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The influence of temperature and humidity on spore formation and germination in *Bacillus anthracis*

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There is a striking contrast between the persistence of the spores of *Bacillus anthracis* in the soils of subtropical countries and the poor survival of the organism in soil of Great Britain. This is in spite of the constant re-infection of the soil here through the extensive reliance of British agriculture on dried bone fertilizers.

An investigation has been undertaken into the effect of temperature and humidity on spore formation and germination in *B. anthracis* to see if these two physical factors could account for the difference in persistance of the organism and the results are presented in this communication.

SPORE FORMATION

Influence of temperature on spore formation in Bacillus anthracis

The temperature effect on spore formation in *B. anthracis* has been fully investigated in the past. Previous work on the subject has been reviewed by Minett (1950) and can be summarized as follows:

Author	Temperature (° F)	Sporing Time (hr.)
Koch (1877)	95	20 (not actually indicated as the shortest time)
	86	30
	64–68	60–72
Stephanidis (1899)	98	8
Weil (1899)	98–88	16
	75	36
	64	50
Jacobsthal & Pfersdorff (1901)	98	6–8
	72	22
	61	62
Preisz (1904)	98	6

These results agree in showing that a rise in temperature lowers the sporing time of *B. anthracis*. No further experimental work appeared to be necessary.

Influence of humidity on spore formation in Bacillus anthracis

In contrast to the literature on temperature, references to the effect of humidity on sporing, especially those dealing with experimental work, are few.

The observations of von Behring (1889) that alcohol vapour and calcium chloride induced sporing in *B. anthracis* suggest that dehydration may be an important factor. Migula (1904) stated, however, that sudden drying hinders sporulation by stopping all development in the bacteria. Daranyi (1927) postulated that any unfavourable conditions, desiccation for instance, might reasonably be expected to increase the time taken for spores to be formed. This is the first reference to sporing time as opposed to the total number of spores produced.

Minett (1950) made some observations on the drying of blood containing anthrax bacilli. He used blood from infected animals diluted with fresh citrated blood as his cultures. These he placed on microscope slides and incubated in jars, above mixtures of water and sulphuric acid, giving a range of humidities from 20 to 100 %. He varied the quantities of blood on each slide from jar to jar, however, according to their rate of drying so that they remained fluid for the duration of his experiments. This might appear to invalidate the assumption that the organisms present in the liquid on the slides were themselves actually exposed to the relative humidities in the jars. The results he obtained are not stated. He was apparently trying to simulate the shedding of blood from infected animals, and was more interested in showing that blood could remain liquid over a whole drying range rather than in the effect of humidity on sporulation. Minett did find, however, in other experiments that the addition of 3 % by weight of water to sterile soil was essential for spore production by *B. anthracis* in 48 hr. at 37° C.

Finally, Lamanna (1952) states that optimum conditions for sporulation are the same as those for growth of the vegetative form, the permissible variation in environment being within stricter limits than those for growth.

These would appear to be the only references bearing directly on the effect of humidity on sporulation. It was therefore decided to carry out some experimental work on this subject.

Materials and methods

Nutrient agar cultures of *B. anthracis* placed on microscope slides were chosen for these experiments.

Approximately 0·3 ml. quantities of molten nutrient agar were pipetted on to the centre of sterile glass slides, spread with a bent glass rod to cover an area of about $\frac{3}{4}$ in. sq., and allowed to set. The slides were then dried completely by incubating at 37° C. for 48 hr. in Petri dishes, the lids being propped open to facilitate evaporation.

A range of constant humidity jars was set up, jars of the Kilner type being used with close fitting glass tops, rubber washers and metal screw caps. These jars were approximately $2\frac{1}{2}$ l. in capacity and were large enough to contain a sufficient volume of liquid to control the humidity of the overlying atmosphere, which was also sufficient in volume for adequate oxygenation of the slide cultures. The series consisted of nine jars of relative humidity range from 20 to 100 %. Buxton & Mellanby (1934) give the composition of sulphuric acid and water mixtures which make up such a series.

