STUDIES ON FACTORS AFFECTING THE RAPID GERMINATION OF SPORES OF *CLOSTRIDIUM BOTULINUM*

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The bacterial endospore has been studied in great detail with respect to its formation, resistance to physical and chemical agents, and germination. However, the treatments of germination have been largely limited to the aerobic, spore-forming species. It was, therefore, the purpose of these studies to investigate the breaking of dormancy, or germination, of spores of an anaerobic organism.

The various phases in the study of spore germination have been reviewed by Schmidt (1955), Stedman (1956), Halvorson and Church (1957*a*, *b*), and Powell (1957*a*). Changes in the heat lability, refractive index, and staining properties of the spore have been used by many investigators as criteria for germination. There seems to be some controversy concerning the point when a spore may be considered as having germinated. Halvorson and Church (1957*a*) have quoted the definition of germination proposed by Campbell, who stated that spore germination is "... the change from a heat resistant spore to a heat labile entity which may not necessarily be a true vegetative cell." Powell (1957*b*) feels that the change in phase contrast appearance should be used as the criterion of germination. On the other hand, Knaysi (1957) has suggested that "complete transformation of the spore into a free germ cell" must be used in studies of the physiology of germination.

Using the change in heat lability as the criterion for germination, Wynne and Foster (1948*a*, *b*) found that carbon dioxide was absolutely necessary for germination of *Clostridium botulinum*. Yeast extract was able to replace this requirement in a synthetic medium, whereas oxalacetate could replace the carbon dioxide only in brain heart infusion. Recently, Hitzman *et al.* (1957) have reported the germination of *Clostridium roseum* in a medium containing only the amino acids, alanine, phenylalanine, and arginine. Wynne *et al.* (1954) have reported germination of *C. botulinum* in a phosphate buffered glucose medium.

MATERIALS AND METHODS

The organism. The culture of *C. botulinum* was originally received from the Western Regional Research Laboratory (U. S. Department of Agriculture, Albany, California) as strain 62A. Biochemical and toxicity tests have confirmed this to be a strain of *C. botulinum* producing type A toxin.

Spore production. The spores were produced on casitone-peptone agar (Sugiyama, 1951) in 6 oz prescription bottles. These were incubated in a 10 per cent carbon dioxide-methane (natural gas) atmosphere for 5 days at 37 C and opened to the air for another 2 days incubation. The confluent growth was harvested from the slants with distilled water and washed once by centrifugation. The suspension was then centrifuged at 150 × G for 10 sec to remove any agar present. After washing again, the suspension was placed in the refrigerator and the spores allowed to sediment for 4 to 10 days. The spore sediment was then washed twice and the final concentration determined by Petroff-Hausser counts and optical density measurements.

Criteria for germination. The spores were stained to determine if germination had occurred by mixing a loopful of the suspension with a loopful of methylene blue on a slide and allowing this to dry. The smear was heat fixed, the excess stain washed off, and a 5 per cent aqueous solution of mercurochrome 220 added. The spore films were also stained with 1:1000 crystal violet solution for 1 hr. Change in heat resistance was determined by diluting the spore suspension in a nutrient medium and heating the final dilution for 20 min at 75 C. This dilution was immediately cooled for 10 min in an ice bath and allowed to come to room temperature before inoculation. Petri dishes, previously containing 0.3 ml of a solution of 5 per cent sodium bicarbonate and 5 per cent dipotassium phosphate, were inoculated with the suspension and the inoculum mixed with 15 ml of heart infusion agar (Difco). The

plates were incubated in Brewer jars (BBL) in the carbon dioxide-line gas atmosphere for 5 days at 30 C before the colonies were counted. Change in per cent transmittance was determined on a Coleman Junior spectrophotometer, model 6B, set at 610 mμ.

The germination studies were carried out in 16 by 125 mm Thunberg tubes (Ace Glass Inc., Vineland, New Jersey). The germination medium was added to the tube to make 5 or 10 ml final volume while any inhibitors or heat labile substances were added to the side arm. The spore inoculum may be added to either the tube or side arm, depending on the treatment desired. A sufficient number of spores was added to give a final concentration of 1 to 3 × 10^8 spores per ml. The side arm was lightly lubricated with Dow Corning stopcock grease and the parts fitted together.

