to the vaccine and the degree of immunity developed in sheep and especially in goats, following different methods of vaccination with Spore Vaccine.

(b) Ascertaining the merits of cuti-vaccination.

Experiment 20.

The vaccine used was the same as that employed on the guineapigs in the previous experiment. It was prepared from a fairly weak vaccine strain and issued for use in goats in a dilution of 1 : 300. In the routine tests on guineapigs, it killed them in a dilution of .01 c.c. while .001 c.c. proved safe. Tested out on sheep and goats all animals remained alive, although in the immunity test 2 out of the 6 sheep died as well as 3 out of 14 goats, showing that the immunizing power of the vaccine was not very strong.

The main aim of these tests was to compare the safety and immunity following: (a) subcutaneous and (b) cuti-vaccination, but since the intramuscular vaccination had given the most encouraging results in guineapigs, it was decided to include this method of vaccination in the tests. As in the guineapig experiments strict precautions were taken in order to avoid skin contamination with the vaccine when injected subcutaneously or intramuscularly. The injection was thus made as far as possible from the puncture of the skin, while the wound was immediately disinfected with the pincet and cotton wool swab soaked in 10% tincture of iodine solution. The vaccination in all cases was carried out on the inside of the thigh, while for the subsequent immunity test the inside of the other thigh was used. Temperature records were kept of all the animals.

The object in using the weak vaccine was to note the differences, if any, in the degree of immunity since with a stronger vaccine it is conceivable that these differences might quite easily have been masked. For the immunity tests a standard fully virulent spore emulsion of

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known strength was used. In this case the injections, unless otherwise stated, were all made subcutaneously into the other thigh of the animal.

A. All vaccine injections made subcutaneously preventing skin contamination:

Animal	Date Vaccine 5/4/1927	Result	Date virulent spores	Dose virulent spores	Result
Sheep 15790	1 c.c. dil 1:300	-	19/4/27	500 M.L.D.	Dead 25/4/27 Anthrax.
" 15546	1 " " "	+	25/4/27	500 "	Remained alive.
" 15584	1 " " "	-	"	500 "	Dead 29/4/27 Anthrax.
" 15776	1 " " "	-	"	250 "	Dead 29/4/27 Anthrax.
" 15474	1 " " "	-	"	750 "	Dead 29/4/27 Anthrax.

B. All vaccine injections made intramuscularly preventing skin contamination:

Animal	Date Vaccine 5/4/27	Result	Date virulent spores	Dose virulent spores	Result
Sheep 10897	1 c.c. dil 1:300	-	19/4/27	500 M.L.D.	Remained alive.
" 15875	1 " " "	-	25/4/27	500 "	Remained alive.
" 15757	1 " " "	-	"	500 "	Remained alive.
" 15467	1 " " "	-	"	250 "	Dead 28/4/27 Anthrax.
" 15643	1 " " "	-	"	750 "	Remained alive.

C. Vaccine applied to skin after scarification of a patch 1½ inch square.

Animal	Date Vaccine 5/4/27	Result	Date virulent spores	Dose virulent spores	Result
Sheep 15649	.25 c.c. dil 1:75	-	19/4/27	500 M.L.D.	Dead 23/4/27 Anthrax.
" 15547	.25 " " "	-	25/4/27	500 "	Dead 27/4/27 Anthrax.
" 10920	.25 " " "	-	"	500 "	Dead 29/4/27 Anthrax.

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Animal	Date vaccine 5/4/27	Result	Date virulent spores	Dose virulent spores	Result
(contd.)					
Sheep 15460	.25 c.c.dil.1:75	-	25/4/27	250 M.L.D.	Dead 29/4/27 Anthrax.
" 15468	.25 " " "	-	"	750 "	Remained alive.

Thus, out of the 5 sheep vaccinated subcutaneously 4 succumbed to anthrax after the immunity test, and likewise 4 of the 5 in which the vaccine was applied on the scarified skin, died after the immunity test. Of the 5 vaccinated intramuscularly, only one died. As mentioned before the vaccine was definitely weak, but still the differences in the results following different methods of vaccination were clearly shown as was the case with the guineapigs where intramuscular vaccination also appeared the most promising. No difference was noted in the results of cuti-vaccination as compared to subcutaneous injection.

It should be mentioned that none of the sheep injected subcutaneously or by scarification showed any temperature reaction after the vaccination, whereas of those injected intramuscularly two showed a well marked rise in temperature up to 106.4°F on the third day after vaccination while two showed a rise to 103.8°F and the 5th only a very slight rise. Thus, the reaction produced by intramuscular injection as gauged by the temperature charts was clearly more marked than with the other two methods.

