THE STRUCTURE, COMPOSITION, AND BEHAVIOR OF THE NUCLEUS IN BACILLUS CEREUS[1]

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The author has recently reviewed the literature on the organization and behavior of the bacterial nucleus (Knaysi, 1951). Since then the situation has been confused by claims (DeLamater, Hunter, and Mudd, 1952) that the organization and behavior of the bacterial nucleus are identical to the organization and behavior of nuclei in the cells of higher animals. These claims have not been confirmed by others (Knaysi, 1952*a*, *b*, *c*, 1954; Robinow and Hannay, 1953; Bisset, 1954).

Observation of the forespore nucleus of *Bacillus cereus* (Knaysi, 1952*a*) on living cells with the phase microscope and on stained preparations indicated the presence of two dumbbell-like bodies, hyaloplasm, and nuclear membrane. Similar observations on *Mycobacterium thamnopheos* (Knaysi, 1952*b*) showed that the nucleus in growing cultures usually contains one to several dumbbell-like bodies interpreted as chromosomes. Occasionally, however, a nucleus is found that contains ellipsoidal bodies. The most common picture is one in which the nucleus consists of two, curved, dumbbell-like bodies separated by dense, homogeneous material interpreted as hyaloplasm. In actively growing cultures many nuclei consist of a single, dumbbell-like body often surrounded by a thin layer of the dense material that we called hyaloplasm.

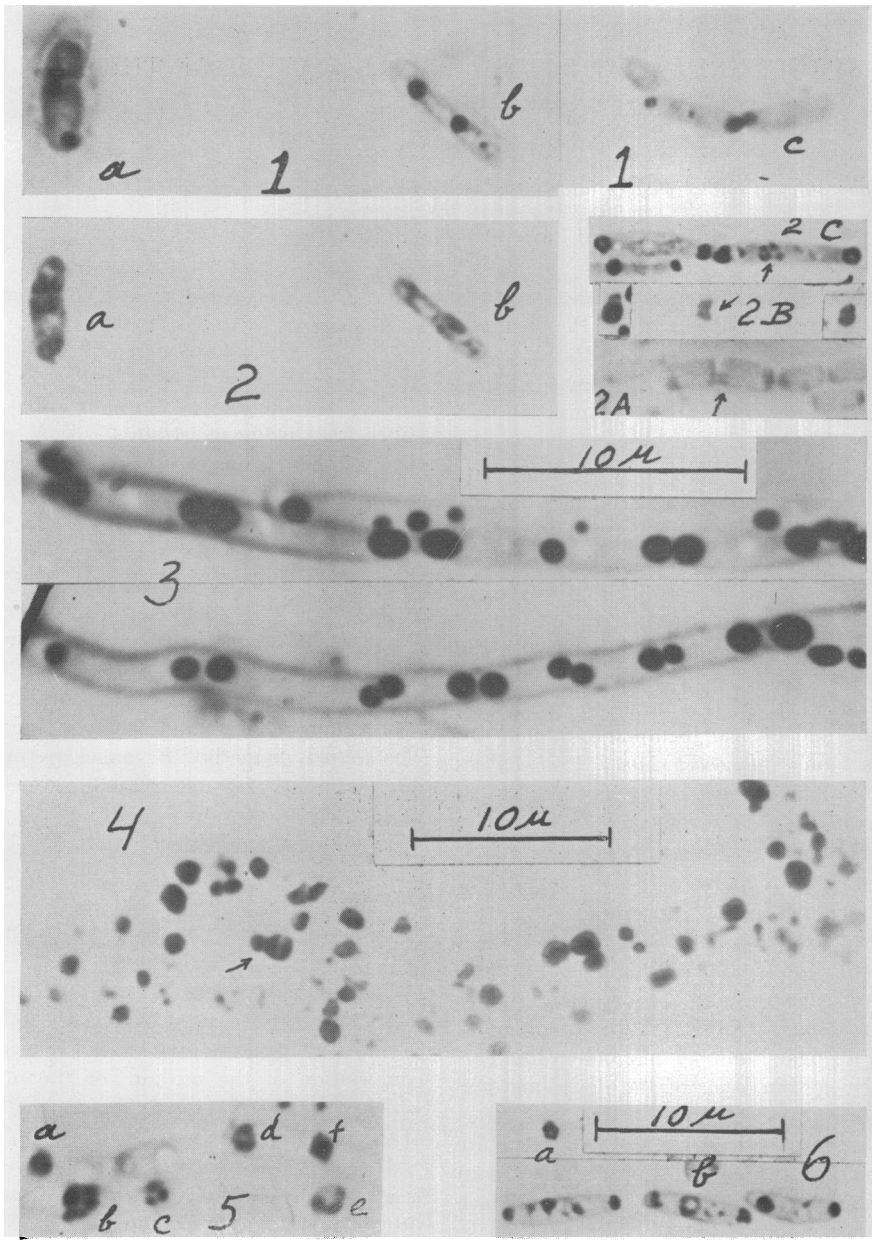
Other recent papers dealing with the structure of the nucleus are those of Murray and Truant (1954) and Truant and Murray (1954) in which those authors report observing vesicular nuclei with peripheral chromatin granules connected by delicate threads, respectively, in *Moraxella* and in *Azotobacter*.

[1] Reports on the progress of this investigation were made at the 52nd meeting of the Society of American Bacteriologists (Boston, 1952), to the Société Française de Microbiologie (Paris, May 5, 1953), and to the 6th International Congress of Microbiology (Rome, September 7, 1953). An abstract of the latter report appeared in the Riassunti delle Comunicazioni of the Congress and was later published in full with only two of the illustrations in Knaysi (1954).

