STUDIES ON THE SURVIVAL TIME OF THE BOVINE TUBERCLE BACILLUS IN SOIL, SOIL AND DUNG, IN DUNG AND ON GRASS, WITH EXPERIMENTS ON THE PRELIMINARY TREATMENT OF INFECTED ORGANIC MATTER AND THE CULTIVATION OF THE ORGANISM.

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Introduction.

One of the most serious problems of the dairying industry at the present time is undoubtedly that of bovine tuberculosis, which not only causes grave losses to the industry itself but is a menace to the health of the human population.

As is well known, segregation of animals which are healthy has been used as one method of eradication, but up to the present it has not been possible to take full advantage of the method, owing to a lack of knowledge of the viability of the tubercle bacillus under practical conditions. The eventual destructive action of the soil on obligate parasitic bacteria, such as the tubercle bacillus, has always been accepted as a *sine qua non*, but little work has so far been done, except upon spore formers such as tetanus and anthrax, to determine the length of time under average conditions which must elapse between the time of infection and the death of the pathogenic organism in question.

There is therefore little information, resting on a sound experimental basis, which allows us to say with certainty that bovine tubercle bacilli in soil, in dung or especially upon the grass in a pasture, will be no longer alive after the lapse of a certain period.

It is not at present even certain that animals are in fact infected from pastures previously grazed by tuberculous animals, but in considering the question of infection by tubercle bacilli, and eradication of tuberculosis in

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cattle, it is clearly a matter of fundamental importance. The experiments about to be described were designed to answer the following questions:

(1) What is the maximum time during which, under field conditions, bovine tubercle bacilli can remain alive and virulent, in (*a*) dung, (*b*) dung and soil, (*c*) soil and (*d*) on grass growing in an ordinary pasture?

(2) After what length of time from infection can pasture be regarded as certainly non-infective?

I. Preliminary work.

Good facilities for housing experimental animals and carrying out the experiments outlined under (1) and (2) above, existed before the work was begun, but the grass needed renovation. This necessarily took some time, which was occupied in an attempt to improve experimental methods for this type of work.

Hoy and Stenhouse Williams (1930) found in their work on the viability of the bovine tubercle bacillus in stored cow dung and liquid manure, that many of their animal experiments were vitiated because of the deaths of inoculated guinea-pigs from causes other than tuberculosis.

They found that deaths were numerous in spite of the most careful treatment of their material to destroy organisms causing intercurrent infections. They used antiformin for this purpose in the first instance but later abandoned it in favour of Petroff's method, but they did not succeed in eliminating the high percentage of deaths.

When the nature of the material necessarily used in such experiments is considered, it appears likely that serious gaps will arise in a series, because of the great probability of deaths amongst the test animals.

Experiments on cultural methods for the recovery of B. tuberculosis.

It appeared, therefore, that any cultural method for the recovery of tubercle bacilli from infected material, which promised any considerable measure of success, merited investigation with a view to its application to the problem in hand.

Corper and Nao Uyei (1929) treated contaminated pathological material, suspected to contain living tubercle bacilli, with an equal volume of 6 per cent. sulphuric acid for 30 min. at 37° C. and spread the material, after washing, on crystal violet (0·0015 per cent.) potato tubes. With human pathological material they reported that the cultural was equal to the inoculation method as a means of diagnosis.

Confirmation of Corper and Uyei using human material.

Before embarking on any extensive series of experiments in the application of the method to the problem in hand, it was thought advisable to test the claims of Corper and Uyei with human material.

Using human tuberculous specimens (bone, sputum, etc.), kindly supplied by Dr Mills, Royal Berkshire Hospital, Reading, no difficulty was found in substantiating the claims of these authors, and the method was then applied to milk samples suspected to contain bovine tubercle bacilli.

Application of Corper and Uyei's method to naturally infected milk samples and comparison with Petroff's method.

In view of the fact that Petroff's (1915) method has been largely used for the destruction in pathological material of organisms other than tubercle bacilli, it was decided to compare its efficiency with that of the method of Corper and Uyei.

Method of testing milk samples.

75 c.c. of milk were centrifuged for 20 min. at 4000 r.p.m. The fat and serum were removed, the deposit thoroughly stirred, and divided into equal parts in sterile tubes.