A retest of the above experiments was carried out on 9 more sheep as follows:

A. All vaccine injections made <u>subcutaneously</u> preventing skin contamination:

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Animal	Date vaccine 28/5/27	Result	Date virulent spores 11/6/27	Result
Sheep 16157	1 c.c.(dil. 1:300)	-	250 M.L.D.	Dead 19/6/27 Anthrax.
15778	1 " "	-	500 "	Remained alive.
16170	1 " "	-	750 "	" "

B. All vaccine injected intramuscularly preventing skin contamination.

Animal	Date vaccine 28/5/27	Result	Date virulent spores 11/6/27	Result
Sheep 16159	1 c.c. (dil. 1:300)	-	250 M.L.D.	Remained alive.
10912	1 " "	-	500 "	" "
16164	1 " "	-	750 "	" "

C. All vaccine injected intradermally.

Animal	Date vaccine 28/5/27	Result	Date virulent spores 11/6/27	Result
Sheep 15769	.25 c.c. (dil. 1:75)	-	250 M.L.D.	Dead 13/6/27 Anthrax.
15831	.25 " "	-	500 "	Remained alive.
10918	.25 " "	-	750 "	" "

From the retest it is seen that one out of the three vaccinated subcutaneously as well as one out of the three vaccinated into the skin succumbed to the immunity test, while those injected intramuscularly remained alive.

A third test on sheep was carried out with the same vaccine although the immunity test dose was increased up to 3000 M.L.D. The object of the experiment was to note the quantity of virulent spore emulsion necessary to break the immunity in each case:

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A. All vaccine subcutaneously preventing skin contamination

Animal	Date vaccine 11/6/27	Result	Date virulent spores 25/6/27	Result
Sheep 15538	1 c.c. (dil. 1:300)	-	1500 M.L.D.	Dead 28/6/27 Anthrax.
16142	1 " "	-	2000 "	Dead 28/6/27 Anthrax.
16151	1 " "	-	2500 "	Remained alive
16139	1 " "	-	3000 "	" "

B. All vaccine intramuscularly preventing skin contamination

Animal	Date vaccine 11/6/27	Result	Date virulent spores 25/6/27	Result
Sheep 15391	1 c.c. (dil. 1:300)	-	1500 M.L.D.	Remained alive
15781	1 " "	-	2000 "	" "
15807	1 " "	-	2500 "	" "
15651	1 " "	-	3000 "	" "

C. All vaccine injected intradermally.

Animal	Date vaccine 11/6/27	Result	Date virulent spores 25/6/27	Result
Sheep 15796	.25 c.c. (dil. 1:75)	-	1500 M.L.D.	Remained alive.
15792	.25 " "	-	2000 "	Dead 28/6/27 Anthrax.
15810	.25 " "	-	2500 "	Remained alive.
15779	.25 " "	-	3000 "	Dead 28/6/27 Anthrax.

Thus, 2 out of 4 vaccinated subcutaneously and another two vaccinated intradermally succumbed after the immunity test, while all 4 injected intramuscularly remained alive.

Seeing that the intradermal vaccination produced no better immunity than the subcutaneous method, the former was discontinued. Comparative tests were then conducted on goats some vaccinated subcutaneously and others intramuscularly.

Comparative immunity tests on Goats.

Experiment 21.

The same vaccine which was employed in the previous experiments was also used on goats as follows:

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A. Vaccine injected <u>subcutaneously</u> preventing skin contamination:

Animal	Date vaccine 12/8/27	Result	Date virulent spores 29/8/27	Result
Goat 17951	1 c.c. (dil. 1:300)	-	500 M.L.D.	Dead 31/8/27 Anthrax.
17967	1 " "	-	500 "	Dead 31/8/27 Anthrax.
17952	1 " "	-	1000 "	Remained alive.
17970	1 " "	-	500 "	Dead 31/8/27 Anthrax.
17947	1 " "	-	1000 "	Remained alive.

B. Vaccine injected <u>intramuscularly</u> preventing skin contamination.

Animal	Date vaccine 12/8/27	Result	Date virulent spores 29/8 /27	Result
Goat 17975	1 c.c. (dil. 1:300)	+	500 M.L.D.	Remained alive.
17972	1 " "	-	500 "	" "
17963	1 " "	-	1000 "	" "
17968	1 " "	-	500 "	" "
17962	1 " "	-	1000 "	" "

<u>Result</u>: Out of the 5 goats vaccinated subcutaneously, three died from anthrax after the immunity test, while all five the goats vaccinated intramuscularly survived the immunity test.

It was then decided to ascertain the degree of safety of a strong cattle vaccine for goats when injected subcutaneously in the one case and deep intramuscularly in the other. For this purpose 1 c.c. of a 1 in 10 dilution of a strong cattle vaccine was used. The vaccine regularly killed guineapigs in a dilution of 1 in $\frac{1}{1000}$.

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A. Vaccine injected <u>subcutaneously</u> preventing skin contamination.

