We have already referred to the variation in the extent to which attenuation takes place in different organisms growing in the same tube and to the fact that to minimize this it is desirable to start attenuation from one single colony. Even then it is likely that a great variation still occurs; e.g., in a tube grown for thirty days one may get individual organisms showing marked differences in the degree to which they have become attenuated. From this it becomes clear that it might be desirable to determine the acquired characters of a large number of individual organisms contained in a tube that had been incubated for a certain period. One would naturally not resort to this procedure unless there were indications that the desired strain might be found in such a group of individuals.

The strain we have so far referred to is one intended for the preparation of anthrax vaccine for general use, for instance, in cattle and sheep. As will be discussed later, goats and equines, owing to their marked susceptibility to anthrax cannot with safety be immunized with a vaccine that, though on the strong side, is perfectly harmless to cattle and sheep. In their case a weaker vaccine is indicated, and in the preparation of such a vaccine the selection of special strains must be taken into consideration. This matter will, however, be dealt with at a later stage.

(5) *Preparation of Spore Vaccine.*—The preparation of a spore vaccine is made possible by the fact that anthrax organisms, whether virulent or attenuated, sporulate easily, rapidly and completely. This fact requires emphasis, because hitherto some authorities have held exactly the opposite view in so far as attenuated bacilli were concerned. Thus, Muir and Ritchie in their text book on Bacteriology, state:—

> "Above 42° C. not only does sporulation cease, but Pasteur found that if bacilli were kept at this temperature for eight days they did not regain the capacity when again grown at a lower temperature. In order to make them again capable of sporing, it is necessary to adopt special measures, such as passage through the bodies of a series of susceptible animals."

In our experience anthrax bacilli sporulate readily, even after they had been kept at a temperature of 42° *C. for periods up to seventy days.* Complete sporulation has been found to be one of the determining factors in the successful preparation of a reliable anthrax vaccine, and, this being so, every effort is made to ensure efficient sporulation of cultures intended for the preparation of a vaccine.

Before the importance of this factor was realised fully, failure to produce a good batch of vaccine was not uncommon. Sporulation on a solid medium commences as early as eighteen to forty-eight hours and may be complete in four or five days. If the medium happens to be fairly moist and if incubation is continued after the initial sporulation is complete, the spores may again go over into the vegetative form; this can be detected macroscopically by the presence of secondary or "daughter" colonies forming on the surface of the original ones. On further incubation these vegetative forms may fail to sporulate, but instead undergo degenerative changes and develop capsules. In such material hardly any spores are present and the vegetative forms die off within a few weeks. Apparently some injurious substances, either derived from the medium or produced by

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bacterial activity, are responsible for the death of the organisms. Further work in connection with this matter is in progress and it is hoped later to find a correct explanation for this phenomenon.

The following steps in the preparation of spore vaccine embody all the up-to-date improvements which it has been possible to make as a result of recent experience.

(a) *Seed Material.*—Our vaccine strains are kept in hermitically sealed tubes (on agar), in order to avoid contamination and drying out. The attenuated organisms in spore form can be kept unchanged in this condition for many years. Subcultures are made in two broth tubes which are incubated at 37° C. for 48 hours. During this time the purity of the growth is ascertained by (*a*) naked eye appearance, (*b*) microscopic examination of stained smears, and (*c*) plating out. If the culture is pure, more seed material is prepared by inoculating a broth flask of ½ litre capacity. In order to avoid contamination later when using this seed material, the flask is fitted out as follows: Two glass tubes are passed through the cotton wool plug, one of these being merely a short straight piece projecting above and below the plug to the extent of two or three inches; it is intended to serve for the introduction of the seed material from the tube of broth. The other reaches to the bottom of the flask and is bent outside to an angle of about 35°; its outside extremity near the mouth of the flask, is fitted with a piece of rubber tubing which is attached to another piece of glass tubing, about six inches in length, which has its end drawn out to a fine point and sealed. Through this tube seed material is drawn off for inoculating other flasks. Before this is done, it is essential to make sure that the culture is absolutely pure, since otherwise it may be the means of infecting a large number of flasks. The test for purity is conducted in the same manner as that previously described for the broth tubes.

