OPTICAL DENSITY OF THE ENDOSPORE OF *BACILLUS CEREUS* AND ITS RELATION TO GERMINATION AND RESISTANCE

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In recent years the bacterial endospore has become a popular object for investigation, and much has been learned about its structure, its chemical composition, the cytological processes and physiological factors involved in its formation and germination, and its resistance to various physical and chemical agents. Unfortunately, fundamental investigations on physiology, composition, and resistance have been handicapped by inadequate information on the state of the spores investigated. Are they all similar? If not, is their dissimilarity inherent or acquired because of environmental influences? Finally, if acquired, what is the nature of the variation, and under what conditions may the spores be kept without immediate danger of a change in their properties. Knaysi (1945*a*) observed germination of the spores in the agar medium where they were formed. There was evidence that it was due to the availability of a fresh supply of food by diffusion from remote parts of the slants and by autolysis of vegetative cells. Knaysi *et al.* (1947) observed with the electron microscope two types of spores in slant cultures, one totally opaque to the electron beams at 50 kv, and the other showing transparent spots. These spots were considered an indication of incipient germination. Two types of spores were also later observed with the phase microscope (Knaysi, 1951, *unpublished data*), one bright and the other dark by dark contrast (figure 1). Dark spores germinated faster than shiny ones and were considered to have undergone incipient germination. Attempts to demonstrate a difference in heat resistance between the two types led to some paradoxical results. The present work was begun with the hope of finding an explanation to these early results. It is evident that a bright spore always turns dark as it germinates, but how early in the germination process does this change take place, and how soon after a bright spore turns dark does its resistance to heat drop to the vegetative level? Furthermore, do all dark spores originate in this manner or are there inherently dark and inherently bright spores?

In an attempt to answer these questions it was soon realized that the endospore is far from being the inert cell it is considered to be, and that its high degree of sensitivity to certain environmental factors requires the development of new and exact methods before the correct answers can be found.

MATERIALS AND METHODS

Throughout this work we employed strain C_3 of *Bacillus cereus*. This organism grows at 40, but not at 45 C, and the vegetative cells are readily killed at 50 C. After developing and testing several procedures, it was decided to adopt the following.

Preparation of the microculture. The organism was grown on collodion membranes. The technique used was generally similar to that of Hillier *et al.* (1948), in which a collodion membrane is deposited on a layer of hardened agar in a petri dish by flooding the agar layer with sterile, distilled water, depositing a drop of collodion solution in amyl acetate on the surface of the water layer and, after evaporation of the amyl acetate, removing the water by means of a pipette. The petri dish is then inoculated by depositing on the surface of the collodion film, with a capillary pipette, a number of droplets taken from a suspension of the organism under study. Composition of the agar and that of the suspension medium may be varied as desired. In the present investigation, the supporting agar layer had the composition of 2 g in 100 ml of distilled water. The inoculum came from a slant of beef infusion, glucose agar, 2 to 10 days old at 30 C, and was suspended in a mixture of 4 ml of a 0.1 per cent tryptone solution and 1 ml of beef infusion, glucose broth. The broth was prepared by diluting normal beef infusion with an equal volume of distilled water and, to a 100 ml of the diluted infusion, adding 0.5 g of tryptone and 0.5 g of glucose. The infusion agar was prepared by adding

2 g of agar to 100 ml of the broth. Henceforth these media will be referred to as beef infusion broth or agar.

After depositing the droplets on the collodion film, the petri dish is incubated 30 min at 30 C; the droplets are then withdrawn and the petri dish reincubated, partly uncovered, for 1 hr at the same temperature. The latter step permits evaporation of the excess free water and results in the formation, within the microculture, of