Animal	Date vaccine 23/8/27	Result	Date virulent spores 15/9/27	Result
Goat 17984	1 c.c. (dil. 1:10)	Dead 29/8/27 Anthrax.	-	-
17974	1 " "	Alive	1500 M.L.D.	Remained alive.
17989	1 " "	"	1500 "	"
17961	1 " "	Dead 1/9/27 Anthrax.	-	-
17944	1 " "	Alive	1500 M.L.D.	Remained alive.
17976	1 " "	"	1500 "	"
17945	1 " "	Dead 26/8/27 Anthrax	-	-
17971	1 " "	Dead 31/8/27 Anthrax	-	-
17941	1 " "	Dead 27/8/27 Anthrax	-	-
17960	1 " "	Alive	1500 M.L.D.	Remained alive.

B. Vaccine injected <u>intramuscularly</u> preventing skin contamination.

Animal	Date vaccine 23/8/27	Result	Date virulent spores 15/9/27	Result
Goat 17964	1 c.c. (dil. 1:10)	Alive	1500 M.L.D.	Remained alive.
17966	1 " "	"	1500 "	"
17982	1 " "	"	1500 "	"
17981	1 " "	Dead 29/8/27 Anthrax.	-	-
17965	1 " "	Alive	1500 M.L.D.	Remained alive.
17942	1 " "	"	1500 "	"
17955	1 " "	"	1500 "	"
17977	1 " "	"	1500 "	"
17969	1 " "	Dead 27/8/27 Anthrax	-	-
17987	1 " "	Dead 26/8/27 Anthrax	-	-

<u>Result</u>: of the 10 goats vaccinated subcutaneously 5 died from anthrax a few days after vaccination.

Of the 10 goats injected intramuscularly 3 died from anthrax after vaccination.

Of the 12 surviving goats every single one withstood the

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immunity test of 1/5 M.L.D. virulent spores, showing that a solid immunity had been established by this strong vaccine irrespective of the method of vaccination. With reference to the relative safety of vaccination as shown by the two results, it would appear that fewer deaths might be expected from intramuscular vaccination than from subcutaneous injections.

The temperature reaction following vaccination was equally well marked in both lots of animals.

Comparative Susceptibility and Immunity experiments on Horses.

Since of the various species of domesticated animals usually vaccinated against Anthrax, horses seem to be the most susceptible to the development of subcutaneous oedematous swellings following vaccination, a series of experiments were conducted with the object of (a) ascertaining what ~~factors~~ factors are involved in the production of swellings. (b) The relative value of different methods of vaccination as far as the incidence of swellings was concerned. As stated before, although the horse shows well marked susceptibility to anthrax under natural conditions, it is very difficult to cause a fatal anthrax infection by the administration of cultures. It is for this reason that immunity tests were not carried out on a large scale, since these could not be regarded as being very reliable.

Experiment 22.

In order to ascertain the cause of the swellings following vaccination, a number of different materials was injected subcutaneously into horses. It was reasonable to suspect either some non living toxic material elaborated by the anthrax organism in the artificial media and collected with the spores in the preparation of the vaccine or alternately its elaboration in the animal body at the seat

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of inoculation. As a second possibility had to be considered the harmful effect that the organism itself might have on the live tissues especially the blood vessels of the part, and allowing an increased capillary permeability.

(a) Injection of broth filtrates.

As mentioned in the latter part of experiment 10 (which see), two horses were injected subcutaneously on the side of the neck with 10 c.c. and 5 c.c. respectively of a sterile broth filtrate prepared by growing fully virulent anthrax organisms in broth for 30 days. As control a third horse was injected with 10 c.c. ordinary sterile nutrient broth. In all three animals the temperature remained undisturbed and not a sign of a swelling was noted. Toxic substances elaborated in the broth were, therefore, excluded.

(b) Fluid from Agar.

It was noticed that a small amount (1 - 2 c.c.) of condensation fluid collected on the surface of the nutrient agar after this had "set" in the flat bottomed Fernbach flasks prior to inoculation with the broth cultures. As some of this fluid at any rate would be collected with the spores and thus be present in the vaccine, it was poured off from a number of agar containing flasks and injected subcutaneously into 2 horses, 5 c.c. each on the side of the neck. They were kept under observation for 7 days without, however, showing any swelling.

(c) Washed Vaccine Spores.

A certain fairly weak batch of vaccine was injected in a dilution 1 in 100 in the ordinary way subcutaneously into 16 horses. Of these 7 showed well marked oedematous swellings and 6 slight swellings within a few days after vaccination. The remaining three did not show any swelling. Seeing that this vaccine definitely produced swellings in horses a small amount of the concentrated glycerinised spore

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emulsion was repeatedly washed in saline and centrifuged. The washed spores were then as near as possible again diluted to 1:100 and injected into horses 1c.c. each subcutaneously on the side of the neck as follows:

Animal	Vaccination	Result
Horse 17605	1 c.c. washed spores subcut.	Small diffuse swelling on 4th day. Disappeared on the 7th day.
" 17970	1 " " " "	Small swelling on 5th day, disappeared on the 8th day.
" 17314	1 " " " "	Negative.
" 17628	1 " " " "	"
" 17717	1 " " " "	"
" 69	1 " " " "	"
" 17793	1 " " " "	"
" 17613	1 " " " "	"
" 17689	1 " " " "	"
" 17541	1 " " " "	"

Thus, out of the 10 horses vaccinated with washed spores subcutaneously, two only showed small swellings at the site of injection.