(b) *Growth on Special (Fernbach) Flasks.*—To obtain maximum sporulation the organisms must have free access to oxygen and to provide for this they are grown on the surface of a solid medium. To obtain abundant growth a large surface has to be provided and this is done by using specially wide flasks, such as Fernbach flasks. Into these flasks is poured a thin layer of nutrient agar, of pH. 7.4, after which they are carefully sterilized. To make sure that the medium is sterile, and to assist evaporation of excessive moisture, the flasks are incubated for two or three days. The number of Fernbach flasks used depends entirely on the amount of vaccine required; for an average sized batch of vaccine, containing about ¼ to ⅓ million doses we use in the neighbourhood of fifty flasks. The Fernbach flasks are now inoculated with the seed material contained in the broth flask, only a small quantity being used for each flask, sufficient to moisten the surface of the medium. This operation is carried out under a flame, every precaution being taken against the possibility of

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contamination. The inoculated flasks are now incubated at 35°-37° C. for varying periods, depending on the rapidity and completeness of sporulation. This period is determined by daily examination of the growth in some flasks for the condition of sporulation; for this purpose a loopful of culture is taken, spread out on a clean glass slide, fixed by heat and, after staining with carbol thionin, examined microscopically. As soon as sporulation is found to be well advanced, i.e. 80 to 90 per cent. of organisms occurring in the form of well developed spores, the flasks are taken out of the incubator and placed in a cupboard at room temperature. Here they are allowed to remain for about a week, in order to allow the spores to develop to their fullest extent.

(c) *Preparation of Spore Emulsion.*—The flasks that have been passed as fit for vaccine production are now taken out of the cupboard into a specially clean and disinfected room where the spores are washed off and suspended in saline. This operation is carried out in the following manner:— Under as aseptic conditions as possible, a dozen or two of sterilized solid glass beads, of good quality, are poured into each flask. This is followed by the introduction of a small quantity of sterile saline, just sufficient to permit of the spores being washed off. The washing off process is, of course, assisted by the action of the beads. The normal saline is kept in a flask specially fitted to allow syphoning off under aseptic conditions; syphoning is assisted by placing this flask on a shelf about one or two feet above the work bench. The spore emulsion so obtained is poured, under as aseptic conditions as possible, into a sterile two litre flask whose weight has been ascertained previously. A piece of sterile gauze is fitted to the mouth of the flask, for the purpose of keeping back any shreds of medium or glass beads that may find their way out of the Fernbach flasks during the act of pouring. As soon as all the emulsion has been collected and thoroughly mixed in this flask pure sterile glycerine is added in the proportion of two parts by weight of glycerine to one part of the original emulsion; the glycerine is an excellent preservative agent that will prevent multiplication of practically all organisms and, in this strength, probably kill off any non-sporulating ones that may be present. The flask is now closed with a sterile cotton wool plug to which has been fitted a sealed sterile glass tube with the object of allowing the emulsion to be drawn off conveniently and aseptically at any time.

(d) *Preliminary Tests for Safety and Efficacy.*—If one works with a well known and properly attenuated strain and prepares the spore emulsion according to the technique described here, there is very little danger of anything being found wanting in either the safety or efficacy of the finished product, but to make absolutely sure a few preliminary tests on small animals should be carried out. The safe and efficient dose of spore vaccine must be

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determined in a susceptible animal which lends itself to immunisation against anthrax. Such animals (sheep) are usually fairly expensive and one does not wish to waste them unnecessarily. This preliminary test is, therefore, also intended to serve as an indication of the potency of the vaccine; for instance, if none of the guinea-pigs succumb to the glycerinized emulsion, sufficient proof is advanced to show that it is too weak and therefore useless as a vaccine; if, on the other hand, rabbits in addition are killed, that serves as an indication that the vaccine is too strong and may have to be discarded altogether. With a properly attenuated strain it rarely happens that rabbits are killed by the spore emulsion. Following are the details of the test referred to:—

One rabbit receives subcutaneously .1 c.c. emulsion.
One rabbit receives subcutaneously .01 c.c. emulsion.
One guinea-pig receives subcutaneously .01 c.c. emulsion.
One guinea-pig receives subcutaneously .001 c.c. emulsion.