TABLE 1

Germination of Clostridium botulinum spores in various media

Medium	Spores Recovered after 4 hr Incubation	Germination
	× 10^6	%
Control*	100.0	
YSI,† 0.1% $NaHCO_3$; pH 7.4	2.4	97
YSI, 0.1% $NaHCO_3$-0.1% K_2HPO_4	1.0	99
YSI, 0.1% $NaHCO_3$; pH 6.3	1.2	99
YSI, 0.1% $NaHCO_3$; pH 7.9	5.0	95
HIB,‡ 0.1% $NaHCO_3$-0.1% K_2HPO_4; pH 7.5	17.0	83
HIB, 1% yeast extract, 0.1% $NaHCO_3$; pH 7.4	4.0	96
2% Glucose in 0.066 M phosphate buffer; pH 6.7	64.0	36
0.066 M Phosphate buffer; pH 7.2	66.0	34
Control*	120.0	
HIB, 1% yeast extract +0.1% $NaHCO_3$-0.1% K_2HPO_4	15.0	87
+0.1% K_2HPO_4	64.0	46
+0.1% $NaHCO_3$	17.0	86

* Spores per ml as determined by Petroff-Hausser counts.

† Heart infusion broth, 2.5 per cent; yeast extract (Difco), 1 per cent; soluble starch (Merck), 0.1 per cent.

‡ Heart infusion broth (Difco).

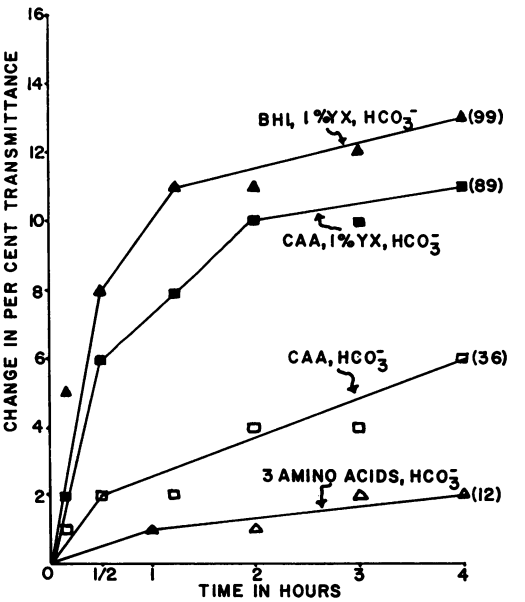

Figure 1. Change in per cent transmittance of a thick suspension (1.4 × 10^8 spores per ml) of spores of *Clostridium botulinum* due to germination in various media. Media and supplements used were: 2.5 per cent brain heart infusion (BHI), 1.0 per cent yeast extract (YX), 0.1 per cent $NaHCO_3$, 1.2 per cent vitamin-free casamino acids (CAA), and three amino acids (1.44 mg per ml of L-alanine, 0.288 mg per ml of L-arginine, and 0.288 mg per ml of L-phenylalanine). All media were adjusted to pH 7.2 before addition of $NaHCO_3$. The numbers in parentheses indicate the per cent germination determined by viable counts.

The tubes were heated in a water bath at 75 C for 15 to 20 min depending on the amount of medium used. The germination tubes were evacuated with a line vacuum, closed and placed in a 30 C water bath to equilibrate. They were then mixed and their initial per cent transmittance read. An uninoculated medium was used as the blank and set at 100 per cent transmittance. Readings were usually taken 5, 10, 20, and 30 min after mixing, and then hourly. The tubes were incubated at 30 C. At any period of incubation, the tube could be opened, sampled, and reevacuated without apparently harming the germination process.

Media and supplements. The vitamin-free casamino acid medium (Difco) is referred to by the letters CAA. The vitamins used are those reported as being present in yeast extract (*per-*

sonal communication from Difco Laboratories). The amino acids used are those reported by Hitzman *et al.* (1957) in the concentrations he recommends. All salts were of reagent grade. The sodium bicarbonate was always added after any heating and cooling of the germination medium.