The desiccated agar slides were then put into the jars, resting on an improvised

rack made from glass rods, above the level of the acid-water mixture. Each jar held six slides side by side.

It was assumed that the dried agar would take up water from the atmosphere to an extent proportional to the degree of humidity in the sealed jar. A period of 72 hr. was allowed for the agar slide cultures to reach the required degree of moisture before they were inoculated. As a source of vegetative forms of *B. anthracis* an overnight culture in 1% glucose broth was used. Koch (1888) thought that glucose had an inhibiting action on spore formation, perhaps because of the accumulation of acid metabolites. Deficient oxygenation in a fluid medium might also play some part in inhibiting spore formation. That no spores were present in the overnight cultures was proved regularly by heating at 65° C. for 5 min. without any residual growth resulting on subculture and also by microscopical examination of stained preparations.

Before use the overnight cultures of the five strains of *B. anthracis* tested (laboratory stock strains originally isolated from cases of human infection in the Cardiff outbreak, Davies & Harvey (1953)) were standardized to tube 6, Brown's opacity tubes (Burroughs Wellcome and Co.), after shaking the suspension well. The spun deposit was then resuspended in 3 ml. of the supernatant fluid. About 0·05 ml. of this was used as an inoculum for each slide. It was considered that the density of the bacterial population in relation to the media for each of five strains was thus approximately equal. One strain only was tested at a time. At fixed intervals a slide was removed and not returned to the jar. The surface growth was emulsified with a moistened loop, and a preparation for staining was made and examined for endospores using Dorner's nigrosin spore stain (*Society of American Bacteriologists Manual*, 1946).

A very large number of organisms, suitably stained for examination, was found on each slide, and within the higher humidity ranges luxuriant chain formation was seen.

Sets of jars at 37 and 26° C. were used. These two common routine laboratory incubator temperatures also simulate to some extent the soil temperatures in India and Britain, respectively, at the season of maximum warmth, though it is probable that 26° C. may not be reached often in soil protected by pasture in this country.

Results

The results are set out in Tables 1 and 2 and show that the sporing time of *B. anthracis* is prolonged considerably as the humidity decreases. This is shown both at 26 and 37° C. The adverse effect of drying on the speed of spore formation is less noticeable at the lower temperature.

SPORE GERMINATION

Influence of temperature on spore germination in Bacillus anthracis

References in the literature to the effect of physical factors on spore germination are few. There are two main ones relating to temperature. Holzmuller (1909) concluded from a study of five strains of *B. mycoides* and four other related specimens

Table 1

37° C. 'S' represents the appearance of endospores in that particular culture. No spores were formed in any case before an incubation time of 6 hr.

R.H. (%)	Culture	Hours 6	8	12	14	16	18	22	24	26	30	34
100	1	S	S	S								
	2	.	.	S								
	3	.	.	S								
	4	.	S	S								
	5	.	S	S								
90	1	.	.	S	S	S						
	2	.	.	.	.	S						
	3	.	.	S	S	S						
	4	.	.	.	S	S						
	5	.	.	S	S	S						
80	1	.	.	.	.	.	S	S	S			
	2	.	.	.	.	.	.	.	S			
	3	.	.	.	.	.	.	.	S			
	4	.	.	.	.	.	S	S	S			
	5	.	.	.	.	S	S	S	S			
70	1	.	.	.	.	.	.	.	S	S	S	
	2	.	.	.	.	.	.	.	.	.	S	
	3	.	.	.	.	.	.	.	.	S	S	
	4	.	.	.	.	.	.	.	.	S	S	
	5	.	.	.	.	.	.	.	S	S	S	
60	1	.	.	.	.	.	.	.	.	S	S	
	2	.	.	.	.	.	.	.	.	.	S	
	3	.	.	.	.	.	.	.	.	.	S	
	4	.	.	.	.	.	.	.	.	.	S	
	5	.	.	.	.	.	.	.	.	.	S	
50	1	.	.	.	.	.	.	.	.	.	.	S
	2	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.
	4	.	.	.	.	.	.	.	.	.	.	S
	5	.	.	.	.	.	.	.	.	.	.	S
40	1	.	.	.	.	.	.	.	.	.	.	S
	2	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.
	4	.	.	.	.	.	.	.	.	.	.	S
	5	.	.	.	.	.	.	.	.	.	.	.
30	1	.	.	.	.	.	.	.	.	.	.	S
	2	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.
	4	.	.	.	.	.	.	.	.	.	.	.
	5	.	.	.	.	.	.	.	.	.	.	.
20	1	.	.	.	.	.	.	.	.	.	.	S
	2	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.
	4	.	.	.	.	.	.	.	.	.	.	.
	5	.	.	.	.	.	.	.	.	.	.	.