For the vaccine to be safe, it would be perfectly in order if both guinea-pigs died, whereas neither of the rabbits should be killed. If only the guinea-pig which received the larger dose should die, the vaccine would still be strong enough, whereas if both survived it is not likely to be of any value in practice. If both rabbits succumbed to the emulsion, one would certainly discard the vaccine, whereas if only the one which received the larger dose were killed, one would repeat the test and only then take a definite decision. These remarks apply only to vaccine intended for use in *cattle and sheep*. At a later stage special reference will be made to tests carried out in connection with vaccines intended for use in *equines and goats*. As previously stated, rabbits and guinea-pigs are not good subjects for use in immunity tests, but experience has shown that with our well known vaccine strains we could form a fairly accurate estimate of the immunizing value of the vaccine by the results of the safety tests given above.

(*e*) *Final Test for Safety and Efficacy.*—Every batch of anthrax vaccine prepared by us must pass this test satisfactorily before it is issued for use in practice. On the results of the test is determined the safe and efficient dose of the vaccine, so that a two fold purpose is served. In America, and elsewhere, the correct dose of vaccine is determined by the number of spores present, but we consider a biological test far superior from every point of view. In order to obtain the most reliable results from this test, it is desirable to carry it out three to four weeks after the spore emulsion was washed off and mixed with glycerine. During this time the glycerine will have acted on any vegetative forms that may have been present, probably killing many of them or rendering them inactive.

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If the test were carried out immediately after the emulsion was made, the vegetative forms might have been present in sufficiently large numbers to influence the results.

Following are the details of the test:—

Each of two sheep receives subcutaneously 20 c.c. emulsion.
Each of two sheep receives subcutaneously 1 c.c. emulsion.
Each of two sheep receives subcutaneously .1 c.c. emulsion.
Each of two sheep receives subcutaneously .02 c.c. emulsion.
Each of two sheep receives subcutaneously .01 c.c. emulsion.
Each of two sheep receives subcutaneously .005 c.c. emulsion.

Such a large dose as 20 c.c. is used, because one wishes to make absolutely certain that the vaccine is perfectly safe, and not likely to kill even a small percentage of animals. With the vaccine strains used at this Laboratory, sheep receiving these doses will survive in practically every case. It has only happened once that a sheep died after receiving 20 c.c. of undiluted emulsion and in that case the vaccine would have been rejected, were it not for the fact that a re-test proved more satisfactory. Extensive practical experience in the field has taught us that for the successful application of vaccination against anthrax one cannot exercise too much care in ensuring the safety of a vaccine. Naturally, the sheep receiving the smaller doses of emulsion must all survive; they will serve to determine the degree of immunity conferred by small quantities of emulsion, to be discussed now.

The *immunity test* is applied to the same sheep used in the safety test, the injection of virulent material being made about three weeks after they received the vaccine. It is, of course, essential to employ virulent material which has been standardized, the M.L.D. for sheep being definitely known. Here again a spore emulsion is the best to use, since the spores can easily be preserved in glycerine and retain all their properties, unaltered in any way, for many months.

This virulent spore emulsion is prepared in exactly the same way as described for the spore vaccine, excepting of course that a virulent anthrax strain is used; we have found it best to employ for this purpose the most virulent strain in our possession.

The M.L.D. is then determined as follows:—

Each of two sheep receives subcutaneously .01 c.c. glycerinized emulsion.
Each of two sheep receives subcutaneously .001 c.c. glycerinized emulsion.
Each of two sheep receives subcutaneously .0001 c.c. glycerinized emulsion.
Each of two sheep receives subcutaneously .00005 c.c. glycerinized emulsion.

With the virulent strain employed by us all the sheep receiving a dose of .0001 and more should die of anthrax while those receiving less might survive. In the latter case .0001 c.c. is accepted as the M.L.D. It stands to reason that the M.L.D. will depend on whether a rich spore emulsion has been obtained, so that with a stronger emulsion than that given above the M.L.D. will be smaller, and conversely with a weaker emulsion it will be larger. From this it

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follows that further tests may have to be carried out to arrive at an accurate determination of the M.L.D. As will be seen later, the standard dose employed by us is 1,000 M.L.D. and hence for the sake of convenience our spore emulsion whose M.L.D. has been determined as .0001 c.c. is diluted with glycerine-saline solution so that 1 c.c. will be equivalent to 1,000 M.L.D. The next step is to fix on a standard dose of virulent spore emulsion against which the vaccine is expected to protect sheep. This is not an easy matter and can only be settled, in an arbitrary manner, by taking into consideration the results obtained from the use of vaccines under different conditions in the field, such conditions being badly infected farms, active outbreaks, etc. It is only after extensive experience in applying vaccination under all possible conditions that one can form an idea of the standard immunizing value required of a vaccine. If it had been known definitely how infection usually took place under natural conditions, what dose of virulent material was required to set up a fatal disease, etc., there would have been no difficulty in fixing a standard. At first we required our vaccines to afford protection against several thousand doses of virus, but this standard was found to be unnecessarily high. Later on an immunizing value sufficient to protect against only 1,000 M.L.D. was decided upon and this has given entirely satisfactory results. Apparently a vaccine of this standard is quite sufficient to protect animals against natural infection under the most varied conditions. The results obtained from vaccination in the field will be discussed more fully at a later stage.