Inhibitors and antibiotics. The cyanide was used either as sodium nitroprusside or potassium cyanide. Sodium azide, monoiodoacetate, 2,4-dinitrophenol, sodium fluoride, subtilin (Chas. Pfizer), penicillin G (NBC) and streptomycin (Chas. Pfizer) were also used.

RESULTS

Some of the early studies on germination media are summarized in table 1. Yeast extract and sodium bicarbonate were found to be necessary for rapid germination, whereas soluble starch and dipotassium phosphate could be omitted without any noticeable effect. A glucose-phosphate medium was unable to support rapid germination under the conditions used.

The necessity of yeast extract is again demonstrated in figure 1. It can also be seen that germination in the amino acids recommended by Hitzman *et al.* (1957) for *C. roseum* was not obtained with *C. botulinum*. Attempts to replace

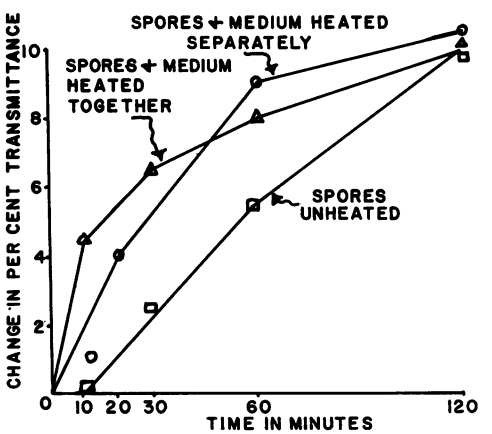

Figure 2. The effect of heat activation (75 C for 20 min) on the change in per cent transmittance fo a thick suspension (3×10^8 spores per ml) of *Clostridium botulinum* spores in 5 per cent yeast extract (pH 6.8). The media were supplemented with 0.1 per cent $NaHCO_3$ after heating. The spores were heated separately in distilled water where indicated. At the end of 2 hr, all tubes showed 99 per cent germination by viable counts.

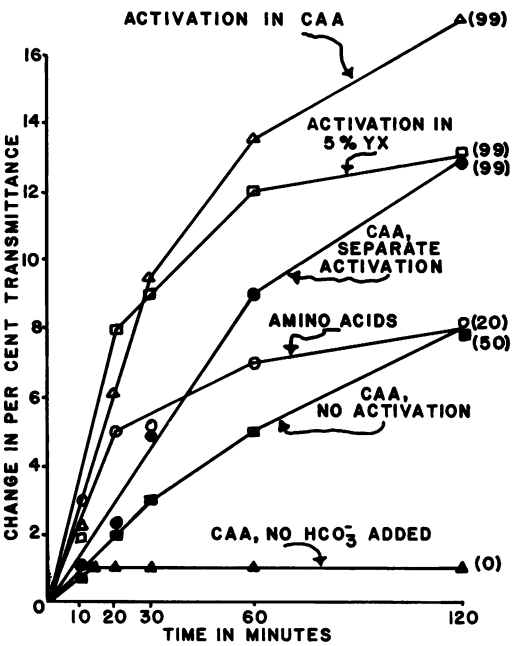

Figure 3. Change in per cent transmittance of a thick suspension (3×10^8 spores per ml) of spores of *Clostridium botulinum* due to germination in 3 per cent vitamin free casamino acids (CAA) plus 0.1 per cent cysteine, 5 per cent yeast extract (YX) or amino acids equivalent to 2.3 per cent CAA. Heat activation was at 75 C for 15 min. Numbers in parentheses indicate the per cent of the spores stained with 1:1000 crystal violet. The pH of the CAA medium before addition of 0.1 per cent $NaHCO_3$ was 5.4. In those cases where the $NaHCO_3$ was not added, the reaction was adjusted to pH 6.8 with NaOH.

the yeast extract with the vitamins known to be present in this substance were not successful. Although L-alanine, calcium, manganese, magnesium, adenosine, and glucose would all stimulate germination in the presence of 1 per cent yeast extract, they would not replace it in the casamino acid medium. During these studies it was found that 5 per cent yeast extract would support rapid germination and complete outgrowth. The effect of heat activation of the spores in this medium is shown in figure 2. It can be seen that the rate of change in per cent transmittance depended upon heating the spores and the medium in which they were heated. Complete germination was obtained in the heart infusion-yeast extract-soluble starch medium