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Table 2

26° C. No spores were formed in any case before an incubation time of 24 hr.

R.H. (%)	Culture	Hours 24	28	40	48	60
100	1	.	S			
	2	.	S			
	3	.	S			
	4	S	S			
	5	S	S			
90	1	.	S			
	2	.	S			
	3	.	S			
	4	S	S			
	5	.	S			
80	1	.	S	S		
	2	.	.	S		
	3	.	.	S		
	4	.	S	S		
	5	.	S	S		
70	1	.	.	S		
	2	.	.	S		
	3	.	.	S		
	4	.	.	S		
	5	.	.	S		
60	1	.	.	S	S	
	2	.	.	.	S	
	3	.	.	S	S	
	4	.	.	S	S	
	5	.	.	S	S	
50	1	.	.	.	.	S
	2	.	.	.	.	S
	3	.	.	.	.	S
	4	.	.	S	S	S
	5	.	.	S	S	S
40	1	.	.	.	.	S
	2	.	.	.	.	S
	3	.	.	.	.	S
	4	.	.	.	.	S
	5	.	.	.	.	S
30	1	.	.	.	.	S
	2	.	.	.	.	S
	3	.	.	.	.	S
	4	.	.	.	.	S
	5	.	.	.	.	S
20	1	.	.	.	.	S
	2	.	.	.	.	.
	3	.	.	.	.	S
	4	.	.	.	.	S
	5	.	.	.	.	S

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that, for any given strain, the optimum for germination and for growth was the same. The temperature range for germination was narrower than that for growth. According to Eurich & Hewlett (1930) *B. anthracis* will grow between 14 and 43° C. Cook (1932), in his review on bacterial spores, states that the spores of *B. anthracis* will not germinate at room temperature; the minimum appears to be in the region of 35–37° C.

Materials and methods

Some experiments were attempted to investigate the influence of temperature on the germination time of *B. anthracis*.

The method chosen used fixed stained preparations. The appearance of a certain arbitrarily chosen proportion of vegetative forms in a preparation originally consisting only of spores is taken as the end-point. Choosing the same end-point throughout, it ought to be possible to make a comparison of the effects of temperature and humidity on spore germination.

Agar slope cultures of *B. anthracis* were stored over granulated anhydrous calcium chloride in a partially evacuated desiccator for 21 days at 37° C. when stained preparations indicated that all the bacterial forms present were spores.

Subcultures of the same five strains of *B. anthracis* which were used in the previous experiment, were prepared in this way. Suspensions were made in peptone water and, using opacity tubes, standardized to equal strengths. An inoculum of 0·5 ml. of each was spread evenly over the surface of a nutrient agar plate which was then incubated. Each agar plate had been prepared so that the surface was as free as possible from irregularities and was well dried before use. All plates had been pre-incubated at the test temperature. An electric incubator with a thermometer reading the temperature of the air in the interior, and not of the jacket, was used; the temperature was adjusted to cover the range required. At timed intervals the surface of the medium was sampled and examined for the presence of vegetative organisms using an adaptation of the technique described by Bisset (1938). Small squares of the medium were removed with a scalpel and forceps, a cover-slip was pressed firmly on top and the whole immersed in Bouin's fixative for about 2 hr. The medium was then carefully peeled away from the cover-slip which was then washed in changes of water for some hours. The attached growth was stained by Dorner's nigrosin method and then inverted and mounted on a slide. A microscopical examination of twenty fields was made under a 2 mm. oil-immersion objective, not less than 200 cells being counted.