MATERIALS AND METHODS

Most of the observations reported in this paper were made on strain C_3 of *B. cereus* although other strains were occasionally employed, in particular strain BMK (Knaysi, 1951) of variety *mycoides* and variant D6 of this strain. At various times three methods were used to render the nuclei visible. The first, or method *A*, previously used in a study of endospore formation (Knaysi, 1952*a*), is based on the principle applied by Knaysi and Baker (1947) that cells that develop in a nitrogen-free medium have poorly stainable cytoplasms and deeply staining nuclei. The second, or method *B*, makes use of the new observation that some partially reduced, basic dyes, such as neotetrazolium and Janus green, stain chromatin more deeply than the cytoplasm. The third, or method *C*, particularly valuable for the demonstration of what we have come to recognize as primary nuclei, is based on the fact that nuclei stained with thionin, unlike the cytoplasm, are not decolorized when mounted in a methylene blue solution of pH 3.1 to 3.2. The second and third methods do not depend on the composition of the medium or the manner in which the cells are grown. The technical details of these methods are as follows:

Method A. The organism under investigation is grown on a collodion film supported by the proper medium, usually medium GA consisting of glucose, 0.2 g; sodium acetate, 0.2 g; agar, 2.0 g; and distilled water, 100 ml; according to the technique of Hillier, Knaysi, and Baker (1948). At the proper time, a colony with the supporting film is floated on distilled water and picked up with a cover glass. The cover glass is placed in an inclined position to dry. The process may be hastened by absorbing the water droplets away from the collodion film with blotting paper. The water droplet which usually collects between the collodion film and the cover glass at the lower



FIGS. 1–6

Figure 1. (53 D-10). Part of a microculture on collodion film supported by GA agar. Age 7 hr + 20 min at 30 C. Fixed with ethanol and mounted in methylene blue solution of pH 3.1 to 3.2. Cells *a* and *c* show simultaneous division of cell and nucleus.

Figure 2. (53 D-12). The same cells *a* and *b* as in figure 1 after treatment with N HCl for 6 min at 60 C and staining for 10 min with Giemsa's solution diluted 5 times with distilled water. Note that the nuclei occupy somewhat similar positions in both figures, but that the morphology of the nucleus was changed by hydrolysis.

Figures 2A and 2B. (54 A-3 and 5). Microculture and treatment similar to those of figure 1. Age 17 hr. The compound nuclei indicated by arrows are dividing transversally at the same time as the cell.

edge of the collodion film may also be absorbed by applying a strip of blotting paper to the edge of the film. The air-dry preparation is then fixed with 95 per cent ethanol for the desired time, usually 2 to 10 minutes, sometimes longer; then it is rinsed with water and dried again as above. At this latter stage complete drying is not necessary; one can absorb the excess water on the colony side of the cover glass with blotting paper; only the underside must be dry. The cover glass is then inverted on a droplet of the methylene blue solution placed on a glass slide, and the excess solution absorbed by applying strips of blotting paper to the edges of the cover glass. The cover glass is then sealed to the slide with vaspar (a mixture of equal volumes of vaseline and paraffine), and the preparation examined through the cover glass with the microscope.

Method B. The bacteria are grown on a collodion film, floated, and picked up with a cover glass as in method *A*. The underside of the cover glass is blotted dry, and most of the excess moisture on the film side is quickly removed. Then, before the microcolony has chance to dry up, the cover glass is inverted over the well of a hanging-drop slide containing a droplet of 0.25 per cent solution of osmium tetroxide in distilled water and is allowed to remain in that position for 40 to 60 seconds. The cover glass is then removed and, without allowing the colony to dry up, is placed, colony side down, on a droplet of a solution of partially reduced neotetrazolium or Janus green. The excess solution is absorbed with blotting paper, and the cover glass is sealed to the slide and examined as in method *A*. It is essential not to allow the colony to dry up during the entire procedure; otherwise staining will be faint, for the chief advantage of using osmic acid vapors is that they allow fixation without drying. A solution of a reduced dye may be prepared by dissolving the desired amount of the oxidized form of the dye in glucose broth (0.5 to 1.0 per cent of glucose) and autoclaving the resulting solution.

Method C. This method was first developed for studying the structure of the forespore, but it was soon found that it was also excellently suited for the demonstration of the primary nucleus and the study of its structure. The cells are grown on a collodion film, floated, and dried as in method *A*. For the study of the forespore, the microculture may be fixed 2 to 10 minutes with 95 per cent ethanol, or about one minute with formaldehyde vapors. For the study of vegetative cells, fixation with formaldehyde vapors is preferable. Fixation with ethanol is followed by

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Each consists of two primary nuclei. At the left-hand side of figure 2B there is a budding nucleus in which the structure of the bud is clearly visible. The right-hand side shows an early stage of nuclear division and the disposition of chromatin in the sister nuclei.

Figure 2C. (52 G-23). Microculture similar to that of figure 1. Age 19 hr + 30 min at room temperature. Mounted in methylene blue solution of pH 3.5 to 3.6. Note transversal division of the compound nucleus indicated by the arrow. Note also a second division of the two nuclei at the proximal ends of the sister cells and the structure of the nuclei at the distal ends.

Figure 3. (52 K-39). Strain BMK. Microculture similar to that of figure 1. Age 9 days + 21 hr + 30 min at 29 C. Mounted in methylene blue solution of pH 2.4. Note the division of nuclei simultaneously with, or in preparation for, division of the cells. Note that sister nuclei are of equal sizes but that different nuclei vary considerably in size.

Figure 4. (53 D-36). Microculture and treatment similar to those of figure 1. Age 3 days + 7 hr + 30 min at 30 C. Note, especially in some of the large nuclei, what appears to be evidence of plasmolysis separating a cortical layer, possibly a nuclear membrane, from a nuclear content. The arrow indicates a dividing nucleus that had previously budded and the bud still attached at the left-hand side. This picture had to be printed lightly and does not show the mother cells of the nuclei.