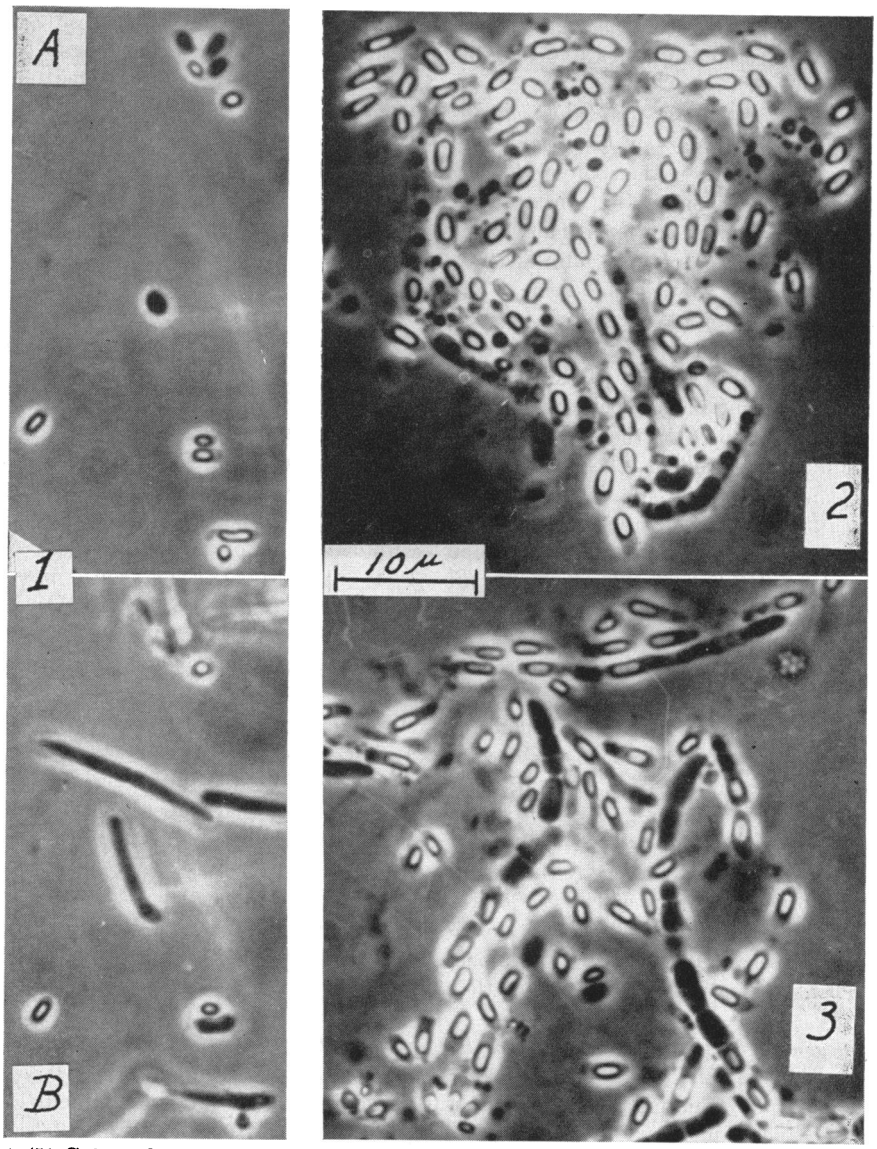


Figure 1. (51-C, 20 and 21). A slant culture on a medium of the composition: yeast extract powder, 0.1 g; glucose, 0.1 g; agar, 2.0 g; and distilled water, 100 ml. *A*, immediately after inoculation. *B*, after incubation of 3 hr + 20 min at 28 C. Note the presence and viability of both bright and dark spores.

Figures 2 and 3 (58-C, 20, and 58-E, 1, respectively). Microcultures on collodion, prepared as described in text. Age, 52 and 48 hr, respectively, at 30 C. Extent of sporulation and type of spores in the untreated microcultures. Note that dark spores are rare.

Note: Figures and numbers in parentheses after the figure numbers identify the negatives in the author's library. Figures 1 to 3 have the same scale magnification.

microcolonies of unicellular thickness. The petri dish is then re-covered and kept at 30 C. In 2 days, sporulation in these microcultures is practically completed (figures 2 and 3). Such cultures may be used as needed, or they may be floated on distilled water, picked up with a cover glass, and stored. In either case the change in the appearance, viability, and heat resistance of the spore is very slow.

Exposure of the endospores to a nutritive environment within the temperature range for germination. The microculture containing the spores is floated on distilled water and picked up with a slide covered with a layer of the agar form of a nutrient medium, usually a layer of beef infusion agar about 0.7 mm thick, so that the underside of the collodion film which supports the colony rests on this layer. The excess free water is quickly absorbed with blotting paper and the slide placed in an incubator at a suitable temperature; in the present case usually 30 C. At the end of the desired time interval, the slide is taken out and the microculture refloated on about 500 ml of distilled water at 50 C where it is allowed to undergo dialysis for 10 min. The microculture may also be refloated at room temperature on about 100 ml of distilled water in a petri dish, and then quickly transferred, with the help of a slide carrying a layer of 2 per cent agar (Difco), to distilled water at 50 C as above. The microculture is then picked up with a cover glass, air-dried, mapped, mounted on agar, as described under Methods of Observation below, and examined. The time intervals used were 5, 10, 30, and 60 min. It will be shown in the following section that floating at 50 C removes the food material taken up by the microculture while preventing further changes of the spores. The time from floating to incubation is usually about 30 sec, and removal from the incubator to refloating may not require more than 5 sec.

Heating endospores to a temperature above the maximum for the organism. (1) In distilled water:—The microculture containing the spores is floated on a beaker containing 400 to 500 ml of distilled water at the desired temperature. The beaker should be covered with a petri dish and should be plunged, almost to the edge of the petri dish, in a water bath with thermostatic control. The microculture is allowed to float for a desired period, after which it is picked up with a cover glass and observed, or air-dried and observed.

(2) In nutrient, liquid medium:—This is accomplished by floating the microculture directly on the nutrient medium in a beaker covered with a petri dish and plunging in a water bath of constant temperature, as described in (1) above. At the end of the desired period, the microculture is picked up with a slide carrying a layer of agar, quickly refloated consecutively on 2 beakers of distilled water at the same temperature, and then on a third, also at the same temperature, where it is allowed to remain for 30 min. It is desirable that each beaker of distilled water contain at least 300 to 400 ml. At the end of the period of dialysis, the microculture is picked up with a cover glass and observed, or air-dried and observed.