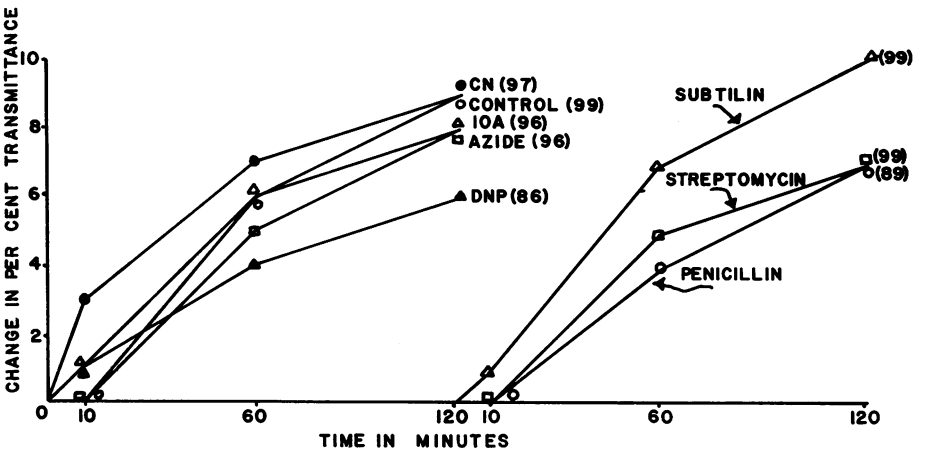

Figure 4. The effect of some enzyme inhibitors and antibiotics on the change in per cent transmittance of a thick suspension (2.8×10^8 spores per ml) of spores of *Clostridium botulinum* in 5 per cent yeast extract. The compounds were used in the following concentrations: 1.6×10^{-4} M CN (as sodium nitroferricyanide), 2.4×10^{-4} M monoiodoacetate, 5.4×10^{-4} M dinitrophenol, 2×10^{-3} M sodium azide, 100 units per ml of penicillin, 20 ppm subtilin, and 0.8 mg per ml of streptomycin. The numbers in parentheses indicate the per cent germination by viable counts.

between pH 6.3 and 7.9. At pH 4.0 or below, no discernible germination occurred as reported by Wynne *et al.* (1954). Change in refractility seemed to be more rapid in the casamino acid-1 per cent yeast extract medium if the spores were incubated at 30 C after heat activation rather than 40 C or 50 C. All subsequent studies were carried out at 30 C.

Later studies indicated that vitamin-free casamino acids supplemented with 0.1 per cent cysteine hydrochloride supported rapid germination if the reaction of the medium before addition of the sodium bicarbonate was near 5.4. The sodium bicarbonate raised the pH to *ca.* 6.8. Figure 3 describes the rates of germination in this casamino acid medium due to heat activation, as compared with 5 per cent yeast extract and an amino acid mixture. When the spores were activated in the casamino acid medium from which the cysteine was omitted, the rate of germination was similar to that when the spores and medium were heated separately. When the spores were activated in the casamino acid medium in which the cysteine was replaced by sodium thioglycolate at the same molar concentration of sulfur, the rate of germination was similar to that when the spores were not heat activated. If the pH of the casamino acid medium was adjusted to 6.8 with sodium hydroxide and no sodium bicarbonate was added after heat activation, germination was not detected.

It was noted that throughout these studies, 50 to 90 per cent of the spores heated in 5 per cent yeast extract were able to take up crystal violet immediately. Similar activation in 1 per cent yeast extract or the casamino acid medium did not produce this change in staining properties. At the same time there was, at the most, a 15 per cent difference in the viable spore counts between the spores activated in 5 per cent or 1 per cent yeast extract.

Preliminary experiments on the effect of inhibitors and antibiotics on germination indicated that rapid germination took place in the presence of 2,4-dinitrophenol, cyanide, sodium azide, sodium fluoride, iodoacetate, subtilin, streptomycin, and penicillin (figure 4). However, all of these compounds inhibited further outgrowth of the germinated cell with the exception of sodium azide and sodium fluoride. The latter two compounds inhibited elongation or cell division. Although no compound completely inhibited germination, dinitrophenol and penicillin seemed to reduce its rate. On the other hand, cyanide seemed to actually increase the rate of change in per cent transmittance. The experiments shown in figures 5 and 6 confirm these results. Different concentrations of the same compound did not seem to alter the effect of the compound upon

the rate of germination. It is interesting to note that the effect of subtilin was altered when the germination medium was casamino acids rather than yeast extract.