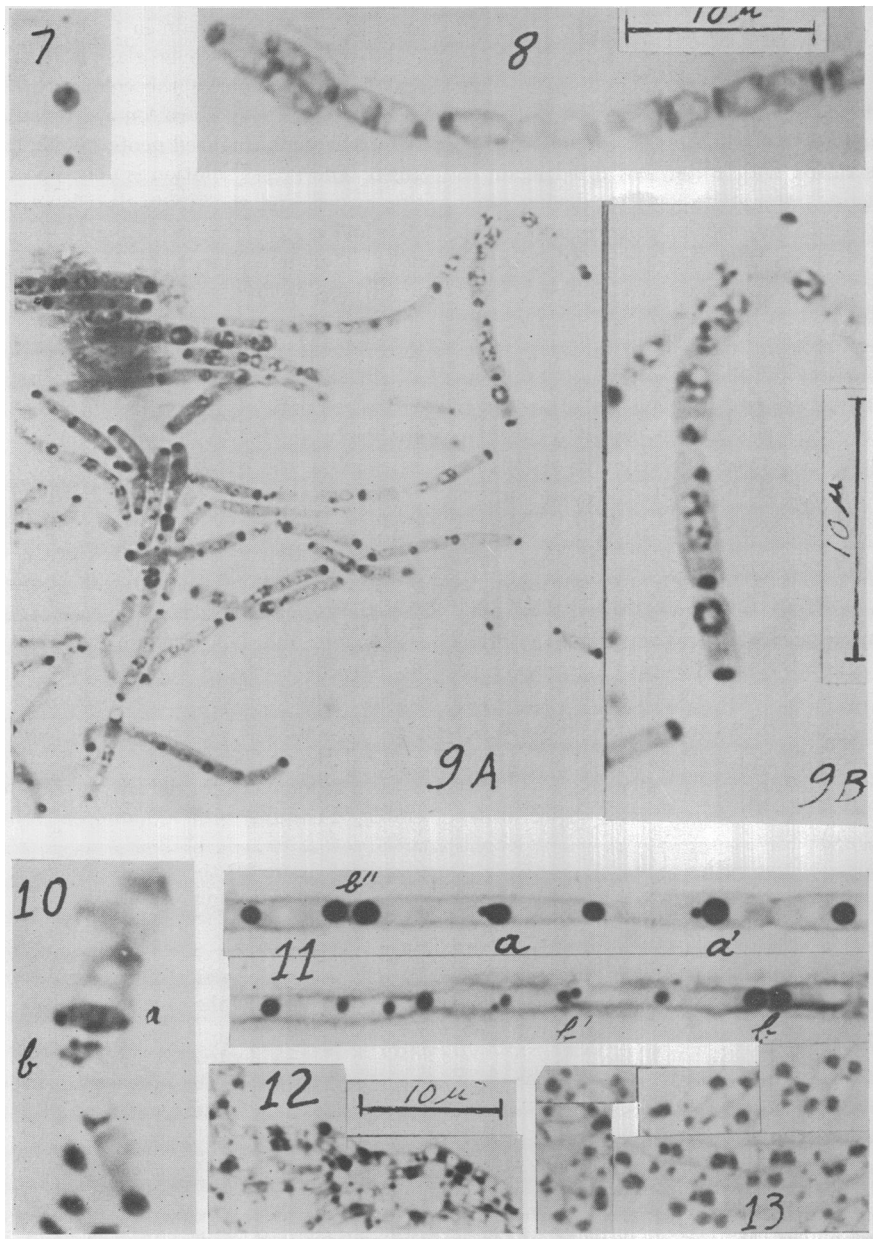
Figure 5. (51 JJ-17). Microculture similar to that of figure 1. Age 2 days at room temperature. Mounted in methylene blue solution of pH 1.64. Cells *a* to *e* illustrate the structure of some compound nuclei. Cell *e* seems to show also a nuclear membrane.

Figure 6. (52 G-28). Same microculture and treatment as in figure 2C. The structure of nuclei *a* and *b* is clearly shown.

Unless the name of the strain is given, descriptions apply to strain C_3 of *Bacillus cereus*.

The numbers and letters in parentheses after the figure number identify the negative in the author's library.

The scale of magnification of figure 4 applies to figures 1 and 2, that of figure 3 applies to 5, and that of figure 6 applies to 2A to 2C.



FIGS. 7–13

Figure 7. (52 M-26). Strain BMK. Microculture similar to that of figure 1. Age 2 days + 20 hr at 29°C. Mounted in methylene blue solution of pH 2.3 to 2.4. This picture clearly shows one primary nucleus, possibly another one along the periphery to the left, and the nucleoplasm. When the original is examined with a hand lens, it also shows a nuclear membrane. The mother cell is not visible because of the necessity of overexposing.

Figure 8. (51 H-8). Microculture similar to that of figure 1. Age 24 hr + 30 min at room temperature. Mounted in methylene blue solution of pH 3.5. Note pleomorphism and terminal location of compound nuclei due to compression by the huge, unstained lipoid inclusions. The internal structure of some distorted nuclei suggests a spindle arrangement.

Figure 9. (52 F-39). Microculture and treatment are the same as those of figures 2C and 6. The struc-

rinsing with water and drying, and the cover glass is then inverted, with the colony side down, on a droplet of a slightly acidified thionin solution. Microcultures fixed with formaldehyde vapors are not rinsed before they are deposited on the droplets of thionin solution. The microculture is allowed to remain in contact with the thionin for 2 to 5 minutes; then the cover glass is lifted by one of its edges with forceps, the adhering droplets of thionin solution carefully absorbed with blotting paper, and the microculture deposited on a droplet of methylene blue solution of pH 3.1 to 3.2. The excess solution is absorbed with blotting paper, the cover glass sealed with vaspar, and the microculture examined as in method *A*. The thionin solution is prepared by mixing 0.1 ml of 0.1 N HCl and 9 ml of 0.1 per cent aqueous thionin solution. The methylene blue solution is prepared by mixing 1 ml of 0.1 N HCl + 2 ml of 0.1 M potassium acid phthalate + 6 ml of distilled water with 1 ml of 1 per cent aqueous, methylene blue solution. We have not yet tested this method outside of the genus *Bacillus*.

The culture media used for methods *B* and *C* are 0.1 per cent tryptone solutions, nutrient agar (MIT/2 agar) consisting of 0.5 g of tryptone + 2 g of agar + 100 ml of half-strength, beef infusion, and glucose agar (MITG/2) which is the nutrient agar just described + 0.5 g of glucose.