Reverting to the sheep injected with different doses of spore emulsion (vaccine), they now receive subcutaneously 1 c.c., i.e. 1,000 M.L.D. of standard virulent spore emulsion. If the vaccine is at all effective, the sheep which had received the higher doses will certainly survive, while those that were injected with the smaller doses may or may not succumb to virulent anthrax, depending on the efficacy of the particular vaccine. With a good vaccine at least those which received .01 c.c. of vaccine will be protected, and consequently in such a case the dose of vaccine for use in practice is fixed at .01 c.c. The emulsion is, therefore, diluted with 60 per cent. sterile glycerine-saline a hundred times, so that the dose for an adult animal is brought up to 1 c.c. This dilution is made in large sterile flasks, specially fitted with the necessary appliances to permit of bottling being carried out under aseptic conditions. As mentioned earlier, the 60 per cent. glycerine-saline solution is an excellent preservative agent, making it possible for the vaccine to be kept for some months without deteriorating to any extent.

In the subjoined experiments are given examples of what may be expected from the biological tests carried out in connection with *very good*, *average*, and *bad* batches of vaccine.

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Experiment No. 5.

Showing a very good *batch* (51).

(*a*) *Small Animal Test.*
(*b*) *Test on Domesticated Animals.*

Animal.	Date and Dose Vaccine. *Safety Test.*		Result.	Date, Virus.	*Immunity Test.*	Result.
a) Rabbit....	·1	4.10.24	Lived.			
Guinea-pig	·1	,,	Died, 14.11.24.			
,,	·01	,,	,, 13.11.24.			
b) Sheep 9605	20	28.11.24	Lived.	1,000 M.L.D.	17.12.21	Lived.
,, 9245	20	,,	,,	,,	,,	,,
,, 9319	1	,,	,,	,,	,,	,,
,, 9526	1	,,	,,	,,	,,	,,
,, 9375	·1	,,	,,	,,	,,	,,
,, 9361	·1	,,	,,	,,	,,	,,
,, 9628	·02	,,	,,	,,	,,	,,
,, 8784	·02	,,	,,	,,	,,	,,
,, 9220	·01	,,	,,	,,	,,	,,
,, 9501	·01	,,	,,	,,	,,	,,
,, 9100	·005	,,	,,	,,	,,	,,
,, 9207	·005	,,	,,	,,	,,	,,
Goat 9903	10	,,	,,	500 M.L.D.	,,	,,
,, 9916	1	,,	,,	,,	,,	,,
,, 9888	·1	,,	,,	,,	,,	,,
,, 9938	·02	,,	,,	,,	,,	,,
,, 9900	·01	,,	,,	,,	,,	,,
,, 9953	·005	,,	,,	,,	,,	,,

Issued for use in cattle and sheep at a dilution of 1 : 200.

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Experiment No. 6.

Showing an average *batch* (49).

(*a*) *Small Animal Test.*
(*b*) *Test on Domesticated Animals.*

Animal.	Date and Dose Vaccine. *Safety Test.*		Result.	Date, Virus.	*Immunity Test.*	Result.
(*a*) Rabbit....	·1	4.10.24	Lived.			
Guinea-pig	·1	,,	Died, 5.10.24.			
,,	·01	,,	,, ,,			
(*b*) Sheep 9183	20	4.11.24	Lived.	1,000 M.L.D.	28.11.24	Lived.
,, 9405	20	,,	,,	,,	,,	,,
,, 9384	1	,,	,,	,,	,,	,,
,, 9310	1	,,	,,	,,	,,	,,
,, 9559	·1	,,	,,	,,	,,	,,
,, 9224	·1	,,	,,	,,	,,	,,
,, 9164	·02	,,	,,	,,	,,	,,
,, 9178	·02	,,	,,	,,	,,	,,
,, 9187	·01	,,	,,	,,	,,	,,
,, 9496	·01	,,	,,	,,	,,	,,
,, 9454	·005	,,	,,	,,	,,	Died, 4.12.24.
,, 9586	·005	,,	,,	,,	,,	Lived.
Goat 9891	10	,,	,,	,,	,,	,,
,, 9906	1	,,	,,	,,	,,	,,
,, 9904	·1	,,	,,	,,	,,	Died, 2.12.24.
,, 9941	·02	,,	,,	,,	,,	Lived.
,, 9884	·01	,,	,,	,,	,,	,,
,, 9887	·005	,,	,,	,,	,,	,,