Methods of observation. Most observations were made on microcolonies that had been air-dried at the surface of a cover glass. An air-dry colony is usually mapped (Knaysi, 1957*a*), inverted on a film of 2 per cent agar, and observed with the microscope. To prepare the agar film, one places on either side of the center of a glass slide, and perpendicularly to its long edges, two strips of no. 1, 22 by 22 mm cover glass coated with a thin film of paraffin. The strips should be 18 to 20 mm apart. The slide is passed several times over a Bunsen burner and then placed in a sterile petri dish. This sterilizes the system and upon cooling cements the strips to the slide. A number of such slides may be prepared and stored, each in a sterile petri dish. When needed, a slide is taken out and two sterile capillary glass tubes 15 to 20 mm long and 0.30 to 0.35 mm thick are placed between, and parallel to, these cover glass strips. The capillaries should be symmetrical to the strips and about 10 mm apart. They are not cemented with paraffin, although a trace of paraffin at some point would help keep these capillary tubes in place. A 12 by 12 to 15 by 15 mm, sterile cover glass is now placed on the capillaries so that two opposite sides are parallel to these tubes. This forms a chamber about 10 mm wide and 0.30 to 0.35 mm deep. This chamber is filled with melted, sterile, 2 per cent agar. After hardening of the agar, the cover glass is slid off, the capillaries are pulled out, and the microculture to be observed is inverted over the agar layer so that two opposite edges of the supporting 22 by 22 mm cover glass are parallel to, and resting on, the paraffin-coated, cover glass strips. The supporting cover glass is now cemented to the strips and to the slide with paraffin or vaspar (a mixture of equal

weights of paraffin and Vaseline) applied with a brush all around. If the preparation is to be used also to test viability, an area about 2 mm long is left unsealed. One may also leave a very small opening at the opposite side of the chamber, for instance, by partly inserting a sterile capillary 0.30 to 0.35 mm into the chamber before sealing. The slide preparation can now be mounted on the stage of a phase microscope and observed in dark contrast. In particular, one can count the number of shiny and dark spores in various fields or groups distributed over the area of the microculture and calculate the proportion of each in a total of 500 to 1000 spores. Although spores of the investigated organism germinate, and the germ cells grow and sporulate on agar (Knaysi, 1945*a*), germination is so slow that at the end of 2 hr no change can be detected in the appearance or behavior of the spores. If the spores are to be tested for viability, the preparation is taken off the stage of the microscope and the chamber filled, or nearly filled, with beef infusion broth injected by a capillary tube through the unsealed area of the preparation or, preferably, by allowing loopfuls of the broth to touch the unsealed area. The broth enters the chamber by capillarity.

When spores are not to be allowed to dry up before observation, mapping may be omitted and, after absorption of the excess free water with blotting paper, the microculture is immediately mounted on a film of agar prepared as described above. Unmapped preparations that are to be subsequently used for viability tests should not be disturbed once groups have been selected and photographed. Filling the chamber with broth and subsequent sealing may be carried out on the stage of the microscope.

Microscopic observation was made with a Spencer phase microscope, and photography with a Contax camera on Microfile film. The primary magnification on the film was 450.

Accuracy of the methods. Accuracy of the methods used in the investigation are of a high order. The spores are formed under a uniform environment. The time between the first contact of the spores with agar and the observation of a selected group is usually less than 2 min. The effect of a given treatment may be stopped instantaneously at any desired stage. The effect of each step has been experimentally determined. The most likely source of error is the method of counting. When one observes a microculture that

TABLE 1

Degree of agreement between duplicate control experiments on the proportion of shiny and dark spores*

Age of Microcultures at 30 C	Per Cent of Dark Spores		Difference
	Expt 1	Expt 2	
24 days	1.26	0.56	0.70
2 days	4.46	2.57	1.89
2 days	3.30	2.45	0.85
2 days + 6 hr	5.24	5.17	0.07
5 days	1.75	1.63	0.12
2 days + 6 hr	4.33	1.66	2.67
2 days + 6 hr	4.33	3.50	0.83
3 days + 6 hr	4.33	2.67	1.66
5 days + 6 hr	5.67	5.50	0.17
6 days	5.20	1.61	3.59
Total			12.55
Mean			1.26
Median			0.84

* Duplicate microcolonies were taken from the same petri dish.

TABLE 2

Velocity of change of the spores from shiny to dark on beef infusion agar at 30 C

Expt	Age of Microculture	Period of Incubation in Min	Per Cent of Dark Spores	Net Per Cent of Dark Spores*
I	3 days + 5 hr	0	5.59	
		5	29.50	23.91
		34	49.47	43.88
		60	54.13	48.54
II	2 days + 5 hr	0	1.40	
		10	43.24	41.84
		30	58.34	56.94
III	2 days	5	22.11	
IV	24 days	0	0.91	
		10	30.68	29.77
V	8 days	5	37.87	
		10	45.58	

* The experimentally determined per cent minus the per cent of dark spores in the control, when a control was run.