One portion was treated with an equal volume of 6 per cent. H_2SO_4 and the other with the same quantity of 4 per cent. KOH and the tubes incubated at 37° C.

After incubation the material was centrifuged, thoroughly washed and then spread evenly on tubes of crystal violet potato (Corper and Uyei) and on Dorset's egg medium. The latter was used because of its excellent qualities in culture work with *B. tuberculosis* and because it was felt that a comparison would be valuable. Its use would also indicate the extent to which the destruction or inhibition of contaminants was due to H_2SO_4 or crystal violet.

It was quickly found that 20 min. incubation at 37° C. with 6 per cent. H_2SO_4 did not kill contaminants found in milk deposits, since heavy growth not only of spore formers but also of cocci occurred in the tubes.

It was therefore decided to increase the time of contact with the acid at 37° C. and various times from 20 min. to 2 hours were tried. The results are shown in Table I.

Table I. *Destruction of contaminants in milk samples by 6 per cent.* H_2SO_4 *and 4 per cent.* KOH.

me of con- ct of milk posits with % H_2SO_4 4 % KOH at 37° C. in min.	Treated with 6 % H_2SO_4						Treated with 4 % KOH					
	Result on Dorset's egg medium			Result on crystal violet potato			Result on Dorset's egg medium			Result on crystal violet potato		
	No. of samples	No growth	Contaminated	No. of samples	No growth	Contaminated	No. of samples	No growth	Contaminated	No. of samples	No growth	Contaminated
20	8	1	7	20	1	19	6	0	6	20	1	19
30–39	10	0	10	10	3	7	10	2	8	10	7	3
40–49	2	0	2	2	2	0	2	2	0	2	2	0
50–59	6	2	4	6	2	4	6	3	3	6	1	5
60–69	20	4	16	20	14	6	20	5	15	20	11	9
70–79	12	4	8	12	4	8	12	2	10	12	9	3
120	2	0	2	2	0	2	2	1	1	2	1	1
ɔtals	60	11	49	72	26	46	58	15	43	72	32	40

Although the experiments were primarily designed to show the extent of the destruction of contaminants, since six of the milk samples used were shown by animal inoculation to contain living tubercle bacilli, they also served to test the efficiency of the method for the direct culture of tubercle bacilli. Table I shows clearly that 6 per cent. sulphuric acid is not effective in killing contaminants in milk deposits and that the growth of the tubercle bacillus is inhibited.

Table II.

No. of samples	Time in contact with 12 % H_2SO_4 in min.	Result on Dorset's egg medium		Result on crystal violet potato	
		No growth	Contaminated	No growth	Contaminated
44	4– 9	7	37	8	36
22	10–19	0	22	5	17
18	20–25	7	11	9	9
Totals 84	—	14	70	22	62

Experiments with 12 *per cent.* H_2SO_4.

The lack of success with 6 per cent. H_2SO_4 led to the use of this acid at a higher concentration. The procedure was exactly as before, except that 12 per cent. H_2SO_4 was used instead of 6 per cent. H_2SO_4 and that the times of contact with the milk deposits were varied. Table II shows that, as with the lower strength of acid, contaminants were not killed in a high percentage of cases. Five of the samples were proved by animal inoculation to contain living tubercle bacilli, but in no case after treatment with 12 per cent. H_2SO_4 did growth of tubercle bacilli occur.

Results of experiments using Corper and Uyei's method with milk deposits.

It is clear that, although the method appears to give good results with human pathological material, it has not, in the hands of the author, eliminated contaminants in milk samples nor even permitted the growth of the tubercle bacillus.

Experiments on the elimination of contaminants by means of compounds containing available chlorine.

It seemed that, if the contaminants in milk deposits were not eliminated by this method, it was unlikely to succeed with such materials as soil and dung, and it became necessary to consider other methods.

Hoy and Stenhouse Williams (1930), working with infected stored faeces and liquid manure, used antiformin for the preliminary treatment, but finding that they lost many guinea-pigs and believing that the chlorine killed tubercle bacilli, abandoned it in favour of Petroff's method. The deaths were still so numerous as to vitiate many experiments, but they believed the tubercle bacilli were not killed to the same extent as when using antiformin. The antiformin used by these workers was variable in composition, and they made

no estimates of the amount of available chlorine present. It is probable that on some occasions they used too great a concentration of chlorine.