Issued for use in cattle and sheep at a dilution of 1 : 100.

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Experiment No. 7.

Showing a bad *batch* (41).

(*a*) *Small Animal Test.*
(*b*) *Test on Domesticated Animals.*

Animal.	Safety Test, Date and Dose.		Result.	Immunity Test, Date and Dose.		Result.
(*a*) Rabbit.....	·1	1.3.24	Lived.			
Guinea-pig.....	·1	,,	,,			
,, ...	·01	,,	,,			
(*b*) Sheep 6863....	20	29.2.24	Lived.	1,000 M.L.D.	21.3.24	Lived.
,, 6300....	20	,,	,,	,,	,,	,,
,, 6351....	1	,,	,,	,,	,,	,,
,, 7846....	1	,,	,,	,,	,,	,,
,, 7794....	·1	,,	,,	,,	,,	Died.
,, 7029....	·1	,,	,,	,,	,,	,,
,, 6520....	·02	,,	,,	,,	,,	,,
,, 5864....	·02	,,	,,	,,	,,	,,
,, 7373....	·01	,,	,,	,,	,,	,,
,, 7368....	·01	,,	,,	,,	,,	,,
,, 6467....	·005	,,	,,	,,	,,	,,
,, 6255....	·005	,,	,,	,,	,,	,,
Goat 8295....	10	,,	,,	500 M.L.D.	,,	Lived.
,, 8292....	1	,,	,,	,,	,,	,,
,, 8407....	·1	,,	,,	,,	,,	Died.
,, 8420....	·02	,,	,,	,,	,,	,,
,, 8275....	·01	,,	,,	,,	,,	,,
,, 8257....	·005	,,	,,	,,	,,	,,

Above vaccine not issued as it did not give *sufficient immunity.*

It should be mentioned again that the standard test discussed here refers to vaccines intended for use in *cattle and sheep* and that certain modifications adopted in the case of vaccines intended for use in goats and equines, respectively, will be considered at a later stage.

(f) *Bottling and Issue of Vaccine.*—Bottling of our vaccine is carried out in a special room, so constructed that it can be thoroughly cleaned and disinfected before the work is commenced. The large flask containing the vaccine is placed on a shelf a few feet above the work bench, and the bottles filled by syphonage under a flame, every care being taken to avoid outside infection. By using standard bottles of known capacity one does away with the troublesome process of measuring off the correct quantity of vaccine into each bottle. After filling, the bottles are first closed with sterile cotton wool plugs and then with sterile rubber corks. To make certain of keeping out contamination and of avoiding corks working loose, the bottles are sealed with sealing wax. To each bottle is affixed an appropriate label, stating the class of animal for which the vaccine is intended, the dose to be employed and the date up to which it may be used. Although it is known with certainty that the spore vaccine will keep its

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properties for many months, the period allowed for use after issue is usually fixed at four months; this is intended to counteract the tendency of some farmers to store vaccines for several years, sometimes under the most unfavourable conditions as regards heat, moisture, etc. As mentioned earlier, the vaccine is prepared, packed, railed or despatched by post, free of charge, to any stock owner in the Union; that the expenses involved, especially in connection with overhead charges, are considerable, will readily be appreciated when it is remembered that the issues may be as high as over two million doses per annum.

(6) *Immunity in Anthrax.*—This question will be discussed more from the point of view of experience gained under laboratory conditions, field experience being reserved for special consideration in a subsequent chapter.

Our remarks will also be directed mostly to the more important practical issues that may be raised in connection with active immunity, our practical experience with *passive* immunity in anthrax not being sufficiently great to entitle us to speak with any authority on this subject.