RESULTS

Let us begin observations at an early stage of spore germination. At that stage one finds that the entire chromatin content is in one to several structures most often filamentous but sometimes round, elliptical, or resembling a double drumstick in optical sections. These various forms may coexist in the same cell. In the filamentous bodies, the chromatin may be localized at both ends of a short, straight or slightly bent, filament or in the form of 3 to 6 beads in longer, curved ones (figure 23, *a* to *c*; also figure 10 in Knaysi, 1955). At a slightly more advanced stage the chromatin tends to be more evenly distributed throughout curved or wavy filaments (figures 8 and 9 in Knaysi, 1955) which gradually divide into shorter ones. During the following period of relatively active growth, the filaments are mostly short, straight or slightly curved, single or in bunches, and the chromatin they contain is either evenly distributed or localized at the ends (figures 24, 25, and 27). It becomes obvious upon careful inspection that even distribution of the chromatin takes place during chromatin increase and that its localization as beads is an expression of chromatin segregation in preparation for division or, as is shown in another report (Knaysi, 1955), in preparation for a stage of rest. During this period the number of filaments per cell may vary from about 3 to about a dozen (figure 27). At less active stages the number may be reduced to one (figure 29). During the active period one begins to note that some of the filaments behave as if they are undergoing organization into groups of mostly two or three (figures 24, *b*, and 25, *a*) which when there is freedom of orientation, form what in optical section suggests a circular, an

ture of a number of nuclei, compound and primary, is clearly shown. Several compound nuclei in terminal positions may be entering the forespore stage. 9B is an enlargement of the group in the upper, right-hand corner of 9A. The upper nucleus of the tip cell contains 3 primary nuclei.

Figure 10. (52 J-1). Strain BMK-D6. Microculture and treatment similar to those of figure 1. Age 1 day + 22 hr + 30 min at 29 C. Note giant nucleus *a* partly liberated from its mother cell and apparently at rest. Note dividing nucleus *b*, containing 2 primary nuclei, which simulates a spindle.

Figure 11. (52 K-38). Strain BMK. Same microculture and treatment as in figure 3. Note budding nuclei *a* and *a'* and dividing nuclei *b* and *b'*. Note also the tremendous difference in the size of nuclei as between those of *b'* and *b''*. Small, compound nuclei are formed by budding, as in *a* and *a'*, or by division of other small, compound nuclei.

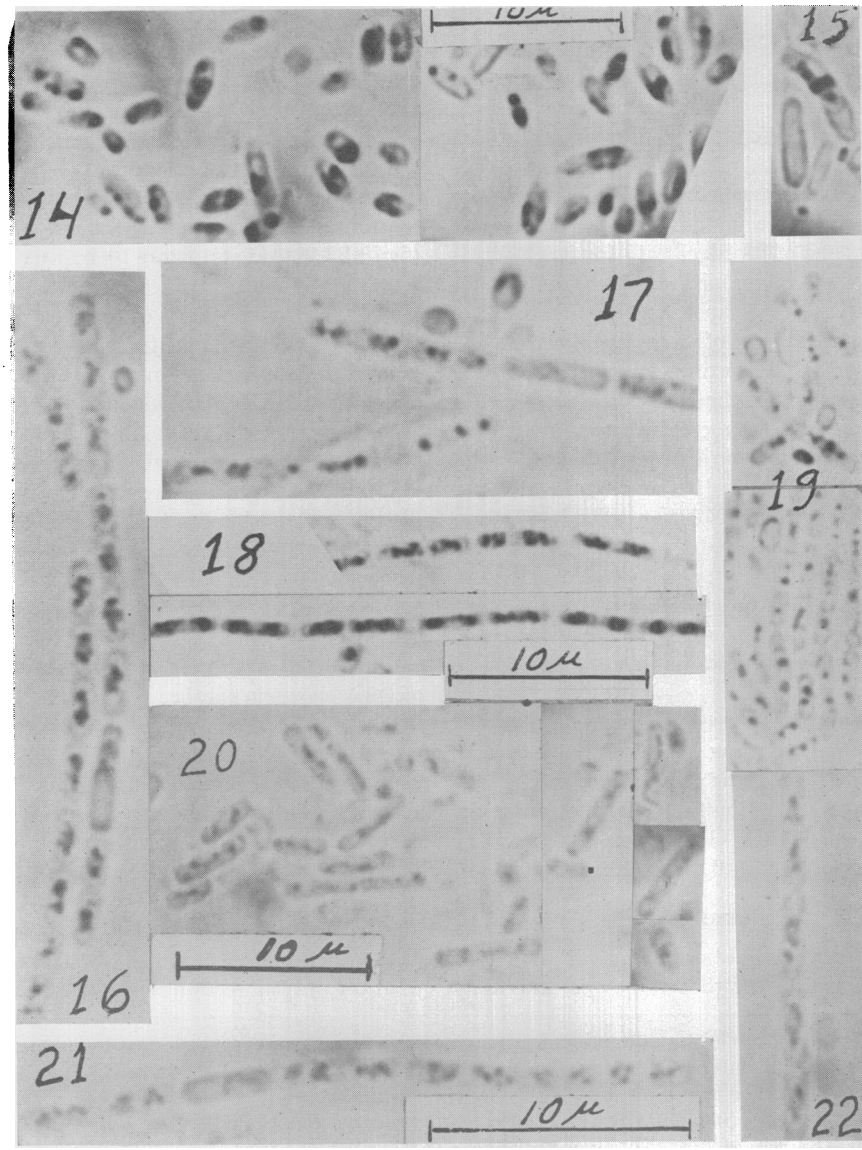
Figure 12. (51 KK-21). Microculture as in figure 1. Age 3 days + 4 hr at room temperature. Mounted in methylene blue solution of pH 2.4. This picture shows several dividing nuclei.

Figure 13. (51 J-23). Microculture and treatment similar to those of figure 2C. Age 3 days at room temperature. This picture shows several dividing, compound nuclei and others preparing to divide. In some of the nuclei one sees an indication of their internal structure.

Unless the name of the strain is given, the descriptions apply to strain C_3 of *Bacillus cereus*.

The numbers and letters in parentheses after the figure number identify the negative in the author's library.

The scale of magnification of figure 8 applies also to 7, that of figure 12 applies also to 9A and 13, and that of 9B serves also for 10 and 11.