In view, however, of the well-established resistance of tubercle bacilli in pathological material to the action of disinfectants, it was felt that considerable strengths of available chlorine might safely be used without killing the organism, and a series of experiments to this end was carried out. If the NaOCl were kept in solution, made strongly alkaline with NaOH, any benefits from the action of the soda (Petroff's method) might be secured, together with the effect due to chlorine.

A solution of NaOCl was made up in 15 per cent. NaOH (A.R.) and the exact amount of available chlorine found by titration. This solution, in various strengths, was then used for treating the material containing living tubercle bacilli.

In every case before use, the stock solution was retitrated, so that the quantity of available chlorine used was always accurately known.

Treatment of milk deposits with NaOCl/NaOH *solution.*

In view of the fact that the efficiency of an oxidising disinfectant, such as NaOCl, depends largely upon the amount of oxidisable organic matter in the treated material, it was decided to begin with considerable concentrations of available chlorine.

The milk deposits prepared as described above were treated with equal volumes of the NaOCl/NaOH mixture in various concentrations and for various times at 37° C. At the end of the incubation period the mixtures were thoroughly washed and centrifuged, until a starch iodide test for free chlorine was negative.

The deposits were then smeared on Dorset egg tubes and incubated at 37° C. Although a number of the samples were known, or later proved, to contain tubercle bacilli, it was only desired, in this experiment, to find to what extent the contaminants were destroyed.

Table III. *Treatment of milk deposits with known strengths of available chlorine.*

No. of samples	Available chlorine, parts per million, final concentration	Time incubated at 37° C. hours	Result on Dorset's egg medium	
			No growth	Contaminated
12	163·65	3	0	12
4	163·65	4	0	4
18	218·20	3	5	13
12	327·3	2	1	11
36	327·3	3	6	30
6	327·3	4	5	1
88	—	—	17	71

The results are given in Table III and show a high percentage of contaminated tubes.

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In fact, however, the actual numbers of contaminants surviving were much smaller than in previous experiments, and large areas, even on contaminated tubes, remained free from growth in 70 out of 88 cases after 6 weeks' incubation.

At the same time as the deposits were smeared on Dorset egg tubes, a 1 c.c. portion from several was plated on dextrose agar and incubated, first for 3 days at 22° C. and then for 2 days at 37° C.

In all cases a very small number of colonies appeared on the plates. These were invariably spore formers of the *B. mesentericus* var. *ruber* or *B. subtilis* type. No non-spore formers survived the treatment, except *B. tuberculosis*, which gave discrete colonies in one case where the sample proved to be naturally infected.

Growth of tubercle bacilli, added artificially to milk samples, after treatment with NaOCl/NaOH *solution.*

Living bovine tubercle bacilli, varying in numbers from about 1200 to 9000 per 50 c.c., were added to fifteen milk deposits prepared as described, treated with various concentrations of NaOCl/NaOH and smeared on Dorset egg tubes. Colonies of tubercle bacilli were obtained in one case only with milk deposits, but emulsions in normal saline, of two infected glands from guinea-pigs, gave good growth of tubercle bacilli after treatment for 3½ hours, with a final available chlorine concentration of 327·3 parts per million.

In spite of the fact that growth occurred in so few cases, the actual survival of tubercle bacilli in the treated material was proved by animal inoculation. It was thus clear that none of the cultural methods tried was in any way likely to supersede the inoculation method, but the experiments clearly confirmed the well-accepted belief that the tubercle bacillus in organic matter is able to withstand very considerable concentrations of available chlorine, and further that contaminants, although not completely removed in many cases, are reduced to such small numbers as greatly to increase the chances of the survival of inoculated animals.

Experiments on the treatment of infected soil with NaOCl/NaOH *solution.*

The proof of survival of the tubercle bacillus, after treatment with alkaline hypochlorite solution, secured in the experiments described, enabled the problem of the effective treatment of soil and dung samples to be approached with much greater hope of success.