At the same time 10 more horses were shaved on the side of the neck and a small patch scarified. Ordinary glycerinised emulsion from the same batch as used above (and which had produced swellings in 13 out of 16 horses) was rubbed in to the scarified parts .1 c.c. of a 1 :10 dilution. In none of these 10 animals was there any sign of the development of a swelling.

In order to check the results obtained in the first test where the animals showed swellings, 5 control horses were again vaccinated subcutaneously with the same ordinary glycerinised emulsion again in a dilution of 1 : 100. The result of the retest was that no swelling developed in any of the 5 horses. It should be mentioned that the

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same aseptic precautions in all these experiments were observed throughout, i.e. the skin was carefully disinfected in each case, and all the instruments boiled. The only difference in these two experiments was that the first lot of 16 horses, that showed swellings, were inoculated under veld conditions, the animals running in a paddock day and night. The experiment was conducted in September 1926. The weather was fairly warm throughout, and no rain fell on the animals. The other animals vaccinated with the washed spores, as well as those done by scarification and the last 5 controls injected subcutaneously, were stabled at night and allowed out into a paddock during the day.

Experiment 23.

It was then decided to ascertain the effect on horses of a strong batch of cattle vaccine that had killed a goat and also guineapigs in dilutions up to 1 in 1000:

(a) Ordinary glycerinised spore emulsion dilution 1 : 100 injected subcutaneously on side of neck 1 c.c. each.

Animal	Result
Horse 17714	Small flat diffuse swellings on 4th day, disappeared 6th day.
" 17876	Negative.
" 17636	"

(b) Ordinary glycerinised emulsion dilution 1 : 100 rubbed on to scarified patch .1 c.c. each.

Animal	Result
Horse 17779	Negative.
" 17441	"
" 17705	"

(c) Twenty-four hour broth culture prepared from the above spore emulsion, repeatedly washed in saline and

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centrifuged. Suspension of washed bacilli only, in dilution 1 : 100 was injected subcutaneously 1 c.c. each.

Animal	Result
Horse 17444	Negative
" 17985	Negative
" 17701	Negative

(d) Ordinary spore emulsion repeatedly washed in saline and centrifuged. Suspension of washed spores only in a dilution of 1 : 100 injected subcutaneously 1 c.c. each.

Animal	Result
Horse 17536	Large round swelling 6 x 4" running down and painful on the 3rd day, subsided on 6th day.
" 17620	Long running down swelling 6 x 2" on third day Subsided on 7th day.
" 17540	Negative.

Thus, out of the 12 horses inoculated in different ways some with ordinary emulsion and others with washed spore and washed bacilli, only the two of the three animals injected subcutaneously with the washed spores showed large and well marked swellings. The others except for the one horse 17714 injected with the ordinary glycerinised emulsion subcutaneously and which showed a small round swelling on the 4th day, all remained in normal health.

Although it is very difficult to interpret these results one point seems certain that the washed spores freed from all extracellular material are capable of producing swellings.

In experiment 17(which see), it was mentioned that the clear fluid collected from the oedematous swelling of Horse 18028 following vaccination was again injected into two other horses in 5 and 10 c.c. amounts without, however,

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producing the slightest swelling in them. From the results obtained with the washed spores it would seem that swellings could be produced without the presence of toxic substances in the vaccine. Furthermore, such substances could not be detected in the oedema fluid as tested out on other horses.

Experiment 24.

The object of this experiment was to ascertain and compare swellings in horses vaccinated (a) subcutaneously (b) intramuscularly with a fairly strong anthrax spore vaccine. All the horses were injected in the side of the neck.

(a) Subcutaneous injection.

Of the 15 horses injected 1 c.c. each of a dilution 1 : 500, 8 showed well marked swellings, 4 slight swellings and three were negative.

(b) Intramuscular injection, preventing skin contamination by swabbing needle wound with tincture of iodine.
Of the 15 horses injected 1 c.c. each of a dilution 1 : 500 8 showed well marked swellings, 3 slight swellings and 5 were negative.

It is thus seen that the number showing swellings were more or less equally divided between the two lots.

Experiment 25.

In this experiment it was decided to attempt to ascertain the degree of immunity established by subcutaneous and intramuscular injections on the same lines as adopted for the sheep experiments. As no reliable minimal lethal dose of virulent spores could be determined for horses, the standard for sheep was adopted.