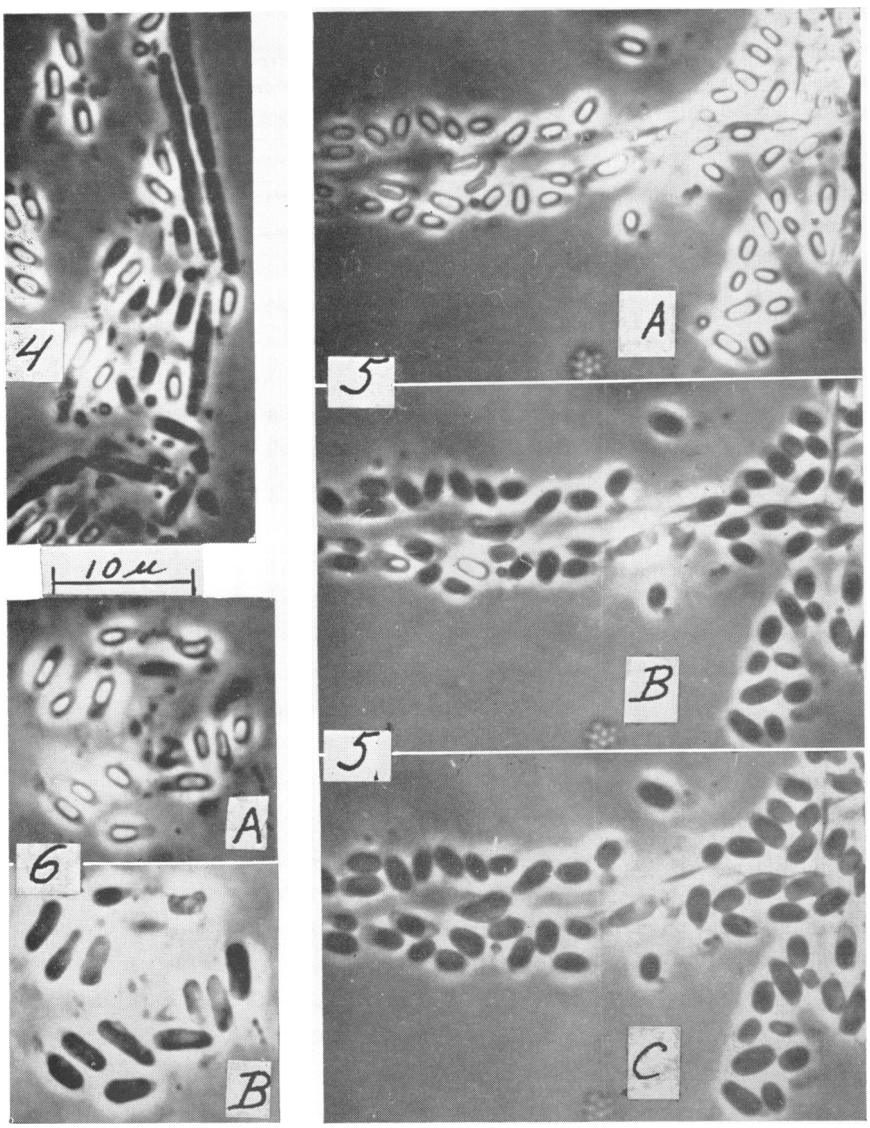


Figure 4. (58-C, 26). Microculture on collodion. Floated on distilled water at room temperature when 52 hr old, incubated on beef infusion agar for 5 min at 30 C, refloated for 10 min on distilled water at 50 C, and observed immediately after mounting on 2 per cent agar. Note that a considerable proportion of the spores turned dark during the 5-min incubation.

Figure 5A to C. (58-D, 33 to 35, respectively). Microculture on collodion. Age, 24 days. Floated on distilled water at room temperature and mounted on 2 per cent agar. *A*, appearance of a microcolony immediately after mounting on the agar, before addition of food. *B* and *C*, appearance of the same microcolony 10 and 45 min, respectively, after the injection of beef infusion broth into the air-chamber surrounding the agar. The quickness and universality of the response rule out any loss of vitality or viability due to age of microculture.

Figure 6A and B. (58-F, 6 and 7, respectively). Microculture on collodion. Age, 2 days + 6 hr. Floated on distilled water at 80 C for 10 min, and mounted on 2 per cent agar. *A*, immediately after mounting on the agar. *B*, 2 hr after injection of beef infusion, glucose broth to the air space surrounding the agar. Note that all bright spores have begun to germinate. Figures 4 to 6 have the same scale of magnification.)

has been incubated, for instance, for 5 min on a layer of beef infusion agar, one often notes wide differences, between different areas of the microculture, in the proportion of spores that turned dark. The differences decrease as the time of incubation increases and they may be attributed, in part, to heterogeneity in the properties of the collodion membrane which separates the spores from the nutritive medium. In order to get reproducible results, it is necessary to count groups or fields in a number of unselected areas distributed over the entire microculture. Table 1 contains counts made on duplicate microcultures in 10 separate experiments. The mean difference between duplicates is 1.26 and the extremes are 0.07 and 3.59 per cent units. The median is 0.84. In analyzing our results, only differences consistently exceeding 5 per cent units were considered significant.

RESULTS

Velocity of change from bright to dark spores in a favorable environment. The general procedure was to float 2 or more spore-containing microcolonies on distilled water at room temperature. One, the control, is picked up with a cover glass, air-dried, mapped, mounted on agar (Difco) and observed as described under Methods of Observation in the preceding section. The others are picked up with slides each carrying a layer of beef infusion agar and further treated as previously described under Exposure of the Endospore, etc. The results of several experiments are recorded in table 2, and the appearance of a microcolony at the end of 5 min incubation on the infusion agar is illustrated in figure 4.

