PHYSIOLOGICAL STUDIES ON SPORE GERMINATION WITH SPECIAL REFERENCE TO CLOSTRIDIUM BOTULINUM[1]

I. Development of a Quantitative Method

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Received for publication September 29, 1947

By far the majority of studies hitherto made on germination of bacterial spores have employed the appearance of visible turbidity as the criterion of germination. Obviously this technique can reveal no quantitative characteristics of the germination process and is therefore of value only in establishing that some germination does or does not take place. Even here its value may be questioned, since it has been clearly demonstrated that germination of spores of various organisms may occur without significant subsequent vegetative proliferation (Itano and Neill, 1919; Knight and Fildes, 1930; Knaysi, 1945; Knaysi and Baker, 1947). Furthermore, various environmental conditions imposed upon germinating spores may have no influence on the germination time but yet may alter appreciably the rate of subsequent vegetative development (Evans and Curran, 1943). Our own experiments confirm this finding.

Direct microscopic counts have been used for quantitative studies of the germination of aerobic spores (Eckelmann, 1918; Curran, 1931), but such a procedure is unduly wearisome and not readily adapted to use with anaerobes. Also, with certain species, for example, *Bacillus anthracis*, it may be very difficult to establish microscopically a criterion of germination, as noted by Fischoeder (1909), Swann (1927), and Cook (1932).

The outstanding physiological difference between spores and vegetative cells of any one organism, namely, heat lability of the latter at a temperature innocuous to the former, has long been employed in quantitative approaches to spore germination, since it is assumed traditionally that when a spore cell is so changed that it becomes heat-labile, germination has taken place (Weil, 1901; Fischoeder, 1909; Evans and Curran, 1943). Although Curran and Evans (1937, 1945*b*) have indicated that the heat-labile state may actually precede rupture of the spore wall and that some morphological changes characteristic of germination may occur prior to the loss of thermal resistance of the spore, heat differentiation of the germinated vs. the ungerminated spore appears to be the most practicable approach. The fact that a definite reproducible standard end point may be selected, viz., survival at a definite temperature for a definite period of time, even though somewhat arbitrary, outweighs the overlapping

[1] This project has been undertaken in co-operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views of or being endorsed by the War Department.

between the physiological and morphological characters which renders the germination process an indistinct one.

Consideration of other possible criteria of germination has repeatedly brought us to the conclusion that changes in heat lability are best from every point of view, and they form the basis of this work. The technique has been designed especially for *Clostridium botulinum*, an important organism in food poisoning, on which apparently no quantitative germination studies of any sort have been made. The method has been applied, with appropriate modifications, to other anaerobic species.

EXPERIMENTAL

Some of the preliminary work was carried out with *C. botulinum* strain 115B; strain 62A was utilized in most of the work. Both strains were obtained from National Canner's Association and were repurified by isolation of colonies from serial shake tubes. Toxin formation was demonstrated for strain 62A by the fact that 1.0 ml of a Seitz filtrate of a 10-day broth culture was lethal for a guinea pig in less than 21 hours, whereas a control animal receiving 1 ml of the filtrate inactivated at 80 C for 15 minutes survived the observation period of 3 weeks.

Spore suspensions were prepared from 15-day cultures in Difco brain heart infusion broth with BBL thioglycolate supplement added. After four washings the cells were heated to 75 C for 30 minutes to destroy vegetative forms and then diluted in sterile distilled water containing glass beads. The final suspensions were shaken 1 hour on a rotary shaking machine to break up clumps and then stored in the refrigerator. The efficacy of the homogenation procedure was shown by repeated comparisons of counts by planting procedures and direct microscopic counts using the Petroff-Hauser chamber. The former averaged about 50 per cent of the latter, which may be considered fairly good correlation.

Other anaerobes used were *Clostridium chauvei*, *Clostridium histolyticum*, *Clostridium perfringens*, and the well-known food spoilage organism designated as putrefactive anaerobe no. 3679.

The dormancy problem. The phenomenon of dormancy or delayed germination has presented a formidable experimental difficulty, which doubtless has been largely responsible for the lack of any really quantitative studies on spore germination in *C. botulinum*. Thus various authors report germination occurring only after incubation periods ranging from 53 days to 5½ years (Burke, 1919, 1923; Starin, 1924; Weiss, 1921; Sommer, 1930; Dozier, 1924; Dickson *et al.*, 1922, 1925; Esty and Meyer, 1922; Dickson, 1928). Dormancy is not restricted to *C. botulinum* spores. It has been established for spores of other clostridia (McCoy and Hastings, 1928), for spores of aerobic species (Burke *et al.*, 1925; Magoon, 1926; Morrison and Rettger, 1930*a*, 1930*b*), and even for cells of *B. coli* (Burke *et al.*, 1925). Vegetative cells of *C. botulinum* have been observed to exhibit a degree of dormancy roughly comparable to that shown by the spores (Starin, 1924).