It was desired to establish the maximum concentration of chlorine which could be applied to infected soil without killing the tubercle bacillus, and the following procedure was adopted in the first instance.

Method.

After a period of excessive rain the soil on the proposed site was deemed fit for experiment on April 13th, 1931.

A representative sample of the surface soil was made up from various places on the site and used for treatment with NaOCl/NaOH.

100 g. of the soil, which was free from stones, was thoroughly shaken with 200 c.c. sterile water at intervals for 2 hours at room temperature.

Dilutions of this emulsion were then plated on nutrient agar, in order to give an idea of the number of viable organisms originally present.

Portions of the emulsion were then centrifuged at 3500 r.p.m. for 20 min., the supernatant fluid poured off and the residue treated with its own volume of NaOCl/NaOH solution. Using a final concentration of 163·65 parts per million available chlorine for 3 hours at 37° C., such large numbers of soil organisms remained alive that the survival of guinea-pigs inoculated with such material was very problematical.

The technique was therefore varied, 100 g. of soil being shaken with 300 c.c. sterile water for 3 hours and a part of the suspension treated with its own volume of NaOCl/NaOH containing 645·6 parts per million of available chlorine.

Separate portions thus treated were incubated for 2 hours and 3 hours respectively, centrifuged, washed and nutrient agar plates poured with the dilutions.

After 2 hours' incubation a large number of contaminants survived (about 10,000 per gram of soil), but after 3 hours the numbers were very small (6–50 per gram).

Treatment for inoculation of artificially infected soil.

The success of the higher final concentrations of available chlorine (327·3 parts per million) in reducing the number of contaminants led to experiments on the ability of tubercle bacilli, added to soil, to survive the treatment.

Therefore, to soil emulsions, treated as described above, a known virulent strain of the bovine tubercle bacillus was added in such numbers that the concentration was about 300 bacilli per c.c. of the final emulsion, which contained 327·3 parts per million of available chlorine.

The mixture was incubated for 3 hours at 37° C. and after washing thoroughly, 1 c.c. was inoculated into each hind-leg of a pair of guinea-pigs.

Both pigs developed tuberculosis within 6 weeks and bovine bacilli were recovered from generalised lesions. It appeared, therefore, that a final concentration of 327·3 parts per million of available chlorine was sufficient to satisfy the requirements of the organic matter in a typical soil. The contaminants were killed to such an extent as to permit the survival of inoculated guinea-pigs and a sufficient number of tubercle bacilli to cause infection remained viable.

Further experiments were however made, by adding known numbers of tubercle bacilli to soil and treating it as before.

To make quite sure of the necessity for the use of such a high concentration of available chlorine as 327·3 parts per million with its accompanying

NaOH, the experiment was repeated, and in addition, one portion of infected soil was treated under the same conditions and for the same time with a concentration of 218·2 parts per million of available chlorine.

The guinea-pigs inoculated with material treated with the lower chlorine concentration died, as the direct result of inoculation, before any evidence of a tuberculous infection could be obtained. Those treated with the higher concentration recovered quite normally from the local injection and one died from tuberculosis in 8 weeks and the other on being killed was proved to be tubercular.

It seems, therefore, that a final concentration of 327·3 parts per million of available chlorine may safely be used for preliminary treatment of average soil emulsions destined for inoculation, and that a concentration of 218·2 parts per million is insufficient to kill contaminants to such an extent as to guarantee the survival of the guinea-pigs. It should be pointed out that since the NaOCl was made up in 15 per cent. NaOH solution, the concentration of NaOH would be 2·205 per cent. with the higher and 1·47 per cent. with the lower concentration. Although no attempt was made to estimate it, there may have been an increased effect due to the greater concentration of alkali.

Summary.

(*a*) The cultural method of Corper and Uyei (1929), involving preliminary treatment of the material with sulphuric acid, although found to be effective with human specimens, is inapplicable to milk samples owing to the survival of contaminants which obscure or prevent the growth of the tubercle bacillus.

(*b*) Milk deposits and samples of average soil may be treated for 3 hours at 37° C. with a solution of NaOCl/NaOH giving a final concentration of 327·3 parts per million of available chlorine without killing the bovine tubercle bacillus. Guinea-pigs inoculated with the washed, treated material, survived but contracted tuberculosis.