Regarding the latter, it may be explained that the reasons for not giving any serious consideration to it are as follows:—

(*a*) The cost of production and distribution of serum, for use either by itself or in combination with vaccine (sero-vaccine) would be far too high. Now that anthrax vaccine is supplied free to all stock owners by the Government, the costs involved in supplying an efficient serum would be too big a burden for the State.

(*b*) Except in very special cases, there is no reason to believe that serum or sero-vaccine will have any advantage over spore vaccine. It may be argued that, on theoretical grounds alone, sero-vaccination is indicated in cases where an active outbreak of anthrax exists. Theoretically this argument is sound, but in practice it does not seem to hold good. We have tested, under laboratory conditions, sero-vaccines imported from Germany and the United States of America and in both cases the immunity produced was on the weak side. Under field conditions we had an opportunity of testing a sero-vaccine, imported from a well known firm in Germany, on horses among which anthrax had broken out, some animals were done with sero-vaccine and others with our spore vaccine, the result being that deaths from anthrax continued for some days in both sets of horses. Apparently no, or not sufficient, passive immunity was conferred by the serum contained in the sero-vaccine to prevent animals from contracting the disease after inoculation. In the case of spore vaccine, of course, one would not expect immunity to be established for some days after vaccination.

Concerning *active immunity* it is proposed to deal with the matter under the following heads:—

(*a*) Immunity following subcutaneous inoculation.

(*b*) Immunity following other methods of administering the vaccine.

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(*c*) Immunity following combined vaccination against both anthrax and blackquarter.

(a) *Immunity following subcutaneous Inoculation.*—This is the routine method of vaccination followed in this country and in most other parts of the world. Owing to its extensive application most of our knowledge concerning immunity against anthrax has been obtained from this method of introducing both vaccine and virus.

The practical points which call for consideration are the following:—

(1) Method of testing immunity.
(2) The time required for immunity to develop.
(3) The duration of immunity.

(1) *Method of Testing Immunity.*—The method employed by us has been described fully elsewhere in this report. It may be of interest to mention here the behaviour of experimental sheep to vaccine and subsequent virulent anthrax injections. In the case of the average batch of vaccine a well marked thermal reaction is set up in the large majority of vaccinated sheep, perhaps over 90 per cent. responding in this way, irrespective of the number of spores contained in the dose of vaccine. The temperature generally rises rapidly about 24 hours after inoculation to anything between 104° and 108° and remains high for a period varying up to four or five days. Now and again the thermal reaction may last longer and belated reactions, occurring a week or fortnight after injection, are occasionally met with. A thermal reaction does not serve as a definite indication of the degree of immunity developed in any particular animal, since quite a good immunity is often developed in animals showing very little reaction. On the other hand, sheep showing marked thermal reactions, may not possess a high degree of immunity when tested subsequently. A thermal reaction may be taken, however, as indicating that the vaccine is a "live" one, and that immunity is likely to develop. Well marked local reactions are hardly ever encountered in sheep, even when large quantities of vaccine are injected into them. Provided the vaccine has been efficient in producing a good immunity, injection of virulent spores three weeks later does not result in the production of either a local or thermal reaction in all cases; in fact, only a minority of sheep, approximately 25 per cent., will show a temperature reaction, varying up to 101° and lasting for only a few days. Where the vaccine had no great immunizing value many more would react, some severely and succumb to an attack of virulent anthrax.

Temperature Charts of Sheep 8627, 9207, and 9319 will serve to illustrate the thermal reactions observed in different types of cases.

