead 19/5/27<br>Anthrax. |
| 1 "         | 1 " "                  | alive                        | .01 " "                             | dead 20/5/27<br>Anthrax. |
| 1 "         | 1 " "                  | alive                        | .01 " "                             | dead 20/5/27<br>Anthrax. |
| 1 "         | 1 " "                  | alive                        | .01 " "                             | Remained alive           |

(b) Deep intramuscular vaccination - preventing skin contamination.

| Animal      | Date vaccine<br>3/5/27 | Result                   | Date stronger<br>vaccine<br>16/5/27 | Result         |
|-------------|------------------------|--------------------------|-------------------------------------|----------------|
| 1 guineapig | 1 c.c. (dil.1:300)     | dead 9/5/27<br>Anthrax.  | -                                   | -              |
| 1 "         | 1 " "                  | dead 11/5/27<br>Anthrax. | -                                   | -              |
| 1 "         | 1 " "                  | alive                    | .01 c.c. Subc.                      | Remained alive |
| 1 "         | 1 " "                  | "                        | .01 " "                             | " "            |
| 1 "         | 1 " "                  | "                        | .01 " "                             | " "            |

(c) Intradermal injection of Vaccine.

| Animal      | Date vaccine<br>3/5/27 | Result | Date stronger<br>vaccine<br>16/5/27 | Result                   |
|-------------|------------------------|--------|-------------------------------------|--------------------------|
| 1 guineapig | .1 c.c. (dil.1:300)    | alive  | .01 c.c. subc.                      | Dead 20/5/27<br>Anthrax. |
| 1 "         | .1 " "                 | "      | .01 " "                             | Dead 20/5/27<br>Anthrax. |
| 1 "         | .1 " "                 | "      | .01 " "                             | Dead 23/5/27<br>Anthrax. |
| 1 "         | .1 " "                 | "      | .01 " "                             | Remained alive           |
| 1 "         | .1 " "                 | "      | .01 " "                             | " "                      |

From this retest it is seen that out of 5 guineapigs vaccinated subcutaneously, one died after vaccination but not from anthrax, while following the immunity test 3 out of 4 died from anthrax. Of those injected intramuscularly 2 out of the 5 died from anthrax after vaccination, while all three the others withstood the immunity test. Of the 5 vaccinated intradermally 3 died after the immunity test.

It should be noted that in the original experiment as well as in the retests the same vaccine was used only in different dilutions, while the same stronger vaccine was used for all the immunity tests. The following points were clear:

- (a) Guinea-pigs could be killed by intraperitoneal injections of vaccine anthrax, while in surviving cases no immunity could be demonstrated.
- (b) Guinea-pigs died from anthrax after deep intramuscular injection of vaccine, while in surviving cases the immunity was fairly definite.
- (c) Guinea-pigs were killed by subcutaneous injection of vaccine, while in surviving cases no immunity could be demonstrated.
- (d) By scarification of the skin or intradermal vaccination, some guinea-pigs died from anthrax, while in surviving cases the immunity was uncertain.
- (e) By intratracheal injections of vaccine, no deaths occurred, although no immunity could be demonstrated afterwards, since all the animals died after the test injection.
- (f) By the oral administration of 10 times the amount of vaccine used in the other tests, none of the guinea-pigs died, although no immunity could be shown afterwards.

Thus, except for the intratracheal and oral administration of anthrax vaccine, guinea-pigs could be easily killed by the different routes, while from the immunity tests the intramuscular vaccination would seem the most promising, while the results of intradermal vaccination were doubtful.

Comparative susceptibility and immunity experiments conducted on sheep and goats.

This series of experiments was carried out with the object of: (a) Ascertaining the differences in susceptibility