Addendum.

During the experiments on the treatment of soil emulsions with NaOCl, to give a final concentration of 327·3 parts per million of available chlorine, two milk samples which showed numbers of acid-fast bacilli microscopically, happened to become available.

The deposit from one of these was treated as described in this paper, that is after treatment it was freed from chlorine by thorough washing and that from the other was not washed. In the latter case some 5 parts per million of chlorine remained. It was found on inoculation that the guinea-pigs in both cases developed tuberculosis. It seems therefore that the presence of some chlorine in the deposits does not inhibit infection.

II. SURVIVAL TIME OF BOVINE TUBERCLE BACILLI IN SOIL, DUNG AND SOIL MIXTURES, AND IN DUNG EXPOSED IN THE OPEN.

Hoy and Stenhouse Williams (1930) showed that in dung plots exposed on pasture the tubercle bacillus survived for 4 months in autumn and 5 months in winter. These workers presented no evidence of the survival time during summer but failed to recover the bacillus after 2 months' exposure. They suggest that a correlation existed between the disappearance of visible organic matter and failure thereafter to recover living tubercle bacilli. However, they suffered such severe casualties with inoculated guinea-pigs that it was decided to carry out a series of experiments similar to theirs, but to treat the material before inoculation by the modified technique described in Part I.

Plan of experiment.

The enhanced resistance to adverse conditions of the tubercle bacillus in naturally infected material, as compared with that of naked cultures, is well known.

Preparation of the emulsion of naturally infected material.

Since pasture and soil infection may be expected to come chiefly from dung containing bacilli enclosed in particles of mucus and from sputum, it was felt that the procedure of Hoy and Stenhouse Williams (1930), who used emulsions of tuberculous lungs, should be followed, as being the most exact reproduction of ordinary conditions which could be devised. These workers showed that to use naturally infected dung is an uncertain method, since they found that they could only recover tubercle bacilli intermittently, though they used a cow suffering from advanced phthisis.

Tuberculous material was, therefore, obtained from the abattoirs and the fat and connective tissue carefully separated from the infected parts, which were ground to a fine suspension by passing repeatedly through a mincing machine. The suspension was then emulsified in normal saline, shaken repeatedly for 48 hours, strained through muslin and then stored in Winchester quart bottles containing glass beads, until required. As autolysis proceeded, the bacilli were dispersed by shaking at intervals until finally a reasonably homogeneous emulsion of tuberculous material, fine enough to pass through a rose nozzle, was secured.

Microscopic examination showed that the distribution of the tubercle bacilli was better than can ordinarily be obtained when attempting to make a homogeneous emulsion from artificial cultures of these organisms.

The numbers of bacilli were estimated by a direct microscopic method and the infectivity proved by animal inoculation. The bacilli were also isolated, grown on artificial media and proved to be of the bovine type in the ordinary way.

During the course of the experiment no post-mortem lesion in inoculated guinea-pigs, however characteristic, was accepted as due to bovine tubercle

bacilli without first demonstrating acid-fast bacilli microscopically and, in key cases, growing out and typing. The possibility that lesions might be due to the saprophytic acid-fast bacteria of the soil was excluded by proving in every case that no growth on media took place within a few days at 37° C.

It is of interest to note that on one occasion a virulent emulsion of naturally infected post-mortem material which was kept tightly stoppered failed to infect guinea-pigs even in large doses, after it had been kept for 2½ months at room temperature in the dark.

It was believed that owing to the rapid establishment of more or less anaerobic conditions in the bottle, the strongly aerobic tubercle bacilli had failed to survive.

In later work this observation was taken advantage of in preserving cultures in sealed Dorset egg tubes, in that air was admitted and saline added at intervals, with obvious benefit to the rate and quantity of growth obtained. The necessity for this procedure has been noted by previous workers, but it is believed that its advantages are not generally realised.

Viability of the bovine tubercle bacillus under field conditions in dung, dung and soil mixtures, and in soil.