FIGS. 14–22

Figure 14. (52 H-6). A microculture on collodion film supported by MITG/2 agar containing 0.005 per cent neotetrazolium. Age 18 hr + 30 min at room temperature. Note that in spite of age the material consists exclusively of germinating spores showing internal, purple bodies, some of which appear to be dividing and others which show the typical position and morphology observed in similar material stained with Giemsa's solution after treatment with HCl. These cells are not viable.

Figure 15. (52 H-42). A microculture on collodion film supported by glucose-acetate agar (GA) was aseptically floated and inverted on MITG/2 broth containing 0.005 per cent neotetrazolium and sealed with vaspar. After 5 hr at room temperature, some of the spores germinated and grew with no internal color. Some, finally, showed internal, purple granules.

Figures 16, 17, 21, and 22. (52 J-21). Strain BMK-D6. A wet smear from a culture 21 hr old at room temperature in 0.1 per cent tryptone solution that had been heavily inoculated with spores. The wet smear was fixed in osmic acid vapors for 1 min and was then mounted in MITG/2 broth containing

elliptical, or a slightly angular figure. This organization is accompanied by the synthesis of a variety of substances. The result is the formation of a body, which stains deeply with basic dyes, at the periphery of the optical section of which are usually located the chromatin containing filaments described above (figure 5, *c* to *e*; 6, *b*; and 9B). When the cells are mounted in methylene blue solutions, these bodies stain deeply and show a dark blue cortex and a purplish-red core over a wide range of pH; the lower limit being about 1.6 to 1.8. The color of the core is characteristic of free ribonucleic acid. There is no reason to believe that when these bodies are at rest or developing slowly and have the freedom of expansion, they do not have a spherical or an ellipsoidal form (figures 3 and 11). On the other hand, when compressed by vacuoles or inclusions, particularly lipoid inclusions, they may assume a variety of forms (figure 8), and when they are actively dividing, they may suggest the cubic, prismatic, or pyramidal form (figure 5; also Knaysi, 1952*a*, figures 5 and 6). It may be significant that the most likely places where these bodies are formed are the terminal regions of the cell, but they may also be found in other regions (figures 2C, 6, and 27). The bodies vary considerably in size (figures 3 and 11), depending on the number or state of the filaments they may contain. Occasionally a body may be formed by a single filament which elongates and bends, assuming the form of a horseshoe (figure 27, *b*). While possibly still occupying s superficial position, the filaments in the large bodies may no longer be at the periphery of the optical section. Sometimes they are found, possibly still at the surface, at other locations (figure 7). It has been shown by the author (Knaysi, 1952*a*, 1955) that the endospore is formed by one of these bodies. Sometimes it is possible to demonstrate directly a membrane at the surface of these bodies (figures 5 and 7), but more often one concludes the presence of a membrane from the immiscibility of the content of these bodies with the cytoplasm. Sometimes, especially in old cultures, one succeeds in demonstrating an osmotic effect that separates these bodies into a cortex and a core (figure 4).

The filaments, independent or associated, as well as the organized body itself multiply by division. In the case of filaments, the chromatin which is at first evenly distributed throughout the filament is resolved into two masses, one at each of the terminal regions of the filament. Division is transversal. In other filaments division may be delayed, and the generally long filament presents the appearance of a beaded thread containing from 3 to 6 beads; such threads may break up at any place between beads and finally resolve themselves into shorter filaments of one or two beads each (figures 23, *c*; 24, *c*; 27, *f* to *j*; see also Knaysi, 1955).

Division of an organized body takes place by the formation of a membrane across that body, followed by splitting or constriction which separates the body into two. We often observed such membrane, especially in the large bodies, but have not succeeded in making a clear photographic record of it. However, figures 4 (arrow), 2B (arrow and right), and 27, *e* show that there

0.005 per cent reduced neotetrazolium. Note the nuclei, some primary but mostly compound, often dividing.

Figure 18. (52 L-6). Strain BMK-D6. Culture similar to that of figure 16, etc. Age 17 hr. Dry smear fixed with ethanol, treated with N HCl for 9 min at 59 C, and stained for 10 min with Giemsa's solution diluted 5 times. Compare with figures 16, 17, 21, and 22.

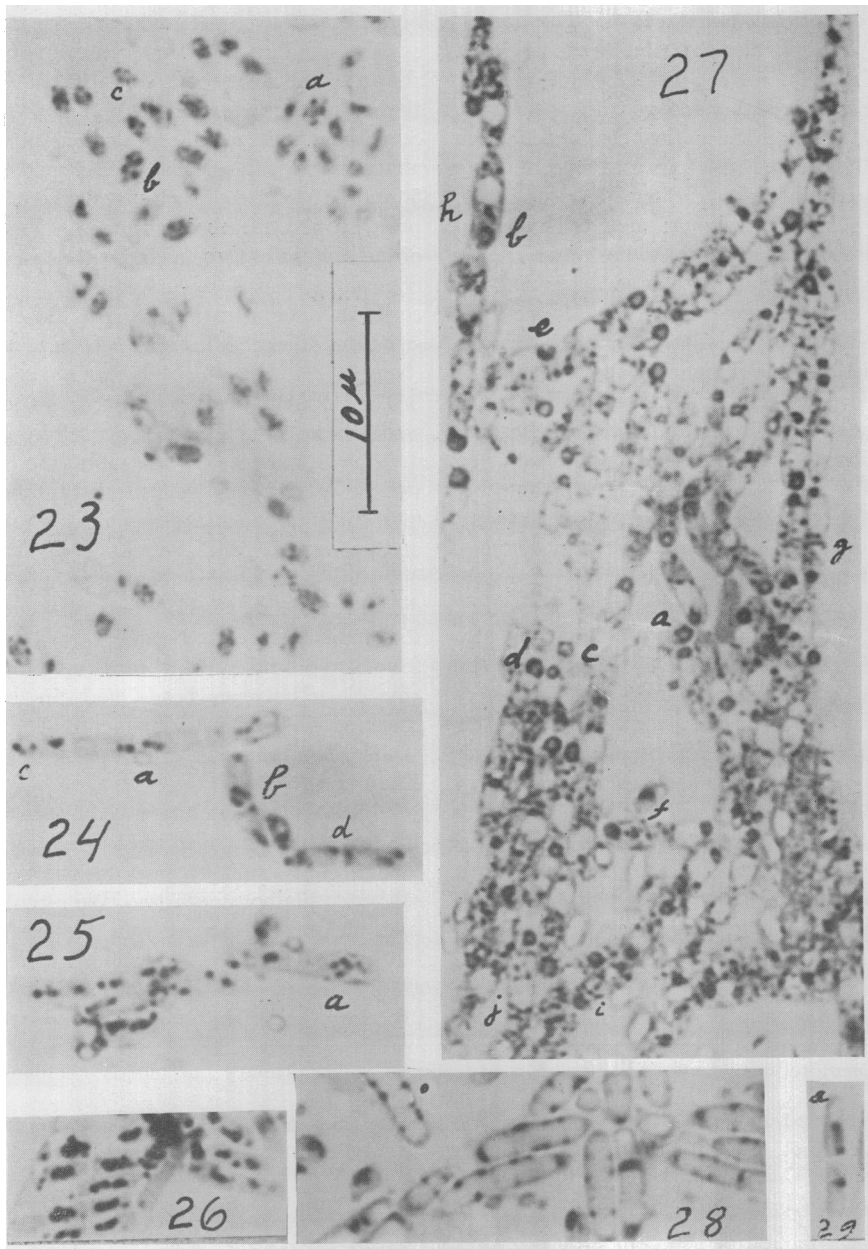
Figure 19. (52 L-11). Strain BMK-D6. Same culture as in figure 18. Droplets of culture on cover glass fixed with osmic acid vapors and the cover glass inverted on a film of MITG/2 agar containing 0.01 per cent reduced (red) Janus green. Photographed after 24 hr of contact. Compare with figures 16, 17, 21, and 22. Note that the stained areas are not as large as in 18.