The arbitrary end-point chosen was when the vegetative cells comprised at least 10 % of the total. A fine film of growth was usually visible to the naked eye when this end-point had been reached. Culture plates were out of the incubator for as short a period as possible during manipulation.

That the cultures incubated at 18 and 46° C. were viable was proved by incubation of the plates at 37° C. after the conclusion of the experimental period. Normal growth then resulted in both cases.

Table 3

'V' represents the appearance of 10 % vegetative forms in that particular culture

Temperature (° C.)	Culture	Hours 1	2	3	4	6	8	10	12	16	18	24
46	1	.	.	.	.	.	.	.	.	.		
	2	.	.	.	.	.	.	.	.	.		
	3	.	.	.	.	.	.	.	.	.		
	4	.	.	.	.	.	.	.	.	.		
	5	.	.	.	.	.	.	.	.	.		
44	1	.	.	.	.	.	.	.	V	V		
	2	.	.	.	.	.	.	.	.	V		
	3	.	.	.	.	.	.	.	.	V		
	4	.	.	.	.	.	.	.	.	V		
	5	.	.	.	.	.	.	.	.	V		
42	1	.	.	V	V	V						
	2	.	.	.	.	V						
	3	.	.	.	.	V						
	4	.	.	.	.	V						
	5	.	.	.	.	V						
39	1	.	V	V								
	2	.	V	V								
	3	.	.	V								
	4	.	V	V								
	5	.	V	V								
37	1	.	V	V	V	V						
	2	.	.	V	V	V						
	3	.	.	.	.	V						
	4	.	.	.	V	V						
	5	.	.	V	V	V						
30	1	.	.	.	V	V	V					
	2	.	.	.	V	V	V					
	3	.	.	.	.	.	V					
	4	.	.	.	.	V	V					
	5	.	.	.	.	V	V					
25	1	.	.	.	.	.	.	V	V			
	2	.	.	.	.	.	.	V	V			
	3	.	.	.	.	.	.	.	V			
	4	.	.	.	.	.	.	.	V			
	5	.	.	.	.	.	.	V	V			
20	1	.	.	.	.	.	.	.	.	V		
	2	.	.	.	.	.	.	.	.	V		
	3	.	.	.	.	.	.	.	.	V		
	4	.	.	.	.	.	.	.	.	V		
	5	.	.	.	.	.	.	.	.	V		
18	1	.	.	.	.	.	.	.	.	.		
	2	.	.	.	.	.	.	.	.	.		
	3	.	.	.	.	.	.	.	.	.		
	4	.	.	.	.	.	.	.	.	.		
	5	.	.	.	.	.	.	.	.	.		

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Results

The results are set out in Table 3. They suggest that there is a temperature span of from 20 to 44° C. which permits the spores of *B. anthracis* to germinate. For the shortest sporing time 39° C. appeared to be the most favourable for the strains tested. Above 39 and below 30° C. sporing time lengthened considerably.

Influence of humidity on spore germination

As the germination process is usually followed in aqueous solution and the system rapidly attains equilibrium with the liquid phase, Cook (1932) points out that little is known about the effect of water on the mechanism.

I can find no reference in the literature directly bearing on any experimental work on the action of humidity on spore germination in bacteria though there are a few dealing with fungi.

Materials and methods

As in the previous experiment two temperatures were chosen, 26 and 37° C., and the humidity was varied at these two temperatures.

The constant humidity jars ranging in steps of relative humidity (R.H.) of 10 %, over a range of 20–100 %, were employed.

Spore suspensions of the same previously used five anthrax strains were employed on a nutrient agar medium on slides. The medium had been evaporated to dryness and then allowed to reach equilibrium with the controlled humidity in the jars in the usual manner. Slides were removed at intervals, sampled and discarded as in the previous experiments.