In April, 1931, a corner of the fenced pasture used throughout the experiments was freed from vegetation and five large (15 in.) porous pots, without bottoms, sunk in the soil to the ground level. Inside each 15 in. pot a 12 in. pot, without bottom, was sunk. All the space between the two was filled with earth until the 12 in. pot reached ground level. This inner pot was then filled with soil. In this way the experimental mixtures could be preserved as far as possible from the depredations of insects and burrowing animals, without detriment to drainage and aeration. From April to June, 1931, observations were made of the condition of the soil in the pots. No substantial physical differences between the condition of the natural soil and that in the pots was noticed and on 2. vi. 1931 the following mixtures were made and placed in the pots:

			Percentage	
Plot	Soil lb.	Cow dung lb.	Soil %	Cow dung %
I	10	—	100	—
II	7½	2½	75	25
III	5	5	50	50
IV	2½	7½	25	75
V	—	10	—	100

10 lb. of soil were removed from each pot and replaced by the experimental mixtures which had an average depth of about 3 in.

The tuberculous emulsion was added to the material for making the plot in such quantity that the final population of tubercle bacilli was about 100,000 per gram at the beginning of the experiment. It was not, of course, known how many of these were alive and virulent.

After preparation, each plot was protected from birds and other animals by covering with wire on a very low frame which did not exclude light and air. The plots and surroundings were kept free from vegetation during the course of the experiment.

Sampling and technique.

At approximately fortnightly intervals from preparation of the plots a sector of 100 g. weight was cut with a knife and transferred to a sterile wide-mouthed bottle.

200 c.c. of sterile water was added and the whole thoroughly shaken and emulsified. The emulsion was strained through a thickness of fine muslin and 50 c.c., representing 25 g. of the original material, was centrifuged at 3000 r.p.m. for 30 min.

The supernatant fluid was poured off and 10 c.c. of sterile water added to the deposit and thoroughly stirred. To this was added 10 c.c. of NaOCl/NaOH solution to give a final concentration of 327·3 parts per million of available chlorine and 2·205 per cent. of NaOH.

The mixture was incubated for 3 hours at 37° C. and shaken at intervals.

Distilled water was then added and the mixture shaken and centrifuged for 30 min. at 3000 r.p.m. The washings were repeated until no available chlorine remained. Two washings generally sufficed. To the final deposit 10 c.c. of distilled water was added and thoroughly stirred and 1 c.c. inoculated subcutaneously into each hind-leg of a pair of guinea-pigs.

Fifteen samples from each plot were taken at intervals over a period of 245 days from the time of laying down. No animal was killed until enlarged glands could be felt or until a tuberculin test was repeatedly negative.

The results are shown in Table IV.

Table IV.

No. of exp.	Date	No. of days infected plot exposed	Plot I Soil only	Plot II Soil 75 % Dung 25 %	Plot III Soil 50 % Dung 50 %	Plot IV Soil 25 % Dung 75 %	Plot V Dung only
1	16. vi. 31	14	+ 25	+ 25	+ 25	+ 23	+ 25
2	7. vii. 31	35	− 281	− 281	− 281	− 281	+ 133
3	29. vii. 31	57	+ 82	− 231	+ 96	+ 82	+ 52
4	12. viii. 31	71	− 217	− 217	+ 96	+ 69	+ 66
5	26. viii. 31	85	+ 122	+ 82	+ 48	+ 48	+ 81
6	8. ix. 31	98	− 190	+ 67	+ 104	+ 86	+ 104
7	2. x. 31	122	− 166	+ 166	− 166	+ 77	+ 90
8	16. x. 31	136	− 153	− 153	− 152	+ 92	+ 76
9	30. x. 31	150	− 139	− 139	− 139	− 139	+ 71
10	13. xi. 31	164	− 125	− 125	Died	− 125	+ 66
11	27. xi. 31	178	+ 53	+ 26	+ 52	Died	+ 29
12	11. xii. 31	192	− 97	− 178	− 178	− 178	− 178
13	5. i. 32	217	− 192	− 192	− 192	− 192	− 192
14	19. i. 32	231	− 178	− 178	− 178	− 178	− 178
15	2. ii. 32	245	− 164	− 164	− 164	− 164	− 164

+ = died or killed with *B. tuberculosis.* − = no *B. tuberculosis.*

Note. The figures after the + or − sign indicate the number of days from inoculation which the guinea-pigs survived.