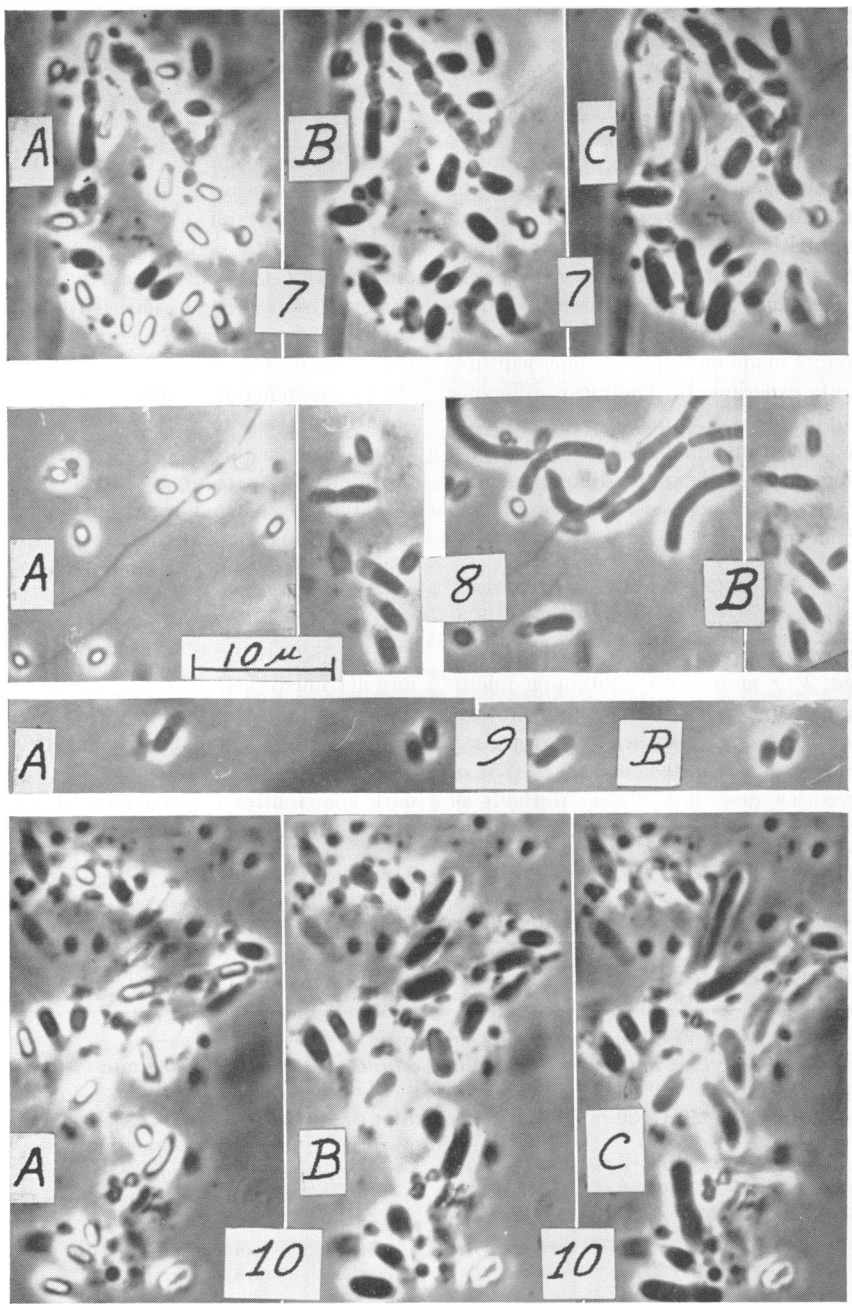
The data of table 2 show, roughly, that under our experimental conditions, between 20 and 40 per cent of the endospores in a microculture changed from shiny to dark with only a 5 min contact in a favorable medium at a favorable temperature. On the other hand, nearly half of the spores remained shiny even after a contact of 60 min. Had there been no collodion membrane between the endospores and the nutritive medium, the percentage of the spores which would have changed in 5 min would probably have been higher, and that of spores that would not have changed in 60 min much lower, than the figures given above.

Effect of age. With the exception of experiment IV, the data of table 2 do not seem to indicate any detrimental effect of age, within the recorded limits, on the rate of change from shiny to dark spore. In the case of experiment IV, where the microcultures are 24 days old, the percentage of dark spores after a contact of 10 min with the nutrient medium is definitely low. Since germina-

TABLE 3

Effect of heating the endospores in a nutrient medium above the maximal temperature for germination

Age of the Microculture	Temp and Time of Heating	Medium in which Heated	Temp and Time of Dialysis	Per Cent of Dark Spores
4 days + 6 hr	50 C, 30 min	Dist. water	—	3.92
		Glucose broth	50 C, 30 min	4.42
4 days + 6 hr	50 C, 30 min	Dist. water	—	2.33
		Glucose broth	Room temp, 10 min	33.23
		Glucose broth	50 C, 30 min	4.42
3 days + 6 hr	60 C, 30 min	Dist. water	—	3.50
		Glucose broth	Room temp, 10 min	15.36
		Glucose broth	60 C, 30 min	3.97
5 days + 6 hr	70 C, 30 min	Dist. water	—	5.59
		Glucose broth	Room temp, 10 min	17.47
		Glucose broth	70 C, 30 min	3.34
2 days + 6 hr	80 C, 10 min	Dist. water	—	5.20
		Glucose broth	Room temp, 10 min	13.18



Figures 7–10

tion tests did not show any loss of viability, or even of vitality (figure 5), we attribute the low figure to clogging of the pores of the collodion membrane by deposits from the drying agar.