The stages of germination and outgrowth of the spores of *C. botulinum* are shown in figure 7. Throughout these studies no gross change in the size of the spore due to swelling was noticed. *A* and *B* of figure 7 are intended to show the similarity in size of ungerminated and germinated spores. In the presence of all of the inhibitors studied (with the exception of sodium azide and sodium fluoride) germination stopped at stage "a." Azide and fluoride prevented stages, "c" or "d." Germination in the casamino acid medium stopped at stage "a." Iodoacetate, penicillin, and streptomycin seemed to cause a swelling of the spores.

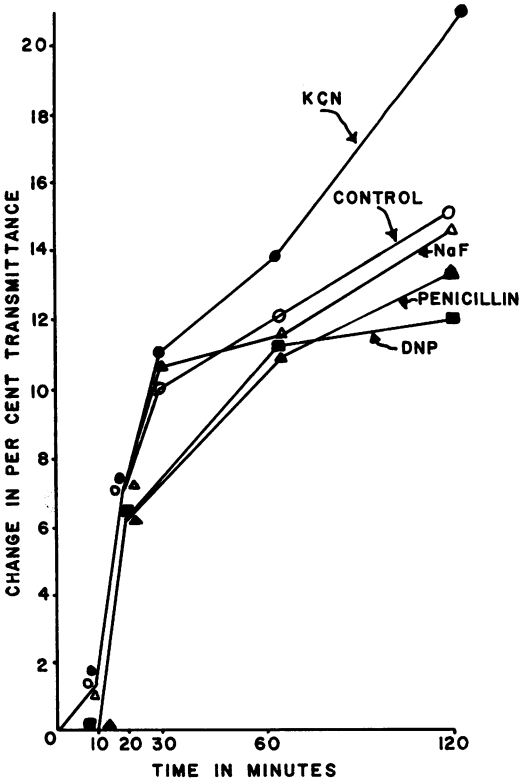

Figure 5. The effect of varying concentrations of KCN, DNP, NaF, and penicillin on the change in per cent transmittance of a thick suspension (3×10^8 spores per ml) of *Clostridium botulinum* spores in 5 per cent yeast extract. The concentrations of compounds used were: 2, 4, and 40×10^{-2} M KCN; 2 and 20×10^{-3} M NaF; 1, 2, and 20×10^{-3} M DNP; and 50, 250, and 500 units of penicillin per ml. The curve for each compound is the average of the separate determinations since there appeared to be no significant difference due to increasing concentrations.

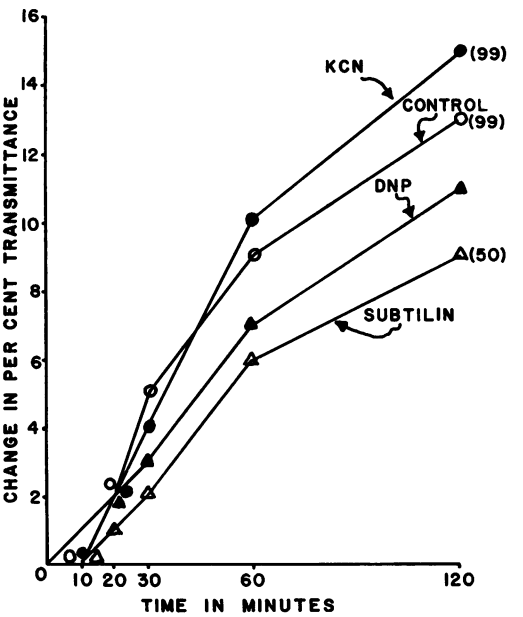

Figure 6. The effect of cyanide, dinitrophenol, and subtilin on the change in per cent transmittance of a thick suspension (3×10^8 spores per ml) of spores of *Clostridium botulinum* in 3 per cent vitamin-free casamino acids. The compounds were used in the following concentrations: 4×10^{-3} M KCN, 1×10^{-4} M DNP, and 100 ppm subtilin.