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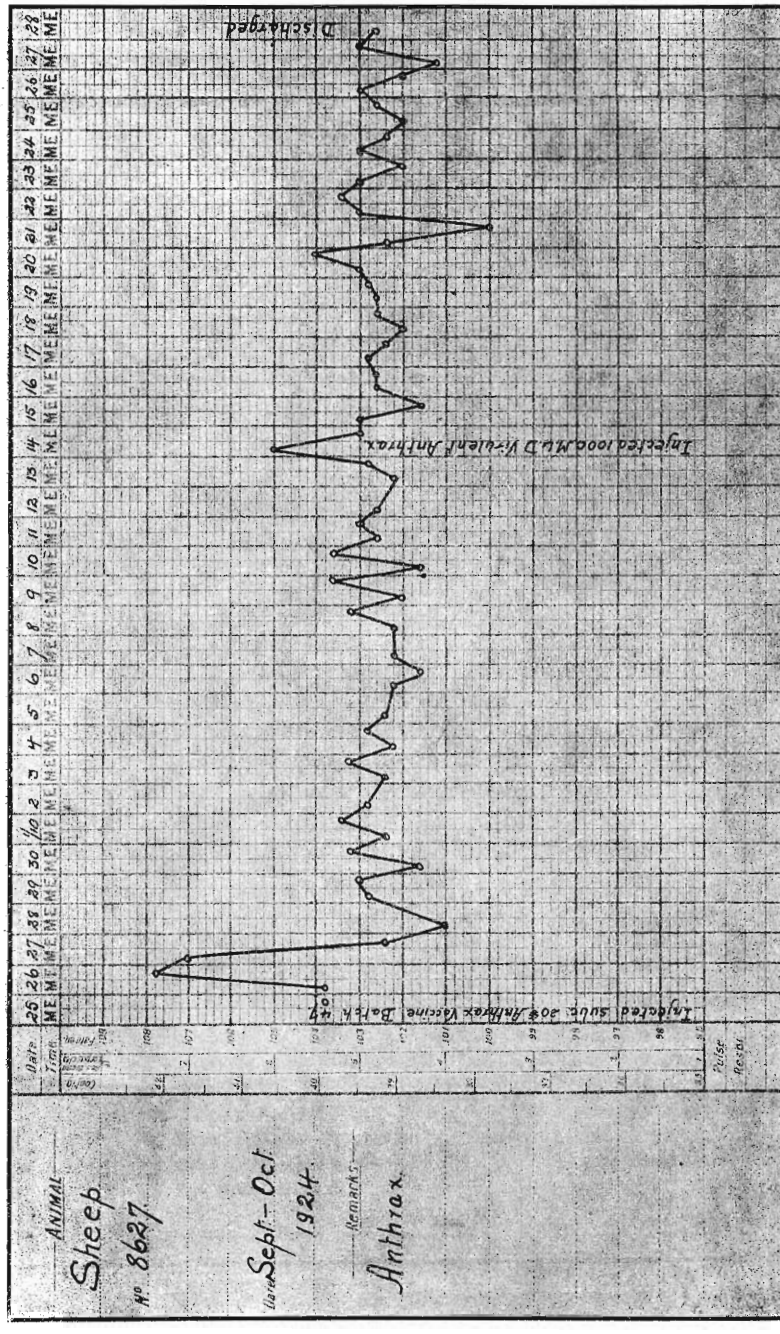

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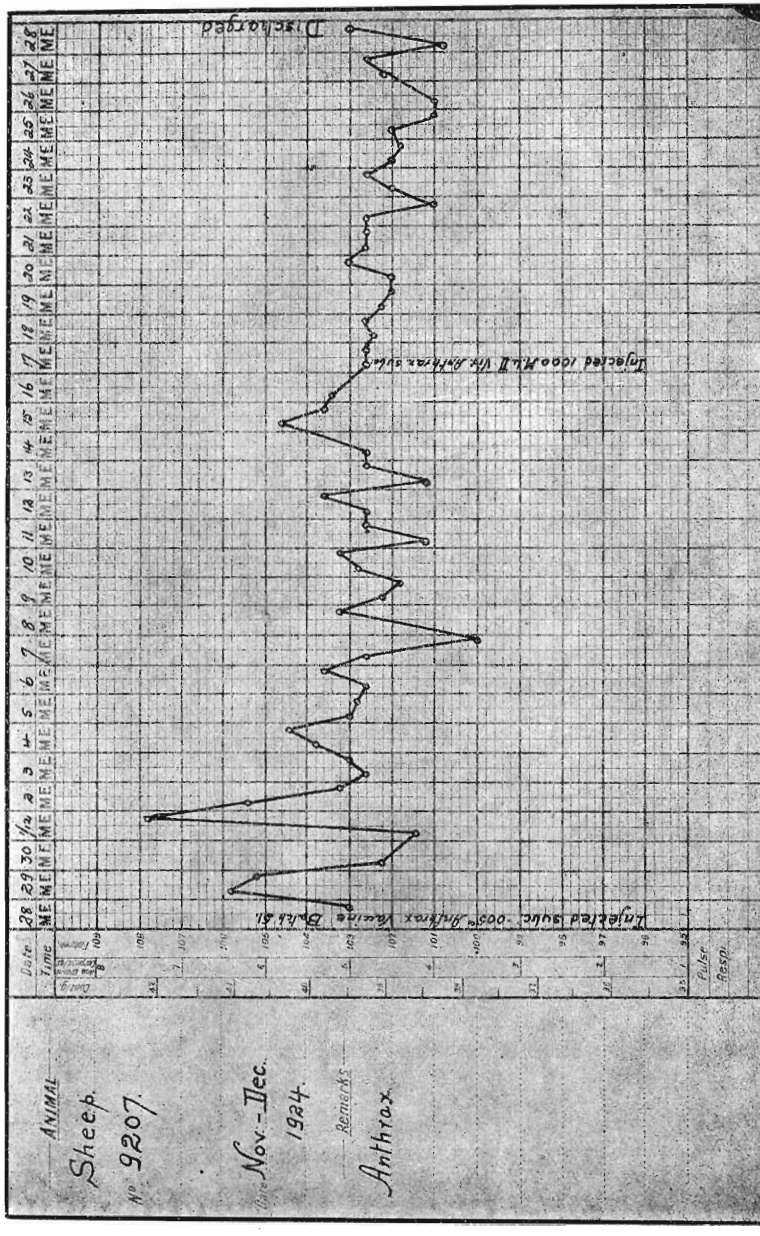