Effect of heating the endospores in a nutrient medium above the maximal temperature for germination. We have seen above that a contact of only 5 min, with a nutrient medium at a temperature suitable for growth, induces the change of 20 to 40 per cent of the spores from shiny to dark. One wonders what would happen if the contact were above the maximal temperature for the organism. We used a broth of the composition: 0.3 g of beef extract + 0.5 g of tryptone + 0.5 g of glucose + 100 ml of distilled water (henceforth referred to as meat extract broth) at temperatures of 50, 60, 70, and 80 C. In the early phases of this investigation, while we were still developing our methods, we floated our microcultures on broth above the maximal temperature and then refloated them consecutively on 4 petri dishes of distilled water at room temperature, allowing them to remain on the last dish for 10 min, in order to remove any food material that may have been held by the microculture or the supporting film. Table 3 shows that under these conditions a notable proportion of the spores turns dark, and that the proportion tends to decrease as the supramaximal temperature increases. At 50 C, as many as 33 per cent of the spores may turn dark; above 60 C the percentage drops to the neighborhood of 15. At first, these observations were taken to mean that the first change from shiny to dark involved a process exclusive to germination, and that this process can go at supramaximal temperatures but tends to be inhibited as the temperature gets higher. However, considerations of resistance (see below) suggested that, in this case, the change from bright to dark might have taken place during dialysis in the presence of food material adsorbed from the broth. Reduction in the percentage of dark spores at the higher temperatures would be explainable by the fact that less nutrient is adsorbed and the amount adsorbed is held less firmly at these temperatures. The correctness of this explanation was shown by the lack of tendency in the spores to change from bright to dark when dialysis was carried out at a supramaximal temperature, usually the same as that of the broth. Here the adsorbed food was completely removed at a temperature unsuitable for germination (table 3).

Resistance of bright and dark spores to heat. Floating at 80 C for 10 min, whether it be on distilled water or on a nutrient medium does not affect the viability of the bright spores of the investigated organism (figures 6 and 7). However,

Figure 7A to C. (58-F, 23, 25, and 30, respectively). Microculture on collodion. Age, 2 days + 6 hr. Floated on beef extract broth at 80 C for 10 min, refloated consecutively on 4 dishes of distilled water at room temperature, as in text, and observed on 2 per cent agar (Difco). *A*, appearance immediately after mounting on the agar. *B* and *C*, 1 hr + 30 min and 3 hr + 50 min, respectively, after injection of beef infusion broth into the air space surrounding the agar. Note that all bright spores have begun, and all dark spores have failed, to germinate.

Figure 8A and B. (58-D, 20 and 24, respectively). Microculture on collodion. Age, 24 days. Floated on distilled water at room temperature, incubated on beef infusion agar for 10 min at 30 C, refloated on distilled water at 50 C for 10 min, picked with a cover glass and kept dry for 3 days before mounting on 2 per cent agar for observation. *A*, group of bright and a group of dark spores in the same microscopic field, immediately after mounting on the agar. *B*, two groups of *A* 18 hr after injection of beef infusion broth into the air space surrounding the agar. Note that only one bright spore, but all dark ones, failed to germinate during the period. Six shed spore coats are seen in *B*. Further growth was limited by oxygen shortage.

Figure 9A and B. (58-D, 27 and 31, respectively). Microculture as in figure 8 except that it remained air dry 4 days before mounting on agar. *A*, immediately after mounting on the agar. *B*, 5 hr after injection of beef infusion broth into the air chamber surrounding the agar. The 3 dark spores show no tendency to germinate.

Figure 10A to C. (58-F, 1, 3, and 5, respectively). Microculture on collodion. Age, 2 days + 6 hr. Floated on beef extract broth at 80 C for 10 min, then, consecutively, on 4 dishes of distilled water at room temperature as in text, was picked with a cover glass and remained air dry for 1 day before mounting on 2 per cent agar. *A*, immediately after mounting on the agar. *B* and *C*, respectively, 1 hr + 10 min and 2 hr after injection of beef infusion broth into the air chamber surrounding the agar. Note that all but one of the bright spores germinated, but none of the 5 dark spores showed a tendency to germinate. (Figures 7 to 10 have the same scale of magnification.)