DISCUSSION

These studies have further confirmed the necessity of sodium bicarbonate and the enhancing effect of heat activation for germination of the spores of *C. botulinum*. Both of these effects have been reported by other authors for *C. botulinum* (Wynne and Foster, 1948*a*, *b*; Wynne *et al.*, 1955; Andersen, 1951). Since soluble starch has been reported necessary for the recovery of drastically heated spores (Olsen and Scott, 1950), it is understandable that it was not found necessary in these studies. The pH requirement before addition of the sodium bicarbonate to the casamino acid medium is interesting. This discovery might explain our earlier negative results with some of the vitamins, amino acids, or glucose.

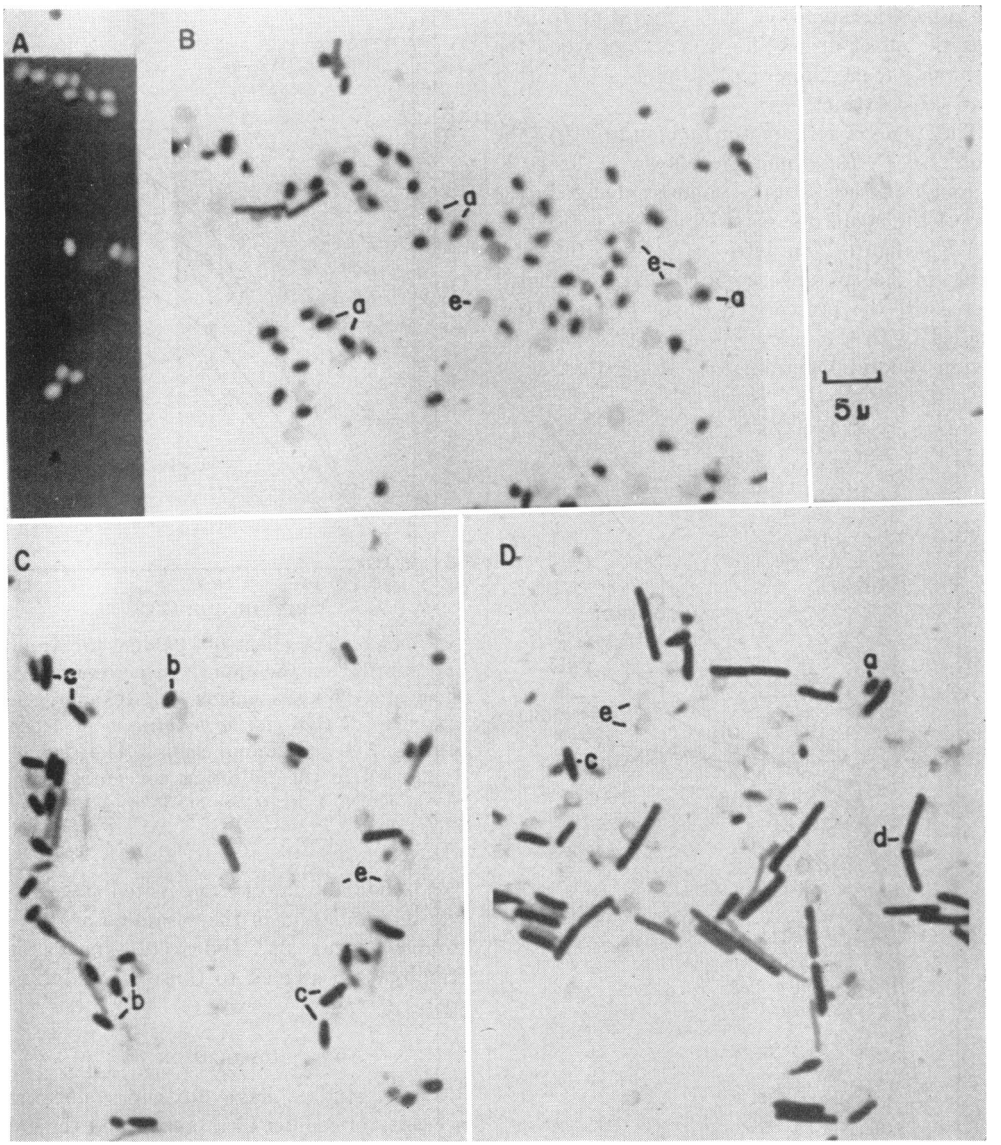

Figure 7. Morphology of germination and outgrowth of spores of *Clostridium botulinum. A.* Negative nigrosin stain of ungerminated spores. The spore wall has taken up the stain leaving the interior clear. *B.* Crystal violet stain of germinated spores after 2 hr incubation in 5 per cent yeast extract. *C.* and *D.* Same treatment as *B* after 3 and 4 hr incubation, respectively.

Stages of germination: *a*, germinated spores having been stained but the vegetative cell remaining within the spore case; *b*, vegetative cell released from the spore case; *c*, elongation of the vegetative cell; *d*, cell division; and *e*, free spore case.

The inclusion of cysteine in the casamino acid medium was not essential but did enhance the rate of germination. Sodium thioglycolate, on the other hand, appeared actually to be inhibitory for germination.

Our results have indicated that heat activation is effective in the casamino acid medium with no discernible change in the staining reaction. Heat activation in 5 per cent yeast extract did cause a change in staining properties without immediately affecting heat lability or refractility of the spores. None of the inhibitors studied sub-

stantially affected the first steps of germination, although dinitrophenol and penicillin seemed to have some effect on the rate of change in refractility. The effect of cyanide is interesting. Cyanide has been reported to reverse the inhibitory effect of metal ions on germination (Murty and Halvorson, 1957). If this were the effect here, one would expect phosphate and thioglycolate to be effective also, as these authors reported. This has not been our experience. Likewise, azide and fluoride did not show this enhancing effect. Cyanide has also been reported to activate certain proteinases (Irving *et al.*, 1942). Stedman (1956) has summarized the possibilities of a heat activated proteinase being implicated in spore germination. It is felt that the altered rates of change in per cent transmittance in the presence of cyanide, subtilin, dinitrophenol, or penicillin can only be explained, at this time, by a binding of these compounds with metals, spore wall material, or exudate. Apparently the effect of these compounds was not altered by changes in their concentration, which would support this hypothesis.