The prime requisite for systematic quantitative germination studies on "bot" spores is the complete elimination of the dormancy that has handicapped virtu-

ally all previous studies with this organism in this connection. Acting on the belief that cultural environment probably conditions dormancy, we felt that the germination medium offered the best prospects for our objective. This had been shown to be the case for aerobic sporeformers in which dormancy could be eliminated by supplying the correct medium (Morrison and Rettger, 1930*a*, 1930*b*; Curran and Evans, 1937). In recent years improvements have been made in media, which now give much higher counts than were possible with the media formerly used, a fact which indicates a high degree of success in eliminating the extremely long incubation period during which the spore count as measured by colony development would continue to increase.

Pork infusion thioglycolate medium[2] has been found to give considerably higher counts on a given spore suspension than do other media ordinarily used for such studies, but no indication is available that maximum counts are obtained in short enough time to be a useful tool for routine studies (Williams and Reed, 1942). Even this medium could be enhanced remarkably (30-fold) in the total count of heated spores obtainable after 23 days by the addition of 0.1 per cent soluble starch directly to the germination medium (Olsen and Scott, 1946).

Our work confirms both the superiority of pork infusion as a germination medium and the striking effect of starch on the germination process, and consequently on the spore counts. We have further demonstrated that starch acts primarily to adsorb and thus render inactive small amounts of substances, present in all media, that repress spore germination. This study is the subject of a separate paper. Finally, the spore-counting procedure ultimately evolved seemingly has eliminated dormancy as a practical obstacle in quantitative germinative studies on "bot" spores and, furthermore, for the first time enables maximum counts to be obtained in an incubation period no longer than that required for sizable colony development of any anaerobe, namely, 3 days.

Comparison of several media popularly employed for counting C. botulinum spores. One spore suspension heated to 75 C for 30 minutes to destroy vegetative cells was serially diluted in triplicate in the various agar media in flat Prickett counting tubes and incubated at 37 C. An effective anaerobic seal was obtained by covering the solidified agar with 3 to 4 ml of 2 per cent agar containing BBL thioglycolate supplement. Prickett tubes are essentially flattened test tubes and, though not generally used, have the decided advantage of permitting colony counts to be made in a thin layer of agar instead of in the entire diameter of a test tube. If maxmum spore counts obtained in the pork thioglycolate starch

[2] The following procedure for preparation of this medium was kindly furnished by Dr. J. Yesair of National Canner's Association:

One pound of finely chopped lean pork is added to a liter of distilled water and boiled 1 hour. After removal of the meat and fat the filtrate is adjusted to pH 7.4 and to each liter are added 5 g of peptone, 1.6 g of tryptone, 1 g of glucose, 1.25 g of K_2HPO_4, and 15 g of agar.

It is our practice to omit the glucose and add 5 g of BBL thioglycolate supplement and 1 g of soluble starch per liter. We also adjust the final medium to pH 7.4. The troublesome precipitate resulting on boiling the final medium may be removed by filtration under negative pressure or discarded after decanting the supernatant.

medium are represented as 100, pork medium without starch gave 75, Difco brain heart infusion 55 to 60, brain heart plus 25 per cent peptone (Bristol, 1925) 45, BBL anaerobic agar in Brewer anaerobic dishes as employed by Curran and Evans (1946) 28, Difco liver veal 20, and Wilson and Blair's (1925) agar 5. Not only were counts consistently maximum in pork medium with thioglycolate starch supplement, but they occurred much earlier, reaching the peak in 3 days' incubation as compared to about 3 weeks for brain heart infusion agar. Actually counts could be made on the second day, but the colonies at this point are really too small to count easily or accurately. Three-day-old colonies provide no difficulties. If dormancy exists at all in the pork starch medium, it is believed to be a negligible interference in quantitative studies. Counts, i.e., colonies originating from germinating spores of *C. botulinum*, have never been observed to increase appreciably on prolonged incubation up to 2 to 3 weeks, whereas with other media the results are meaningless before that time. Counts of spore suspensions have regularly been around 50+ per cent of direct microscopic counts (Petroff-Hauser chamber), a not too unsatisfactory correlation considering the tendency of the spores to clump in various degrees and the fact that viability doubtless is not 100 per cent. A special experiment to detect dormant spores (i.e., ungerminated viable spores) in this medium after 3 days' incubation failed to reveal any that germinated up to the time of writing, a period of 4½ months. Typical dormancy under these conditions manifests itself as gradually increasing counts over the entire incubation period.

Detailed procedure for studying germination of C. botulinum spores. The following procedures typify our approach to the quantitative study of the germination process and of factors influencing it. One-ml portions of the appropriately diluted spore suspension were transferred to tubes containing 9 ml of Difco brain heart infusion broth with BBL thioglycolate supplement. This particular germination medium was chosen because the relatively moderate rate of germination occurring in it allows the study of factors both stimulatory and inhibitory to germination. The tubes were then heated to 75 C for 20 minutes to expel dissolved oxygen and to effect any possible "heat activation" of the spores (Evans and Curran, 1943; Curran and Evans, 1945*a*). After appropriate intervals of incubation at 37 C in air or other atmosphere, replicate (usually triplicate) tubes were reheated to 75 C for 20 minutes to destroy any vegetative cells that had developed as a result of germination. Residual spore counts were made as above in pork thioglycolate starch agar. Available data indicate that germination is somewhat faster at 30 C than at 37 C, but for convenience 37 C was used in all these germination studies.