A. Subcutaneous injection of the same vaccine as used above.

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Horse	Date vaccine 3/8/27	Result	Date virulent spores	Result
17967	1 c.c. subcut.	Negative	15/9/27 - 20,000 M.L.D.	Alive
18230	1 " "	Small flat swelling	28/10/27- 40,000 "	"
18213	1 " "	Slight nodular swelling	25/8/27 - 10,000 "	"
17789	1 " "	Slight swelling	28/10/27- 50,000 "	"
18097	1 " "	Negative	15/9/27 - 20,000 "	"

Result. Three out of the five horses vaccinated showed small swellings. All the animals withstood an immunity test as high as 50,000 M.L.D. (sheep standard).

B. Deep intramuscular preventing skin contamination.

Horse	Date vaccine 3/8/27	Result	Date virulent spores	Result
17737	1 c.c.	Negative	25/8/27 - 10,000 M.L.D.	Alive
18089	1 c.c.	Negative	28/10/27 - 40,000 "	"
18079	1 c.c.	Negative	" - 40,000 "	"
17860	1 c.c.	Very slight swelling	" - 50,000 "	"
18464	1 c.c.	Very slight swelling	" - 50,000 "	"

Thus, 2 out of the 5 horses showed very slight swellings after vaccination while all withstood the immunity test which in some cases was applied practically three months after vaccination.

Control. Horse 18100 was injected with 10,000 M.L.D. (for sheep) of the virulent spore emulsion used above. The animal died from anthrax within 5 days. Horse 16621 was given by the mouth 100,000 M.L.D. (for sheep) of the same spore emulsion, without, however, showing any ill effect.

From the preceeding experiments, as well as from a number of other tests conducted on horses, it seems very difficult to predict the development of swellings, since in many cases it may result from the use of a weak vaccine and not from a stronger one. Further, it frequently happens that a retest reveals totally different results. With regard to the different methods of vaccination, swellings

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have been noted with each, in practically the same proportion of cases so that no definite advantages could be ascribed to any particular one method from this respect. Although it is difficult to ascertain within narrow limits the M.L.D. of virulent material for horses, there is little doubt that a strong immunity is developed, e.g. the vaccinated horses withstood as much as 50000 Sheep M.L.D. whereas one control died of 10,000 M.L.D. (Sheep).

5. Summary and General Discussion.

As mentioned before the main object of this work was to ascertain (a) to what extent the metabolic products elaborated by the Bacillus anthracis either in vitro or in the animal body could be utilised as active immunizing agents against the disease. (b) To compare the degree of immunity developed by different methods of vaccination.

In the foregoing report an account was given of the various experiments conducted and the results that were obtained. It is now intended to summarize and discuss these results:

1. Introduction. Mention was made of the great importance of the anthrax problem in most countries of the world, and as a result of it the tremendous amount of research work carried out. Although much valuable knowledge had been gained in combating the disease, numerous points in connection with the biology of the organism both in nature and in the animal body were still unsolved.

Practically every make of anthrax vaccine used, is composed of the living but attenuated organism either in the vegetative or in spore form. Vaccine prepared from a well selected strain and carefully prepared, as a general rule affords a good active immunity in most of the domestic animals, and is indeed together with the proper disposal of anthrax carcases, our only means of eradicating the disease.

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There seems to be strong evidence that once a particular strain had been attenuated to the vaccine stage, subsequent increase in virulence does not take place, although animals may occasionally die from the attenuated strain following vaccination. A more ideal vaccine, however, would be one from which the living organism has been excluded.

2. Selection of Experimental Animals. Before conducting immunity work on anthrax, as on any other disease, it is essential that the experimental animals are carefully selected especially where there are marked differences in susceptibility and immunity exhibited by the various species to the disease in question. Failure to appreciate this fact may easily lead to erroneous conclusions. The small laboratory animals, although markedly susceptible to anthrax, are difficult to immunize, hence their unreliability for such work. Horses and cattle on the other hand, although susceptible to natural infection with anthrax, are difficult to kill by artificial infection. Outbreaks of anthrax among these animals may be stopped by vaccination clearly showing that immunity is developed, although it is difficult to ascertain the degree of immunity since many animals show an inherent resistance to artificial infection.

The ideal species of animal for the work is one showing a practically uniform susceptibility to artificial infection combined with the quality of developing an active immunity after vaccination, and which can be fairly accurately tested within a suitable period by the administration of virulent material. From the various experiments conducted it would seem that the merino sheep and to a lesser extent the ordinary domesticated goat, are more suitable for this work than any of the other species tried out. It is for this reason that practically all the immunity experiments were conducted on sheep and in some cases also on goats.