Figure 20. (52 L-19). Strain BMK-D6. The same culture as in figures 18 and 19. Dry smear fixed with ethanol; treated with N HCl for 9 min at 59 C, and observed mounted in a film of Schiff's reagent (Feulgen reaction). Note similarity of stained bodies to those of figure 19.

Unless the name of the strain is given, descriptions apply to strain C_3 of *Bacillus cereus*.

The numbers and letters in parentheses after the figure number identify the negative in the author's library.

The scale of magnification of figure 14 serves also for figures 15 and 18 to 20. The scale of figure 21 applies also to figures 16, 17, and 22.



FIGS. 23–29

Figure 23. (54 F-38). Microculture on collodion film supported by glucose-acetate agar (GA). Age 2 hr at 30 C. Germinating spores. Stained by method *C*. Note that the nuclei are practically all primary as in *a* to *c*.

Figure 24. (54 F-31). Microculture and treatment similar to those of figure 23. Age 4 hr + 30 min at 30 C. Note dividing sister nuclei at *a*. Previous division apparently was synchronized with cell division as at *d*. Note at *b* what seems to be the association of 2 primary nuclei into a compound nucleus.

Figure 25. (54 F-30). The same microculture and treatment as in figure 24. Note at *a* the association of 3 primary nuclei into a compound nucleus.

Figure 26. (54 H-5). Microculture on collodion film supported by MITG/2 agar at 30 C. Treatment as in figure 23 except that fixation was made here with ethanol instead of formaldehyde vapors. Age 3

is differentiation of a thin layer across an organized body before it divides. Instances of division of the organized body by direct constriction may also be occasionally observed and have recently been recorded by the author (Knaysi, 1955). Division of the organized body is sometimes synchronized with cell division (figures 2A and 2B, arrows; 3, 8, and 11). In old cultures, an organized body sometimes multiplies by budding (figure 11, *a* and *a'*). An organized body that has produced a bud may subsequently multiply by division (figure 4, arrow). Figure 2B (left) shows that a bud contains two chromatin beads; this was also confirmed by observation of living cells with the phase microscope.

A schematic representation of the structure and division of organized bodies has recently been published by the author (Knaysi, 1954).

DISCUSSION

Compound and primary nuclei. The organized bodies described above are nuclei. This conclusion is based chiefly on the behavior of these bodies during the development of the investigated organism. They are mostly absent during the very early stages of spore germination (figure 23), but after that they are found in variable number in practically all vegetative cells (figures 6 and 9A). They divide, often initiating cell division (figures 3 and 11); they also initiate the formation of the endospore of which they constitute the principal part within the coat (Knaysi, 1955). These criteria of behavior are the ones on which we relied chiefly for identifying these bodies as nuclei although from time to time we were able to show in the same cell that a body of this type is identical with the one which stains deeply with Giemsa's solution after hydrolysis with N HCl (figures 1 and 2, *a* and *b*). It must be admitted, however, that in cells grown on glucose-acetate agar and fixed with ethanol the deeply staining material of these nuclei was usually removed by the acid treatment except at certain stages, namely, the early stage of spore germination and the late vegetative stage. Outside of these two stages, the nucleic acids disappear, leaving a faintly stained matrix corresponding to the morphology and position of the component filaments. This and the fact that the entire forespore stains homogeneously with Giemsa's solution after the acid treatment (Knaysi, 1955) show that the HCl-Giemsa method cannot always be trusted for the demonstration of the bacterial nucleus. We are inclined to believe that the Feulgen negative reaction of the nuclei of certain plant cells (reported by Gulick, 1944) is not due to the absence of desoxyribonucleic acid but to the presence of this acid in a form not sufficiently polymerized to withstand the acid treatment.

The present work shows that the filaments described above are also nuclei since they divide and, especially in cells containing only one of these filaments, the filament apparently divides with the cell, each half remaining in one of the

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hr + 40 min at 30 C. Note that the primary nuclei tend to run together and fuse into irregular mass. Note also the similarity with figure 18.

Figure 27. (54 I-13). Microculture and treatment similar to those of figure 23 except that age is 16 hr and duration of the fixation with formaldehyde vapors was 3 min instead of 1 min. Note at *a*, *c*, and *d* the structure of the compound nucleus, and at *b* what may be the initiation of a compound nucleus by a single, primary one. Note at *e* what appears to be the division of a compound nucleus. The nuclei below *f* are typical 2-beaded ones. At *g* and *h* the primary nuclei appear homogeneous; *i* shows 3 beads and upper *j* shows an early stage in the segregation of chromatin.