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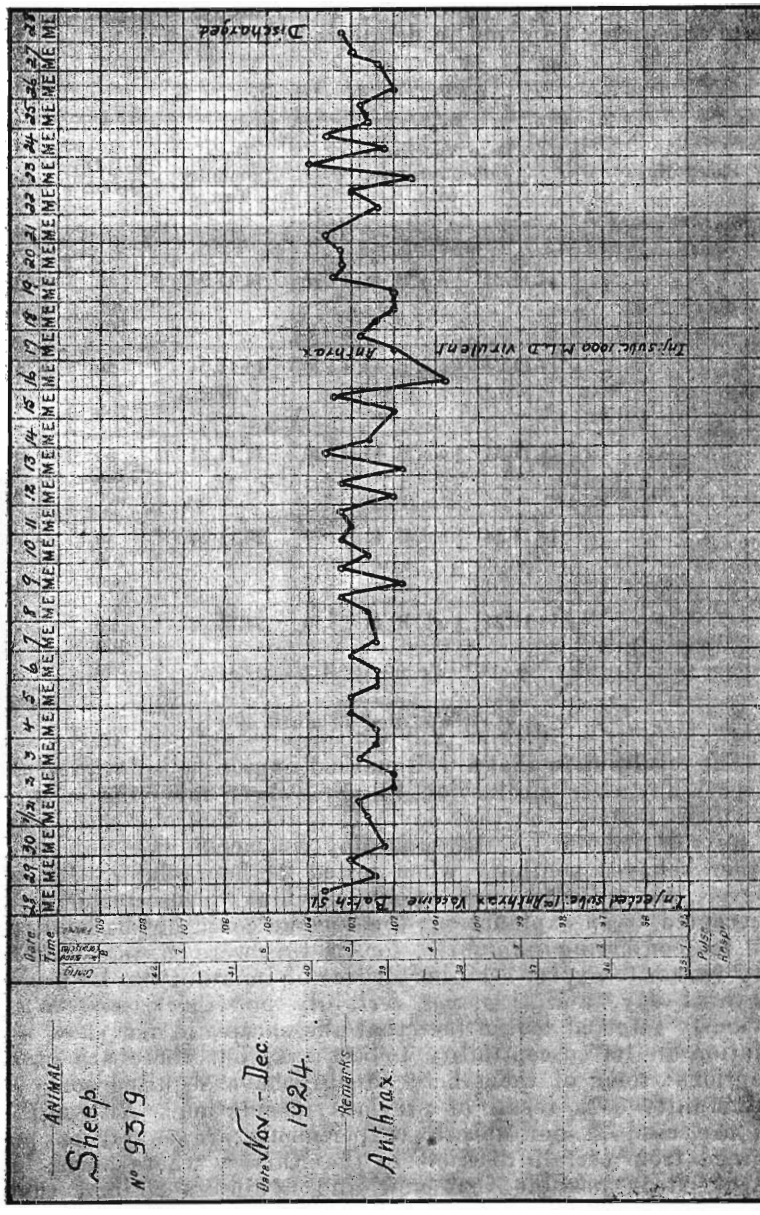

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