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3. Attenuation of virulent strains. Several strains of virulent anthrax were attenuated by the Pasteur method, i.e. the prolonged incubation of broth cultures at a temperature of 42°C. Subcultures on to nutrient agar were made every 5 days, and test batches of spore vaccine prepared from different attenuations and also from different colonies of the same attenuation. The virulence of the test vaccines was ascertained by injecting small animals. From the results obtained, it was clear that some strains took much longer to become attenuated than others, while even in the same strain there was an unequal attenuation of the organisms, some showing marked virulence even after 30 days incubation at 42°C. Although the vaccines prepared from many of the attenuations proved safe, the immunizing power in many instances, was unsatisfactory as shown by tests on sheep and goats. The only reliable method of obtaining an efficient vaccine strain seems to be the preparation and testing out of numbers of test batches of vaccine prepared from the different attenuations and even from different colonies, of several strains. It would appear that there exists a marked individuality of strains and perhaps even of the organisms in the same strain.

Attenuation was also attempted by cultivating virulent organisms for long periods in various uncommon liquid media. From small animal tests it would appear however, that attenuation is in no way hastened by this method. The repeated alternate freezing and thawing of young virulent Anthrax bacilli was not found to cause any decrease in virulence for guineapigs, rabbits and white mice, and hence unsuitable for the production of a vaccine strain. The Pasteur method, although frequently very tedious and disappointing, seems to be the best method of attenuating anthrax strains.

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4. Disintegrated Anthrax Cultures. Saline emulsions prepared from the surface growth of young anthrax cultures were disintegrated either by physical or ~~clinical~~ chemical means. Sterile germ free filtrates ~~were~~ were obtained by passing this material through bacterial filters. Such filtrates when injected into animals were neither toxic to guineapigs and white mice nor did they provoke immunity in sheep and goats against the disease. These experiments tend to exclude the presence of any endocellular toxins or immunizing substances within the anthrax bacilli cultivated on artificial media and subsequently disintegrated.

5. Washed Anthrax Cultures. Vaccine spores as well as young vegetative forms derived from these spores were thoroughly washed free from all extracellular substances by repeated shaking up in saline and centrifugalising. Both the washed spores and the washed bacilli when injected into guineapigs and mice, produced death from anthrax the same as did the unwashed material. In this respect it differs markedly from the B. chauveaui where the presence of its metabolic products is essential in causing the death of an animal. Sheep and goats developed a solid active immunity when vaccinated either with the washed anthrax spores or the washed bacilli.

6. Metabolic products elaborated by the Bacillus anthracis in vitro. Both virulent and vaccine strains of anthrax were cultivated in 15 different liquid media for periods up to 5 weeks, after which time the material was passed through bacterial filters and a sterile filtrate obtained. The object of these experiments was to ascertain the toxic or immunizing properties of such filtrates. Varying amounts of each filtrate were injected into guineapigs and mice, without producing any ill effect on any of the animals. This excludes the presence of any extracellular toxins elaborated in these different media. When tested out on sheep and goats, these filtrates were completely safe but

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of no immunizing value since with one or two exceptions all the animals died from anthrax after the immunity test. In two sheep injected with filtrate prepared by growing B. anthracis in ascites fluid from a dog mixed with an equal quantity of ordinary nutrient broth, the immunity test was withstood. It would thus appear that no metabolic products comparable to the "aggressin" of the B. chauveaui or to the toxin of B. tetani, were elaborated by the B. anthracis in artificial media. Such products, if present, were neither toxic nor of any real immunizing value.

7. Metabolic products elaborated by the Bacillus anthracis in the animal body. An imported product so-called "true anthrax aggressin", prepared from the juices extracted from anthrax carcases, was tested out on sheep and goats. Although completely safe, its immunizing value was negligible since all 8 animals vaccinated with it died from anthrax after the immunity test. In order to ascertain the presence of any such substances, susceptible sheep and goats were infected with virulent material. From these animals, blood was collected shortly before death when numerous bacilli had appeared in the peripheral circulation. Peritoneal fluid and bile, as well as certain organs for the preparation of extracts, were collected after death. Sterile germ free filtrates prepared from some of the diluted blood, peritoneal fluid, bile and organ extracts, were injected into small animals without producing any toxic effects. The same products when tested out on sheep and goats showed practically no immunizing value since out of a total of 14 sheep, 12 died from anthrax after the immunity test. Instead of filtering, some of the blood samples mentioned above were treated with (a) high dilutions of neosalvarsan, (b) with anilin gentian violet immediately after the blood was drawn. (Previously conducted experiments had shown that these substances had a marked bactericidal effect on the B. anthracis). Subcultures

