THE DUPLICATION OF BACTERIAL CHROMATIN

Interpretations of Some Cytological and Chemical Studies of the Germinating Spores of Bacillus cereus and Bacillus megaterium

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In a separate paper, the changes in the P fractions of bacterial spores during germination were correlated with the morphological changes which took place at the same time. Germination has been defined as the over-all period, some 50 to 60 min, extending from the resting state of the "mononucleate" spore to the vegetative or "binucleate" cell. Thick suspensions (10^9 spores/ml) of synchronously growing spores, suitable for chemical analysis, were obtained by heat activation and adjustment of the spore concentration to the medium. It was observed that during germination the basophilic nuclear material (chromatin) of the spore and the amount of polymerized desoxyribonucleic acid increased simultaneously and continuously. The desoxyribonucleic acid had doubled only when the growing chromatin had separated into two structures, each of which was similar in size and arrangement to the single chromatin structure seen during the initial stage of germination (Fitz-James, 1954 unpublished results).

However, recent histochemical studies of cell division in higher forms have indicated that in a typical mitosis the uptake of phosphorus into the desoxyribonucleic acid (Taylor, 1953) and the doubling of the desoxyribonucleic acid occur well before the separation processes of metaphase and anaphase (Pasteels and Lison, 1950; Swift, 1950; Fautrez and Fautrez-Firlefyn, 1953). Thus, in spore germination the continuous rise of desoxyribonucleic acid, which occurs simultaneously with the separation of the chromatin, makes one seriously question the suggestion of Hunter and DeLamater (1952) that spore nuclei divide by a typical mitotic process.

In the present paper, the arrangement of the chromatin in germinating spores of *Bacillus cereus* and *Bacillus megaterium* is examined, and alternative processes of separation are suggested which are consistent with both the cytological and the chemical findings.

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METHODS

The methods of growing the spores of *B. cereus* and *B. megaterium* as well as the various media and techniques used to study thick cultures of rapidly germinating spores are fully described elsewhere (Fitz-James, 1954 unpublished results). The amounts of the nucleic acids in germinating spores were estimated by applying the methods of Schneider (1945) and of Schmidt and Thannhauser (1945) with some modifications (Fitz-James, 1954 unpublished results). Smears of germinating cells fixed in osmium vapor were cleared by N HCl hydrolysis (60 C for 7 to 10 min) and then stained in either azure A reduced with sodium metabisulfite (SO_2-azure A) as recommended by Huebschman (1952) or with thionin reduced with thionyl chloride (SO_2-thionin) as described by DeLamater (1951). The basophilic structures so revealed in the growing spores were considered to be the chromatin, i.e., the site of the desoxyribonucleic acid.

The projection and tracing procedure for measuring the cross-sectional area of the chromatin images in the photomicrographs is described in a separate paper (Fitz-James, 1954 unpublished results).

RESULTS

In figure 1 are grouped photomicrographs showing the chromatin structures of germinating *B. cereus* at different times after initial contact with a complete medium. (Figures 1 to 3 are taken, in part, from a separate study of germinating spores, Fitz-James, 1954 unpublished results.) Differences in intensity of staining are largely due to slight variations in technique. As the aim was to disclose the arrangement of the

chromatin, the preparations presented were selected to show the finest detail. In germinating *B. cereus*, the better detail was often present in the more faintly stained and poorly contrasting preparations. With *B. megaterium*, except during the initial stages of germination, detailed pictures of chromatin could be obtained with a high degree of contrast. Line drawings are shown opposite the photomicrographs. These drawings were taken from both the original preparations and the photographic negatives; their purpose is to emphasize the detail and depth lost in printing and enlarging.

The arrangement of chromatin, when first seen during germination (figure 1—5′), closely resembles the open ring structure found in sliced or smashed resting spores (Robinow, 1953; Fitz-James, 1953). In the first 5 to 10 min, although the spore increased in volume and the amount of ribonucleic acid increased, no change occurred in the amount of desoxyribonucleic acid phosphorus or area of chromatin. During this period, however, the circular band of resting chromatin seemed to contract into a string of three connected granules (triad). Both side and edge views of this structure are seen in figure 1—10′. By 20 min both the desoxyribonucleic acid and the area of stainable chromatin showed a significant increase. Photomicrographs of the chromatin at 20 and 30 min showed an increase in number or size (or both) of the granules. Many cells contained bent "Y" forms. By 40 min the desoxyribonucleic acid of the culture was 1.45 times that of the original spores and the chromatin, which had shown a similar increase, was arranged in a variety of patterns. However, careful study indicated that the chromatin was arranged in a similar manner in most cells, and that the variations were largely the result of observing a three dimensional figure on a variety of two dimensional planes. By 50 min separation of the granular network or chain was progressing and by 60 min was complete in most cells. Following separation, the daughter triads continued to grow, and at 60 min quadrangular structures could be seen in many of the "binucleate" cells. This continuous growth of recently separated bacterial chromatin has been reported previously (Neumann, 1941; Robinow, 1945). The same granular net of chromatin was found in the young vegetative rods (figure 1—120′).