Bisset's technique is not suitable for the thin layer of agar essential in a controlled humidity experiment, so smears prepared from the surface growth, heat-fixed and stained by Dorner's nigrosin method, were made. The appearance of 10 % of vegetative cells in twenty fields examined under the oil-immersion objective was again taken as the end-point.

Results

At a temperature of 26° C. and a R.H. of 100 % all five strains germinated within 12 hr. With R.H. at 90 % all five strains germinated within 18 hr., but at a value of 80 % and below no germination could be detected. At a temperature of 37° C. and a R.H. of 100 % all five strains germinated within 6 hr. With the R.H. at 90 % all five strains germinated within 10 hr., but at a value of 80 % and below no germination could be detected.

DISCUSSION

The importance of the composition of the medium in all experiments with spores is well known. Nutrient agar based on ox-heart infusion broth, as used in these experiments, contains the essential elements for sporulation and spore germination, and is sufficiently constant in composition to eliminate gross nutritional varia-

tions that might serve to complicate the interpretation of the results of these experiments.

There is no apparent reason to believe that the relative humidities set up were not those actually operating in the cultures under study. The initial thorough evaporation of the medium and the period allowed for it to reach equilibrium with the atmosphere in the constant humidity jars should have ensured this. It is admitted that these jars had to be opened to insert slides and to remove them for examination during the experiments, but this was done as rapidly as possible. The introduction of a small amount of liquid in the form of the inoculum in some of the experiments was also considered as a possible source of error. However, the small volume used, the large controlling volume of the acid and water mixture, and the length of time the experiments required for completion all probably rendered this unimportant.

It is conceded that there may be limitations of accuracy associated with the use of staining methods for the demonstration of spore formation and germination, though such methods have been used by many reputable workers in the past (see Brunstetter & Magoon (1932), Tarr (1932), and Kaplan & Williams (1941)). In addition, Knaysi (1952) states that staining methods, though dependent on technique and judgement, are best for revealing changes in bacterial structures.

It was found possible to recognize similar end-points in each series of experiments and hence, I believe, to evaluate fairly the effect of varying the particular physical factor chosen.

An alternative to staining would have been the use of heat resistance as an indication of the presence of spores. However, Magoon (1926) demonstrated that thermal resistance of spores was influenced by the humidity of the environment and was not a fixed property within any given bacterial species; hence a staining method might be preferable.

Continuous direct microscopical examination of living spores presented too difficult a technical problem for it to be considered feasible.

As a general conclusion, from the experiments performed it is considered that temperature and humidity effects may play a large part in explaining the persistence of anthrax infection in the soil of subtropical countries. Special points here are the rapid sporulation in warm soils and lack of spore germination in dry ones, both tending to help the organism to survive.

In Britain, the main infection of the soil takes place in the winter months, in the form of *B. anthracis* spores derived from imported fertilizers which are mainly used at this season. During the winter it is probable that the low soil temperature prevents spore germination and the infection tends to persist. With the advent of summer, however, soil temperature is sufficient for the spores to germinate, soil humidity usually being adequate. The vegetative forms resulting are eliminated by the action of other soil bacteria, and hence the infection tends to die out.

SUMMARY

Experiments performed on spore formation in *B. anthracis*, in a wide range of closed atmospheres of chemically controlled humidity, showed that the sporing time of *B. anthracis* is prolonged considerably as the humidity decreases, both at 26 and 37° C. The effect is less noticeable at the lower temperature.

Experiments performed on spore germination in the same organism showed that a temperature range of 20–44° C. permitted germination of the five strains tested. Above a temperature of 39° C. and below 30° C. germination time lengthened considerably.

Experiments performed on the influence of humidity on spore germination, both at 26 and 37° C. showed that no germination could take place at a relative humidity of 80 % or below. Germination time was considerably prolonged when the humidity value fell below 100 %.

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