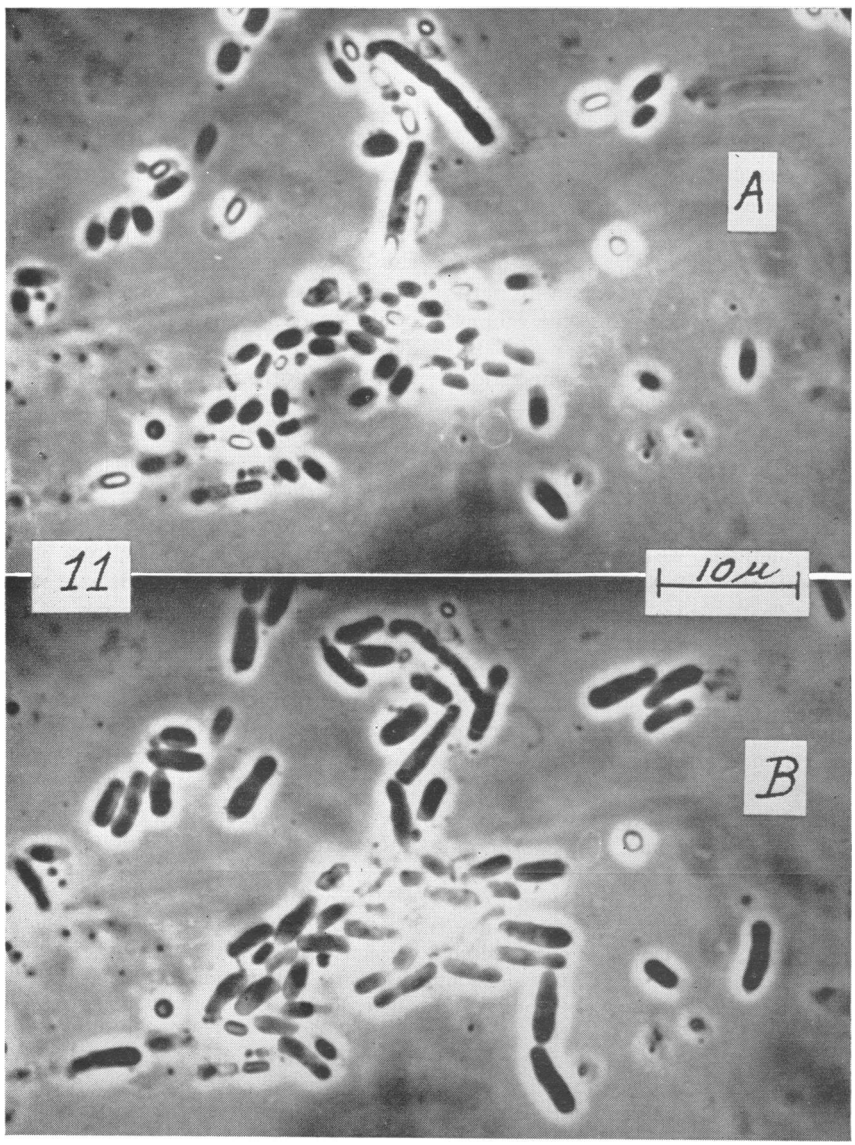


Figure 11A and B. (58-G, 17 and 19, respectively). Microculture on collodion, prepared as in text. Age, 5 days. Floated on meat extract broth at 70 C for 10 min, refloated consecutively on 4 dishes of distilled water at room temperature as in text, picked with a cover glass, and mounted while still moist on 2 per cent agar. *A*, immediately after mounting on the agar. *B*, 2 hr + 45 min after injection of beef infusion broth into the air chamber around the agar. Note that most of the dark spores are at various stages of the germination process.

the dark spores are readily killed by this temperature and, indeed, even by a temperature of 50 C for 10 min (figures 8 and 9). In the early stages of this work, we occasionally noted, in microcultures that had been floated on glucose broth at a supramaximal temperature and dialyzed at room temperature, that some of the dark spores were viable. We soon noted, however, that in microcultures that had been air-dried after dialysis, the number of dark spores that were viable usually was very small, often nil (figure 10). In microcultures that were not allowed to dry, the number of viable, dark spores was much higher; sometimes practically all the

dark spores in a microcolony were viable (figure 11). It was obvious that most of the dark spores were killed by air-drying. Having become sensitive to drying, dark spores were also probably sensitive to heat, which suggested that they were not formed while the microcultures were floating on the broth above the maximal temperature but during dialysis at room temperature, as pointed out and confirmed in the preceding part of this section.

CONCLUSIONS AND DISCUSSION

The normal resting endospore of *B. cereus* strain C_3 appears bright when observed in dark contrast with the phase microscope, and it has little tendency to change in appearance in a medium devoid of nutrients, like distilled water. In a nutritive medium, a bright spore tends to turn dark. In a medium of good nutritive qualities at a favorable temperature, many spores can undergo this change in less than 5, probably in 3 to 5 min; in a poorly nutritive medium like agar (Difco) the change is very slow, requiring several hours. The endospores have no tendency to change in the medium where they were formed, when that medium is no longer nutritive for the particular organism. In the present work, the appearance and viability of the spores were still perfectly preserved in microcultures 24 days old at 30 C. It must be recalled, however, that under ordinary conditions a culture medium may have supported sporulation and still contain some food material in remote parts of the culture or liberated by autolysis (Knaysi, 1945*a*). Consequently, a slant culture may contain both bright and dark spores. Preparing a spore suspension from such cultures requires special care so that residual food is not incorporated in the suspension, otherwise more bright spores may turn dark.

The normal bright spores of *B. cereus* are heat resistant. They are not injured by exposure to 80 C for 10 min, but as soon as they turn dark their resistance, to heat as well as to drying, drops to the vegetative level. This is in agreement with the recent observation of Powell (1957).

Neither the appearance nor the viability of bright endospores are affected by an exposure of 10 to 30 min to the supramaximal temperatures of 50 to 80 C whether it be in distilled water or in glucose broth. In the case of the broth, however, the spores may adsorb nutrients and, when the microcolony is subsequently floated on distilled water at room temperature, the spore may turn dark before dialysis of the adsorbed material is complete.