Figure 28. (54 G-29). Microculture similar to that of figure 23. Age 2 days at 30 C. Treated with N HCl for 6 min at 60 C and stained for 10 min with Giemsa's solution diluted 5 times. Note that large lipoid inclusions reduced the cytoplasm to a thin peripheral layer and thinner, transversal films. The nuclei are relegated to the periphery, usually at the points of intersection of the transversal and peripheral layers. This arrangement is reminiscent of some of the nuclei of the early bacterial cytologists (*cf* Swellengrebel, 1906).

Figure 29. (54 H-9). From a dry smear prepared from a culture, 25 hr old at room temperature, that had been heavily inoculated with spores. Method *C*. Note that the structure of the primary nucleus in cell *a* is clearly seen although a slight vertical tilt in the orientation of this nucleus tended to reduce the degree of contrast in the photograph.

Figures 23 to 29 represent strain C_3 of *Bacillus cereus*, and the scale of magnification is the same for all these figures.

daughter cells (figure 24, *a* and *d*). Furthermore, it can be seen that the nucleus we have been calling the organized body contains filaments morphologically and tinctorially similar to the free filaments, and when the "organized body" initiates formation of the endospore, these filaments may be clearly seen in the forespore and later in the germinating spore where they regain their independence (Knaysi, 1955).

We are thus faced with the surprising fact that the bacterial cell may contain two kinds of nuclei: the filamentous one for which we propose the name of *primary nucleus*, and the organized one which is formed by the association of primary nuclei, usually 2 to 6, for which we propose the name of *compound nucleus*. A primary nucleus containing two or more chromatin beads may be called *complex primary nucleus*.

The formation of compound nuclei by the association of primary ones, concluded from the present work, is reminiscent of the process of fusion of nuclei recently observed by the author (Knaysi, 1952*b*) in living cells of *M. thamnopheos*. It was further seen in that organism that in a given cell two nuclei may be fusing while another one may be dividing, which is also in harmony with the observations reported here. These observations indicate that the association of primary nuclei into a compound nucleus is a normal and general process in the bacteria, a process that seems to take place in response to environmental factors tending to slow down the rate of growth. It seems to be a protective process that, in spore formers, leads to the formation of an endospore. In bacteria that do not form endospores, compound nuclei rich in ribonucleic acid and possibly rich in other substances are formed, but these organisms are unable to synthesize the characteristic substance from which the spore coat is formed. This suggests that the gap between sporulating and nonsporulating bacteria may not be very wide. The conclusion that compound nuclei are formed in response to influences that hinder growth finds confirmation in the observations of Delaporte (1952) and DeLamater *et al.* (1953) of giant nuclei in cells exposed to certain antibiotics and other toxic substances.

Nucleus or chromosome? One of the questions which come to one's mind is whether the filamentous, chromatin containing structures we called primary nuclei are in reality nuclei or chromosomes. This may be as academic as whether some primitive microorganism is a plant or an animal. The idea that the bacterial nucleus is a "naked chromosome" is not new to bacterial cytologists. Badian (1933) and others who consider the bacterial nucleus as an undifferentiated mass of chromatin believe this to be the case. We, on the other hand, prefer to apply the term nucleus to any chromatin containing, organized body that grows, multiplies, actively participates in the development of the organism throughout all phases of its life history, and seems to lead an independent existence within the cytoplasm. A nucleus containing a single chromosome would have all these characteristics, but a "naked chromosome", according to this concept, would not lead an independent existence although it might have all the other characteristics. Our primary nuclei contain chromatin, are organized into a membrane, chromatin, and hyaloplasm, a term we reserve to the nonchromatin content of the primary nucleus to distinguish it from what we called the nucleoplasm of the compound nucleus, actively participate in the formation of the endospore as well as in vegetative reproduction, and are able to exert their activity singly within the cell (figures 23 to 27, and 29; see also Knaysi, 1955). They are primary because by their association they can form another nucleus, the compound nucleus. The nucleoplasm of the compound nucleus is undoubtedly formed by the component primary nuclei as a result of the intensification of certain normal activities of primary nuclei since we observed in living cells of *M. thamnopheos* that primary nuclei are often surrounded by a thin, dense layer, morphologically resembling a thin slime layer (Knaysi, 1952, *unpublished data*).

On the phenomenon of budding. Ever since we started this investigation in 1950, we were surprised and at times dismayed by the observation of genuine budding in the large nuclei. We observed the process both in the living state with the phase microscope and in stained preparations, so it was not a question of an artifact caused by the treatment we gave the preparations. We were also confident that we were observing genuine nuclei. The discovery that budding nuclei are compound nuclei in which several primary ones may be associated rationalized the budding process. Since a bud seems to contain two beads of chromatin (figure 2B, left), it is a midget compound nucleus potentially equivalent to two

primary nuclei and contains the entire complement of hereditary factors transmitted by means of the nucleus. Indeed, the budding process stamps the primary nucleus as the nuclear unit of the cell. If it were not, budding could not be a means of nuclear multiplication since a bud represents only a small fraction of the mother nucleus. The simplest primary nucleus is one containing a single bead of chromatin. Primary nuclei containing two or more beads of chromatin are complex primary nuclei ready to divide or fragment. It may be concluded from this that the chromatin bead of the primary bacterial nucleus is equivalent to what in more highly developed organisms is called a chromosome.

Concerning mitosis. Throughout this investigation we have kept our eyes and mind open for any evidence of mitosis. The methods we used here as well as other methods previously used, including observations on living cells of *B. cereus* and *M. thamnopheos* (Knaysi, 1952), have given us clear and similar pictures of the processes of nuclear multiplication. At no time were we able to uncover any evidence of mitosis. Indeed, realizing as we do now that what we (Knaysi, 1952*b*, 1953, *unpublished data*) and others have called chromosomes are in reality primary nuclei, it is difficult to expect that division of the compound nuclei would take place by mitosis. In the division of primary nuclei there undoubtedly are duplication and segregation of the chromatin, but there is no resolvable evidence that segregation takes place mitotically. We are inclined to believe that what DeLamater *et al.* call mitosis is an impression given by the primary nuclei within certain large, compound nuclei and by groups of free, primary nuclei during active growth. We further believe that a compound nucleus to which a bud is attached would tend to give the impression, when studied by methods involving the usual treatment with acid, that one is observing a nucleus with centriole.