Expression of results. Most workers have utilized absolute numbers of residual spores as a basis for interpreting the effect of a particular treatment on the germination process. We feel that *percentage* of germination is to be preferred as a more reliable basis for interpretation of results, because of the large populations employed, and especially is this true when germination is largely complete. For example, on the comparative basis of residual, relatively small spore counts, Evans and Curran (1943) concluded that a considerable accelera-

tion of germination of aerobic spores had resulted from preheating the spores in glucose broth. If, however, the residual spores are considered as a fraction of a large population and calculated as percentage of that population, the stimulatory effect for 4 out of the 7 positive cases would be less than 6 per cent and in one instance less than 0.1 per cent. Certainly the magnitude of the effect is much different when expressed percentagewise, the only valid way in our estimation. A simple numerical example of this point seems worth while. Suppose a germination test is run under two treatments on a spore suspension containing 5,000 spores per ml and the residual spore count shows 100 and 200 per ml, respectively. While the 100 per cent difference between the residual spore counts seems striking, the values for *germinated* spores are the design of the experiment and the more important data. These would be 4,900 and 4,800, respectively, or 98 and 96 per cent germination, an insignificant difference in work of this nature.

Application to germination under stimulatory and under inhibitory treatments. Though germination curves may be employed for determining the effect of a given factor throughout the time course of germination, the effect taken at any one significant incubation time is usually sufficient.

TABLE 1

Effect of starch on germination

STARCH	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION (%)
	hours			
	0	575		
	24	400	175	30
0.1%	24	60	515	90

If a stimulatory factor is being studied, the time selected should be such that germination is relatively small in the control in order to allow the treatment to manifest itself to the maximum. An example is the effect of 0.1 per cent soluble starch in the germination medium (brain heart broth) shown in table 1. The spore-counting medium was the usual pork thioglycolate starch agar.

On the other hand, an inhibitory effect is best demonstrated at an incubation time when germination is nearly maximum in the controls. Table 2 demonstrates that germination in brain heart broth is considerably retarded by momentary contact with air during the removal of sample tubes for counting from a desiccator made anaerobic with an inert gas phase (natural gas, CH_4), even though re-exhaustion with a Hyvac pump and replacement with inert gas is done without delay. In the unopened desiccator 87 per cent of the spores germinated, whereas in the desiccator opened briefly at 20 and 24 hours only 29 per cent germination was obtained—a striking inhibition.

Accuracy and reproducibility of spore counts. The degree of accuracy obtainable with this method depends, of course, on the number of replicates used for determining the "average" counts. For zero controls triplicate tubes were generally used, with triplicate dilutions plated for each tube, or a total of nine

counts. For other averages triplicate tubes with duplicate or triplicate platings of dilutions were usually employed. The over-all reproducibility and accuracy of counts on a *C. botulinum* spore suspension stored in the refrigerator are illustrated in table 3.

Agreement between replicate counting tubes seems to depend on several factors, including scrupulous chemical cleanliness of glassware, the presence of

TABLE 2

Effect of momentary contact with air on germination in natural gas

	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION (%)
	hours			
Desiccator unopened	0	560		
	28	74	485	87
Desiccator opened twice to remove...	0	535		
samples at 20 and 24 hours	28	380	155	29

TABLE 3

Reproducibility of spore counts on a single suspension

DATE OF COUNT	SPORES PER ML
3/10	535
3/15	560
3/27	540
4/2	575
4/9	530
4/20	560
4/20	520
4/24	600
4/27	500
4/30	535
5/22	540
6/1	580
Average	548

Extreme deviation from mean

$$\frac{50}{548} = 9\%$$

soluble starch in the counting medium, and the atmosphere in which germination takes place.

Factors conducive to variability. Considerable evidence has been accumulated that germination of "bot" spores is extremely susceptible to minute amounts of substances in the general category of impurities. As mentioned earlier, these occur in all organic media and possibly in tap water. At any rate, a high order of variation was experienced between replicate tube counts of a given dilution of the suspension until a rigorous cleaning procedure was adopted. A marked re-

duction in count variation followed when the cleaning was done with "dreft," followed by thorough rinsing with distilled water. However, the best means of minimizing this tube to tube variation proved to be the additional feature of incorporation of the starch. The adsorption effect mentioned above explains this leveling action of the starch.

Finally, even though the cultivation of the germination tubes in an atmosphere of ordinary air gives good growth, use of an inert atmosphere of natural gas further reduced appreciably the count variation in replicate tubes.

These procedures work equally well with the four other anaerobic sporeformers tested.

SUMMARY

A simple, reasonably accurate method for quantitative study of spore germination in *Clostridium botulinum* and other anaerobes is described. The obstacle of dormancy has been eliminated, maximum counts appearing in 3 days. Illustrations are given of the application of the method.

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