Once an endospore has been in contact with the nutrients, germination may be initiated even after the material has apparently been removed. This is of considerable practical importance to the survival of the organism and to anyone investigating the bacterial endospore. Certainly, knowledge of this fact explains the unexpectedly low resistance of endospores to heat reported by Hodge and Knaysi (1937).

According to Evans and Curran (1943) and Curran and Evans (1945), heat shocking not only kills the vegetative cells but also affects the spores, and the effect varies with the medium in which the spores were heated. The present work confirms this conclusion and indicates the danger of an indiscriminate practice of heat shocking.

In view of the instability of the endospore in a nutritional environment, study of the mechanism of spore resistance to various deleterious agents becomes fascinating. It appears that in the survival of a sporeforming organism, the value of the endospore is greatest when food is absent, as in distilled water, or present but not utilizable, as in a dry environment or at temperatures outside the limits for the germination of the organism. In the presence of available food, resistance of the endospore is transitory and, for many spores, drops to the vegetative level in a few minutes to a few hours, depending on the quality and concentration of the food and the ambient temperature. In the presence of food, the initial degree of resistance is, in the long run, less important for survival than variation among spores in their tendency to germinate. In our experiments, the survival of *B. cereus* at 50 to 80 C depended not on the 20 to 40 per cent which turned dark in 5 min on infusion agar, no matter how resistant they were, but on the spores which remained bright after 60 min on that medium.

At the present time bacteriologists are in disagreement on the definition of germination (Campbell, 1957). To some, germination is complete only when the germ cell emerges from the spore coat. To others, germination is complete as soon as the spore acquires vegetative characteristics, particularly sensitivity to heat. By the first standard, an endospore may take 90 min or more to complete germination; by the second it may take less than 5. The verb *to germinate*

has no reference to any sort of sensitivity. It derives from the latin verb *germinare* which means to sprout, and germination is usually understood to include all the changes that a spore undergoes in its development from the state of rest to the growing form typical of the organism. According to present knowledge, the acquisition of sensitivity to heat takes place at a very early and important stage in the germination process. It indicates the incipience of germination which, however, under certain conditions, may not be followed by further development (Knaysi, 1945*b*). Consequently, it would not be correct to make sensitivity to any deleterious agent represent the whole germination process (Knaysi, 1957*b*).

SUMMARY

The normal heat resistant endospores of *Bacillus cereus* strain C_3 appear bright when observed in dark contrast with the phase microscope. In a nutritive medium, and when the ambient temperature is favorable for germination, the endospores tend to turn dark and, simultaneously, their resistance to heat and drying drops to the vegetative level. In glucose-infusion media at 30 C, 20 to 40 per cent of the endospores undergo this change in less than 5 min. In the absence of nutrients and at supramaximal temperatures in the presence of nutrients, bright endospores have no tendency to change in appearance or in resistance. When endospores that had been in a nutritive medium at a supramaximal temperature are transferred to distilled water at a temperature suitable for germination, they tend to become dark and to acquire vegetative characteristics before the absorbed food material is completely removed. The importance of these observations in the study of spore resistance and their bearing on the survival of sporeforming bacteria is pointed out, germination is discussed, and the new and exact methods used in this study are described.

REFERENCES

CAMPBELL, L. L., JR. 1957 Bacterial spore germination—definitions and methods of study. In *Spores*, pp. 33–44. Am. Inst. Biol. Sci., Washington, D. C.

CURRAN, H. R. AND EVANS, F. R. 1945 Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. J. Bacteriol., **49**, 335–346.

EVANS, F. R. AND CURRAN, H. R. 1943 The accelerating effect of sublethal heat on spore germination in mesophilic aerobic bacteria. J. Bacteriol., **46**, 513–523.

HILLIER, J., KNAYSI, G., AND BAKER, R. F. 1948 New preparation techniques for the electron microscopy of bacteria. J. Bacteriol., **56**, 569–576.

HODGE, H. M. AND KNAYSI, G. 1937 *Elements of bacterial cytology*, pp. 249 and 251, by G. Knaysi. Comstock Publishing Associates, Ithaca, N. Y.

KNAYSI, G. 1945*a* A study of some environmental factors which control endospore formation by a strain of *Bacillus mycoides*. J. Bacteriol., **49**, 473–493.

KNAYSI, G. 1945*b* Investigation of the existence and nature of reserve material in the endospore of a strain of *Bacillus mycoides* by an indirect method. J. Bacteriol., **49**, 617–622.

KNAYSI, G. 1957*a* Les granules des mycobactéries et le siège de l'acido-résistance dans leurs cellules. Ann. inst. Pasteur, **92**, 197–201.

KNAYSI, G. 1957*b* Structure of the endospore and the cytological processes involved in its formation and germination, with remarks on criteria of germination. J. Appl. Bacteriol., **20**, 425–430.

KNAYSI, G., BAKER, R. F., AND HILLIER, J. 1947 A study, with the high-voltage electron microscope, of the endospore and life cycle of *Bacillus mycoides*. J. Bacteriol., **53**, 525–537.

POWELL, E. O. 1957 The appearance of bacterial spores under phase-contrast illumination. J. Appl. Bacteriol., **20**, 342–348.