Discussion of results.

It will be seen that, after exposure for 178 days, living and virulent tubercle bacilli were recovered from all the plots (except plot 4, where the guinea-pigs died soon after inoculation). Thereafter all results were completely negative.

It is, perhaps, significant that on every occasion up to the 178th day when a test was made, the plot containing dung only was always positive.

If the number of plus signs in Table IV be taken as a criterion of infectivity, it will be seen that the greatest number were obtained with plot V, followed in descending order by plots IV to I. Even allowing for the deficiencies of the technique used, which is admittedly far from perfect, support is given to the contention of Hoy and Stenhouse Williams (1930) that there is a correlation between the amount of organic matter in an exposed plot and the survival of the tubercle bacillus. It was recorded that visible organic matter had disappeared from plot II after $17\frac{1}{2}$ weeks, from plot III after $23\frac{1}{2}$ and from plot IV after $25\frac{1}{2}$ weeks. A surface scab remained on plot V after 35 weeks. As a matter of interest it may be said that the samples of July 7th were taken at the end of the only really fine-weather period of the summer, and that negative results were secured in all plots except No. V. Even there the virulence was so low as to allow inoculated guinea-pigs to survive for 133 days as compared with much shorter times when the samples were taken in less fine weather.

The long periods between positive results in the case of the soil only, may have been due to failure to mix the bacilli thoroughly at the time of exposure. This was certainly difficult, but it is hardly likely that so large a sample as 100 g. would escape without bacilli. It is perhaps legitimate to advance the tentative conclusion that the virulence of tubercle bacilli in soil is more rapidly lost than in soil and dung mixtures and in dung.

Summary.

It is certain that some virulent *Bacilli tuberculosis* can survive a period of 6 months' exposure in soil, in soil and dung mixtures and in dung.

Since the results thereafter were consistently negative, it appears that in the south of England, exposure for a period of about 7 months (June-December inclusive) suffices to kill the *B. tuberculosis* in soil, soil and dung mixtures, and in dung.

III. Viability of the bovine tubercle bacillus on grass growing in a pasture.

The ultimate object of the experiments on the viability of the bovine tubercle bacillus, is to determine whether bovine animals can be infected by feeding on infected pastures and what length of time, after infection, pastures must remain unpopulated to ensure safety. The following experiments were therefore designed.

Preliminary experiments on the recovery of virulent tubercle bacilli from infected growing grass.

It was proposed to use emulsions of tuberculous material similar to those previously described, but before starting it was necessary to devise a method of infection which would be as nearly as possible uniform over the area of grass used.

Using as an indicator 1 gallon of an emulsion of lime delivered through a fine rose from a watering can, it was found that over an area of 150 sq. ft., uniformly wetted, only the upper surfaces of a high proportion of the grass blades showed lime particles. For thorough wetting of the grass a quantity of 1 gallon on 50 sq. ft. was found to be necessary.

It was further necessary before embarking on the experiment proper, to devise a technique for the direct recovery from infected grass of a sufficient proportion of the tubercle bacilli.

An emulsion of 0·25 g. of very finely divided carmine was suspended in 60 c.c. of water and sprayed over 2 sq. ft. of grass. After 3 days the grass was cut from the plot, taken to the laboratory, cut into lengths of about 1 in. and suspended in 1600 c.c. of water.

The mixture was thoroughly beaten with a long heavy wire egg whisk and strained through muslin under slight negative pressure. The fluid recovered was centrifuged, the deposit collected and the amount of carmine estimated chemically. It was found that 38·48 per cent. of the amount originally sprayed on the grass was recovered. Since the carmine used was very finely divided and approximated to the size of a bacillus, it was felt that a recovery of nearly 40 per cent. was sufficient to warrant the use of the same technique for infection with, and recovery of, the tubercle bacillus.

Infection of pasture.

On April 25th, 1932, an area of grass which was beginning to grow, was marked out in three plots of 50 sq. ft., each plot being isolated from its neighbour by a path on all sides. A fine emulsion of tuberculous bovine lungs prepared as previously described was used for infecting the plots.