It seems apparent that heating produces a change in the spore itself rather than simply affecting the suspending medium. The first discernible change due to germination appears to be an uptake of stain or a change in the permeability of the spore coat. Franklin and Bradley (1957) have described the swelling of spores due to heat activation in distilled water and feel that this causes the change in permeability. We have found that the effectiveness of heat activation is dependent upon the menstruum in which the spores are suspended. The same conclusions were reached by Curran and Evans (1945). These results would suggest that heat activation is more than a physical process. The change in refractility and the increase in heat lability could be explained by the leakage of spore materials into the surrounding medium. This hypothesis is supported by the reports of loss of dry weight and appearance of amino acids, peptides, and other compounds in the germination medium (Powell, 1957*a*). Electron micrographs have also shown the disappearance of material between the spore coat and the vegetative cell wall (Mayall and Robinow, 1957).

The change in permeability of the spore does not appear to be affected by the inhibitors or antibiotics tested. This may be due to the inaccessibility of these compounds to the sensitive areas or the absence of a reaction involving an exogenous source of energy during the initial steps of germination. Immediately upon germination, these same compounds inhibit further development of the sensitive vegetative cell still present within the spore case. Subsequent changes after germination follow the steps described by Schmidt (1955) and Stedman (1956).

SUMMARY

The germination of spores of *Clostridium botulinum* has been studied using changes in heat lability, refractive index, and staining properties as criteria for germination. Complete germination has been obtained within 2 hr in 5 per cent yeast extract, with outgrowth and multiplication in 4 hr. Complete germination has also been obtained in vitamin-free casamino acids. The necessity of sodium bicarbonate and the effect of heat activation on germination of this organism has again been demonstrated. A number of enzyme inhibitors and antibiotics have been shown not to be effective in the initial steps of germination. The implications of these results have been discussed. It is suggested that the first step in germination is a change in the permeability of the spore coat, possibly mediated by a heat activated proteinase. Leakage of spore material then causes the change in refractive index and heat lability.

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