Mechanism of staining with reduced dyes. The staining red of certain intracytoplasmic bodies when living cells are placed in a medium containing small concentrations of neotetrazolium or of Janus green (figures 14 and 15) has been observed by a number of investigators (Bielig *et al.*, 1949). It has been assumed by these investigators that the dyes are reduced to their red forms in the stained bodies where the reduced dyes accumulate. On the other hand, the author showed (1952, *unpublished data*) that assumption of the color of the reduced dyes by these bodies does not necessarily mean reduction *in situ* since in cells suitably fixed these same bodies stain with solutions of the reduced dyes prepared in the test tube. Furthermore, the morphology and apparent positions of these bodies correspond to the morphology and positions of nuclei when similar cells are stained by conventional methods of staining nuclei or by our method *A* described above (figures 16 to 22). Regardless of the medium in which the culture was growing, the reduced dyes stain the primary nuclei less faintly than the cytoplasm and in compound nuclei less faintly than the nucleoplasm (figures 16, 21, and 22). It appears from this that staining with the reduced dyes does not depend on nucleic acids but possibly on a basic protein with which the desoxyribonucleic acid may be united. Indeed, the reduced form of a basic dye may not remain a basic dye since by reduction the colored cation may be changed into a neutral compound or even to an anion (Clark, 1928) which, if colored, would mean that the originally basic dye has been changed to an acid dye. We did not attempt to determine the extent of change involved in the formation of the red dyes used, but we noted a considerable decrease in the ability of the reduced dyes to permeate the membranes of dry cells, hence the importance of not allowing the cells to become dry before mounting in the staining solution.

Nuclei or mitochondria? The bodies which assume a red or purple color when living cells are placed in an environment containing small concentrations of dyes like Janus green or neotetrazolium, and which are dense to the electron beam have been considered as mitochondria by Mudd *et al.* (1951). The evidence is based on certain staining reactions given by phospholipids or ribonucleic acid, which are present in mitochondria, and on the fact that these bodies take the color of reduced Janus green or neotetrazolium under the conditions given above. The latter reaction is attributed to the reduction of these dyes by enzymes said to be present in these bodies and known to be concentrated in mitochondria (Hogeboom *et al.*, 1948). It has been pointed out in the preceding section that taking the color of the reduced dyes does not necessarily mean reduction *in situ*, for these bodies can be stained red or purple even when the dye is reduced in the

test tube as in our method *B* described before. Furthermore, cells in which these granules become colored are dead; only cells in which all or some of these granules are colorless are able to grow, and growth ceases as soon as all of the granules become colored. Thus, the reaction appears to be essentially a staining reaction, and the reduced dye may have been formed in other parts of the cell. The author has also pointed out (Knaysi, 1952*b* and *unpublished data*) that large, dense, terminal bodies are not present in all cells of *M. thamnopheos* and that, when present, they often give a strongly positive, Feulgen reaction. It may now be added that nothing morphologically resembling what Mudd *et al.* consider mitochondria has been observed in the forespore of the strain studied, nor during the early stages of spore germination as illustrated in figure 23. Recently Weibull (1953) showed that bacterial extracts from which the red bodies were removed by centrifugation still reduced triphenyltetrazolium, and he claimed that the red bodies are artifacts produced by conglomeration of smaller particles of the reduced dye. Since we were able, by operating on the same cell, to stain red bodies by our method *A*, and since the morphology and location of some of the red bodies closely resemble those of bodies demonstrable by other means (figures 14 and 15), we are led to interpret Weibull's observation on the artificial formation of red bodies only as indicating possibilities and inviting caution. A recent report bearing on the question of mitochondria is that of Chapman and Hillier (1953) who did not see any "organules having the characteristic internal structure of mitochondria".

In view of the recent report by Mudd (1953) that bacterial "mitochondria" vary in their staining reactions, it is not possible to make a general statement as to which, if any, of the bodies observed in the present work correspond to these "mitochondria". Since Mudd and his co-workers have always emphasized terminal position, large size, and optical and electronic density, we are inclined to believe that their most typical "mitochondria" correspond to our compound nuclei which are large, tend to occupy terminal positions, and contain a dense nucleoplasm that is rich in ribonucleic acid and may well contain phospholipid, accounting for the staining properties of "bacterial mitochondria". The high density of the nucleoplasm to the electron beam is clearly visible in some of the micrographs published by Chapman and Hillier. Furthermore, compound nuclei are not found in all cells at all times, which is also true of the large, dense, terminal "mitochondria" of Mudd and his co-workers. The resemblance is indeed striking.

SUMMARY

Strain C_3 of *Bacillus cereus* is able to form two types of nuclei, called *primary* and *compound nuclei*. The primary nucleus is rodlike and consists of a variable number (1 to 6) of chromatin beads and hyaloplasm within a nuclear membrane. The chromatin beads appear to be homologous with the chromosomes of more highly developed nuclei. During active growth the primary nucleus appears to become filled with chromatin which segregates usually into two terminal beads, and the nucleus divides transversally toward its middle. Primary nuclei containing 2 or more chromatin beads are called *complex primary nuclei*; this condition results when segregation of the chromatin is not immediately followed by division of the nucleus.

A compound nucleus is formed by the association of two or more primary nuclei. It may also be formed by a complex primary nucleus. When freely formed, a compound nucleus is a body with a round optical section. The primary nuclei are at the periphery, and the space within is filled with dense *nucleoplasm*, apparently rich in ribonucleic acid. The compound nucleus multiplies by division or budding. The present observations are in close agreement with those recently made by the author on living cells of *Mycobacterium thamnopheos* with the phase microscope.

Three methods of demonstrating the nuclei are described and discussed.

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