Figure 2 illustrates the results of some confirmatory studies on a rough variant of *B. cereus*. The chromatin structures had the same form as those described above. The first increase in their size was observed at 20 min (figure 2a), and by 35 min (figure 2b) they were growing apart in the elongating cells. By 50 min (figure 2c) the duplication and separation of chromatin were largely complete. Similar structures were encountered when the germinating spores (alcohol or osmium fixed) were cleared with ribonuclease rather than HCl. This enzyme was ineffective, however, in producing clear results until some 40 to 50 min of germination. Figure 2d shows the nuclear structures of 60 min germinating cells from a separate experiment in which acid hydrolyzed cells were stained with unreduced azure A (without sodium metabisulfite). Although some detail (see drawing) could be seen in the original preparation and in the negatives, in the photomicrographs most of the separating pairs of triads appeared as solid, butterfly shaped masses.

Corroborative studies were done on the spores of *B. megaterium* with similar results. During the first 15 min of germination, the contraction of the resting chromatin ring into a granular triad was again apparent. Similar condensations were previously observed in *B. megaterium* by Hunter and DeLamater (1952) who considered them to be contracted chromosomes. During the 20 to 40 min period of germination, the chromatin was partly obscured by diffuse acid-persistent basophilia, and sharp photomicrographs of the structures were difficult to obtain (Fitz-James, 1954 unpublished results). After 40 min, however, the spore coats were shed and the arrangement of the chromatin could be seen clearly. Figure 3a shows a smear of *B. megaterium* germinated for 60 minutes in heart infusion broth (Difco). Although the cells vary slightly in degree of development, many of the arrangements seen correspond to those observed in the *B. cereus* studies between 40 and 50 min of rapid germination. The amount of desoxyribonucleic acid began to increase during the 20 to 30 min interval and again showed a remarkably steady rate of increase. In the 60 min sample (figure 3a) it was 1.4 times the spore amount. By 120 min, in a more synchronously growing subculture, the amount of desoxyribonucleic acid was 3.2 times and the average area of chromatin approximately