The infectivity of the emulsion was proved by inoculation of guinea-pigs and the bacilli proved to be of the bovine type.

A direct count showed that the emulsion contained 5,180,000 acid-fast bacteria per c.c.

Of these 3,000,000 per c.c. were assumed to be living and dilution made to give the following weight of infection per sq. ft. on each of three plots of 50 sq. ft. of grass:

Plot	I.	120,000
,,	II.	1,200,000
,,	III.	120,000,000

B. tuberculosis per sq. ft.

Preliminary practice with non-infective emulsions of the same consistency

allowed the emulsions to be delivered from the can in such a way that uniform infection of each plot was secured.

1 c.c. of the highest dilution of the emulsion, used on plot I, caused the rapid death of inoculated guinea-pigs from tuberculosis, proving the infectivity of the emulsion.

Actual technique employed in recovering tubercle bacilli direct from grass.

At intervals from the time of infection, 1 sq. ft. of grass was enclosed by dropping over it a wooden frame of the required area, and cut to within ¼ in. of the ground with shears. Great care was taken to avoid contact with the soil. The grass was transferred to a sterile pail, taken to the laboratory, cut into lengths of 1 in. and then thoroughly beaten for half an hour in a pail with tap water. The amount of water which had to be added varied (from 600 to 1800 c.c.) with the quantity of grass, which of course differed enormously from experiment to experiment. The amount of water used was such that it allowed every particle of the cut grass to be thoroughly beaten in a small pool at the bottom of the pail.

The grass was removed as far as possible by filtering through a Buchner funnel under slight negative pressure and 400 c.c. of the fluid was centrifuged at 3000 r.p.m. for 30 min. The reaction of the fluid was always about neutral.

The deposit was stirred with 10 c.c. of NaOCl/NaOH and 10 c.c. of normal saline to give a final concentration of 327·3 parts per million of available chlorine and the mixture incubated for 3 hours at 37° C. Washings and centrifuging were carried out as before and 1 c.c. of the deposit, suspended in saline, inoculated into each hind-leg of a pair of guinea-pigs. Tests for free chlorine before inoculation were always negative. Table V gives the results of this experiment to date, together with the rainfall registered at a station within 300 yards of the experimental plots.

In spite of the abnormal rainfall, which was torrential on some occasions, it will be seen that the grass remained infective on all plots to such an extent as to cause the death from tuberculosis of all the guinea-pigs inoculated up to 14 days from the time of infection.

The results are of course incomplete, but they show that the technique used is suitable for the purpose.

Table V. *Survival of* B. tuberculosis *on infected grass. Results to date.*

No. of exp.	Date	No. of days after infection	Rainfall since infection of grass in.	Plot I	Plot II	Plot III
1	28. iv. 32	3	0·16	+14	+21	+ 28
2	2. v. 32	7	1·69	+61	+68	+ 23
3	9. v. 32	14	2·65	+70	+65	+ 53
4	16. v. 32	21	3·25	0	0	+ 61
5	23. v. 32	28	5·30	0	+87	+103
6	30. v. 32	35	6·05	0	0	+105
7	13. vi. 32	49	6·47	0	0	+ 43

The figures after the + sign indicate the number of days from the time of inoculation elapsing before the death of the test guinea-pigs.

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Discussion of results.

It will be seen that in spite of the exceptionally heavy rain after infection, followed by great heat and dryness, all the plots gave positive results after 14 days' and plot III after 49 days' exposure. Tuberculin tests on surviving guinea-pigs indicate that this period is likely to be considerably extended.

Summary.

For the present it may safely be stated that on growing grass, bovine *B. tuberculosis* remain alive and virulent, for a period of at least 49 *days in summer*, in the south of England. Simultaneously with the experiments described above, a feeding experiment on guinea-pigs has been carried out, to discover whether the bacilli, proved to be infective for these animals by inoculation are, after exposure on grass for varying times, capable of setting up infection by the alimentary route. For this purpose a number of colonies of a dozen guinea-pigs each is being fed on grass at increasing intervals after the time of infection. These experiments, which are in progress, are it is hoped preliminary to the feeding of calves on similarly infected pasture.

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