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prepared from this blood 24 - 48 hours later showed it to be sterile while on microscopic examination the bacilli showed marked disintegration. Guineapigs and mice injected with this material showed no ill effect. Sheep and goats were then injected with 2 c.c. and 5.c.c. amounts and tested with virulent spores 3 to 4 weeks afterwards. As all the animals remained alive, immunity had undoubtedly been produced by the disintegrated blood. After 5 months standing this blood was again tested on sheep and goats. In order to ascertain the thermostability some of the blood was pasteurised as high as 92°C for 30 minutes. The immunity produced was in no way inferior to that noted in the previous test. Out of a total of 20 sheep and goats vaccinated with the disintegrated anthrax blood 18 withstood an immunity test ranging from 100 to 250 M.L.D. Further, this immunizing substance was still potent after a period of 5 months and was not destroyed by heating to 92°C for 30 minutes. These results were directly opposite to that obtained with the filtered blood and other body juices where 12 out of the 14 sheep died after the immunity test. The only explanation for these conflicting results would seem to be that the immunizing substance undoubtedly present in the blood was not filterable, i.e. it is not of the nature of a soluble filterable aggressin as found in blackquarter, but that it is closely associated with the organisms present in the blood, probably either in the bacterial body itself or in the capsular material surrounding it. It thus seems probable that the bacillary debris present in the blood contains in itself an active and reliable immunizing agent. As mentioned before, this could not be established for artificially cultivated organisms which when disintegrated either by physical or chemical means, or even killed by pasteurisation, did not exhibit any immunizing powers. In this respect the anthrax

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bacillus found in the circulating blood compared to the organism cultivated on artificial media seems to possess totally different qualities.

It should be mentioned that of all the inert non-living anthrax products obtained, either from artificial cultivation or from the animal body, or from any other sources, this disintegrated unfiltered blood was the only material which produced a definite and reliable active immunity. The fact that in practice more or less all anthrax vaccines used consist of the attenuated living organisms shows that "dead" or sterile vaccine does not produce the same strong immunity.

The oedema fluid collected from horses showing swellings after vaccination was not found to be toxic for small animals, nor did it produce swellings in other horses. Its immunizing value, as tested out on sheep and goats was negligible.

8. Comparative Safety and Immunity experiments with anthrax Spore vaccine. Under this heading an account is given of a number of different experiments. In the first series of experiments an attempts was made to ascertain the susceptibility of guineapigs to different routes of infection. For this purpose a fairly weak spore vaccine was used. Except where cuti-vaccination was deliberately intended, careful precautions were taken to prevent contamination of the skin with vaccine, e.g. on subcutaneous or intramuscular injections the needle wound was immediately disinfected by being swabbed out with a fine pincet, the points of which were rolled in cotton wool and steeped in 10% tincture of iodine. By this prodedure it was found that these animals readily succumbed from anthrax after subcutaneous, intraperitoneal and intramuscular injections, while with intradermal injection death was uncertain. By oral and intratracheal administration, none of the animals were killed.

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In order to ascertain if any immunity was developed by different methods of inoculation, a number of guineapigs were injected with smaller amounts of the same vaccine that had killed before. Most of the animals survived, and after 2-3 weeks the immunity was tested by injecting small amounts of a stronger vaccine. Those vaccinated intraperitoneally, subcutaneously, intratracheally and orally all died after the immunity test showing that no immunity had been developed. It, however, seemd fairly definite that some vaccinated deep intramuscularly had developed immunity since those not killed by the vaccine all withstood the immunity test. The result of the intradermal vaccination was doubtful since only 2 out of the 5 withstood the immunity test. It thus seemed clear that guineapigs showed well marked susceptibility to most of the routes of infection except to oral and intratracheal administration, while in only those vaccinated deep intramuscularly was the development of immunity fairly definite.

Using the same weak vaccine several experiments were conducted on sheep, and the degree of immunity compared, as shown by subcutaneous, intramuscular and intradermal vaccination. Contamination of the skin was prevented except in intradermal vaccination. After 2-3 weeks the immunity was tested with virulent spores ranging from 250 to 3000 M.L.D. Of the 12 sheep vaccinated <u>subcutaneously</u> 7 died from anthrax after the immunity test. Of the 12 sheep vaccinated <u>intradermally</u> 7 died from anthrax after the immunity test, while of the 12 vaccinated <u>deep intramuscularly</u> only one died after the immunity test. These results were obtained from three separate experiments carried out. It thus seems definite that where a weak spore vaccine is used the immunity established by intramuscular inoculation is much stronger than either with the subcutaneous or the intradermal vaccination. No difference was noted in the degree of immunity developed by the latter two methods.

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This experiment was repeated on goats, using the same weak vaccine. The results confirmed those obtained with sheep. Three out of the five goats vaccinated subcutaneously died after the immunity test while all 5 vaccinated intramuscularly survived the test. In a subsequent experiment where a strong cattle vaccine was used on goats 5 out of 10 goats died from vaccination while the surviving 5 all withstood an immunity test of 1500 M.LD. Of 10 goats vaccinated deep intramuscularly 3 died from the result of vaccination while the surviving 7 all withstood an immunity test of 1500 M.L.D. This clearly shows the susceptibility of goats to a strong vaccine. As all the goats which survived the vaccination also survived the immunity test, it would appear that where a strong vaccine is used the difference in the degree of immunity following (a) subcutaneous and (b) intramuscular vaccination, is not so striking as when a weak vaccine is used. The immunity may even be the same with the two methods where the strong vaccine is used.

A number of experiments were then conducted on horses, the main object being to ascertain what factors were responsible for the development of oedematous swellings so frequently noted in this species after vaccination. Subcutaneous injections were made with sterile broth filtrate in which anthrax had been cultivated for long periods. In none of the animals were swellings noticed. Oedema fluid collected from horses showing such swellings when injected into other horses, also gave negative results. Washed vaccine spores, freed from extracellular material produced swellings in some cases, while in others no swellings were noticed. By subcutaneous, intramuscular and intradermal vaccination, using a glycerinised spore emulsion, swellings were obtained in some cases and not in others.

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Further it was noticed that whereas marked swellings followed vaccination in a troop of horses, a retest of the same vaccine on a different lot of horses frequently resulted in none or only a few of the animals showing swellings. Certain experiments conducted also clearly showed that while a definitely weak vaccine may cause swellings in horses, a much stronger vaccine as e.g. used for cattle and sheep may be completely safe for horses, i.e. no swellings are developed. Thus the strength of the vaccine itself cannot be considered as the sole factor determining the development of swellings; neither could the method of vaccination be the cause since swellings were noted in practically equal proportions following different methods of vaccination. Since anthrax broth filtrates as well as oedema fluid from other horses did not cause swellings, it would seem that extracellular toxins cannot be incriminated as being the cause.

The possibility of ideosyncrasy of animals combined perhaps with certain environmental conditions may account for these inconsistent results.

Conclusions appended.
See page 73 (a) etc.

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Conclusions.

From experiments carried out the following conclusions would appear to be justified:-

1. The small laboratory animals, although markedly susceptible to Anthrax, are difficult to immunize, hence their unreliabilityin a study on anthrax immunity. Horses and cattle on the other hand, although susceptible to natural infection with anthrax, are difficult to kill by artificial infection. The Merino sheep, which shows a practically uniform susceptibility to artificial infection combined with the quality of developing an active immunity after vaccination, seems to be an ideal species for immunity work on anthrax.

2. Attenuation of different Anthrax strains according to the Pasteur method shows that some strains take much longer to become attenuated than others, while even in the same strain there is unequal attenuation of the organisms, some showing marked virulence even after 80 days incubation at 42°C. Thus there appears to be a marked individuality of strains and even of single organisms in the same strain.

3. Cultivation of B. anthracis in various uncommon liquid media does not hasten attenuation. Similarly repeated alternate freezing and thawing of anthrax cultures fails to cause any decrease in virulence.

4. By physical or chemical disintegration of artificially cultivated anthrax organisms the sterile cell substance is found to be free from both toxic and immunizing properties.

5. Anthrax spores as well as the vegative forms when washed free from all extracellular material are by themselves capable of either causing a fatal infection or of producing an active immunity.

6. Prolonged cultivation of both virulent and vaccine anthrax strains in different uncommon liquid media,

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followed by subsequent filtration fails to reveal either toxic or immunising properties in the filtrate.

7. Blood drawn at the time of death from animals infected with virulent anthrax and sterilised by chemical means, is seen to possess well marked immunizing properties. This quality is maintained in blood kept for 5 months and resists heating at 92°C for 30 minutes. Such blood produces an active immunity. Filtrate obtained from freshly drawn anthrax blood fails to reveal any such immunizing powers. Thus the unfilterable bacillary debris itself seems to be the immunizing substance in the blood. The debris obtained by disintregrating artificially cultivated organisms does not show the same antigenic properties.

8. By utilising anthrax spore vaccine on guinea pigs, lethal infections can be produced by subcutaneous, intraperitoneal or intramuscular injections while the results of intradermal application are uncertain. With oral or intratracheal administration these animals are not easily killed.

9. The nature and degree of immunity developed in sheep following <u>intradermal</u> vaccination with anthrax spore vaccine is no stronger nor more permanent than that following <u>subcutaneous</u> inoculation. Following the use of fairly weak anthrax spore vaccine, the immunity established by <u>intramuscular</u> vaccination seems to be definitely superior to that produced by either subcutaneous or intradermal injection. With a strong vaccine these differences are less marked.

10. In horses the strenth of the vaccine cannot be considered as the sole factor determining the development of swellings since weak vaccines may provoke swelling whereas stronger ones may fail to do so. Further the method of vaccination cannot be the responsible cause, since approximately the same per-

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centage of animals show swellings after intradermal, subcutaneous or intramuscular vaccination.

Lastly anthrax broth filtrates as well as oedema fluid from other horses showing anthrax swelling do not provoke swellings in susceptible horses, and hence extracellular toxins cannot be incriminated as being the cause.

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