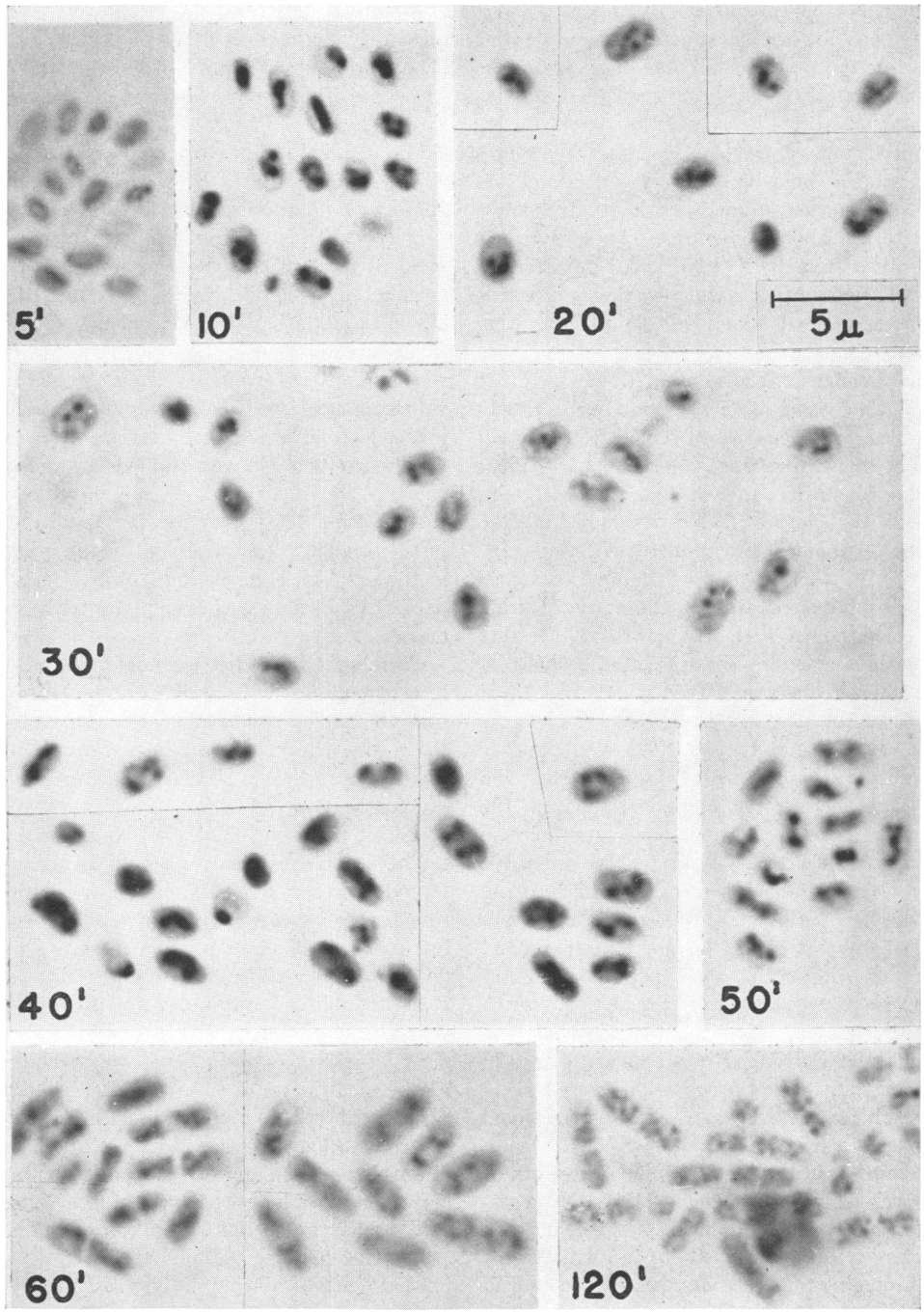


Figure 1. The chromatin of *Bacillus cereus* (S) spores rapidly germinating in semisynthetic medium, showing time in minutes after inoculation (osmium fixed, HCl hydrolyzed, and stained with SO_2-azure A after Huebschman, 1952). Interpretive line drawings are shown opposite.

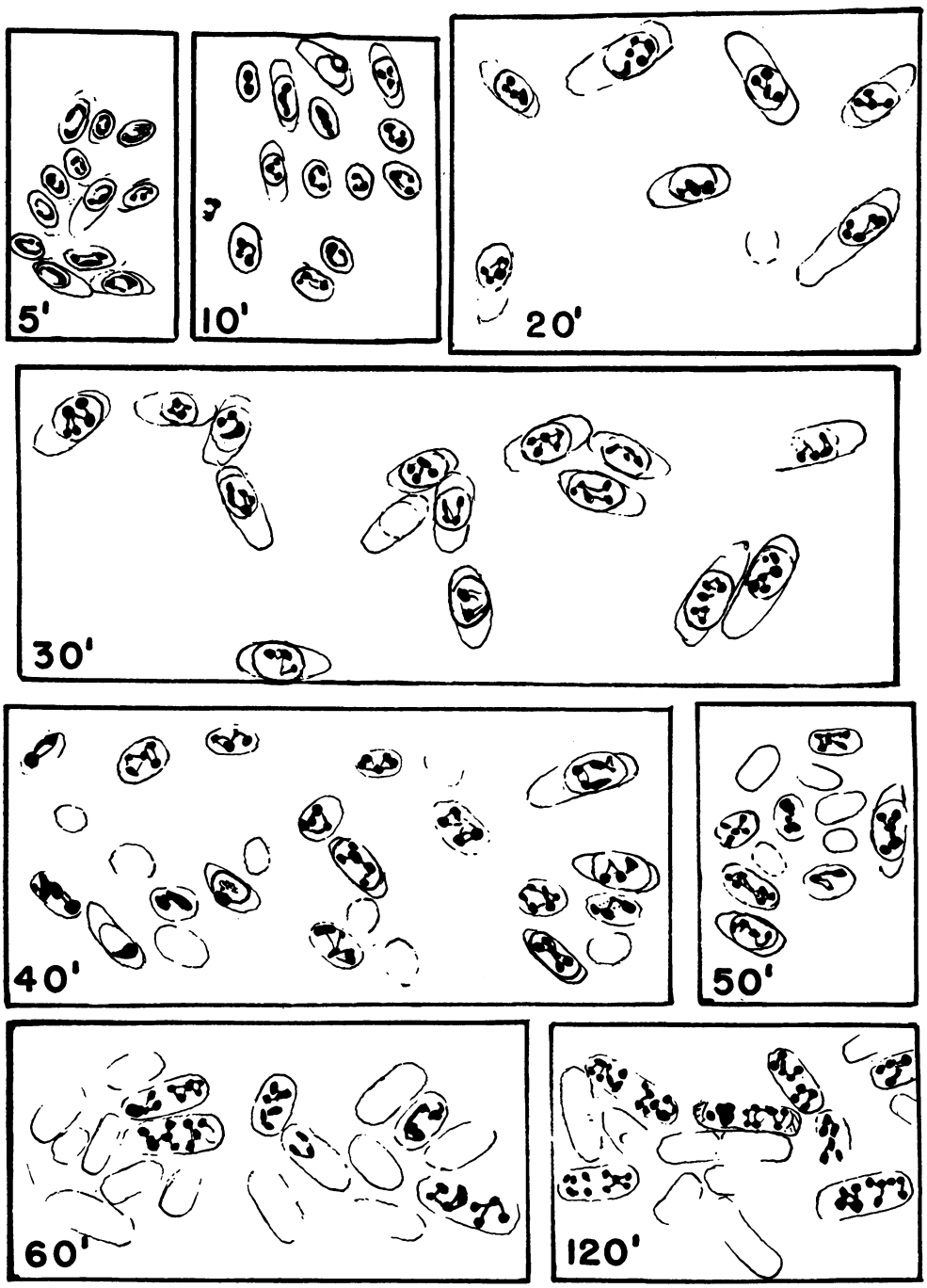


FIG. 1—Continued

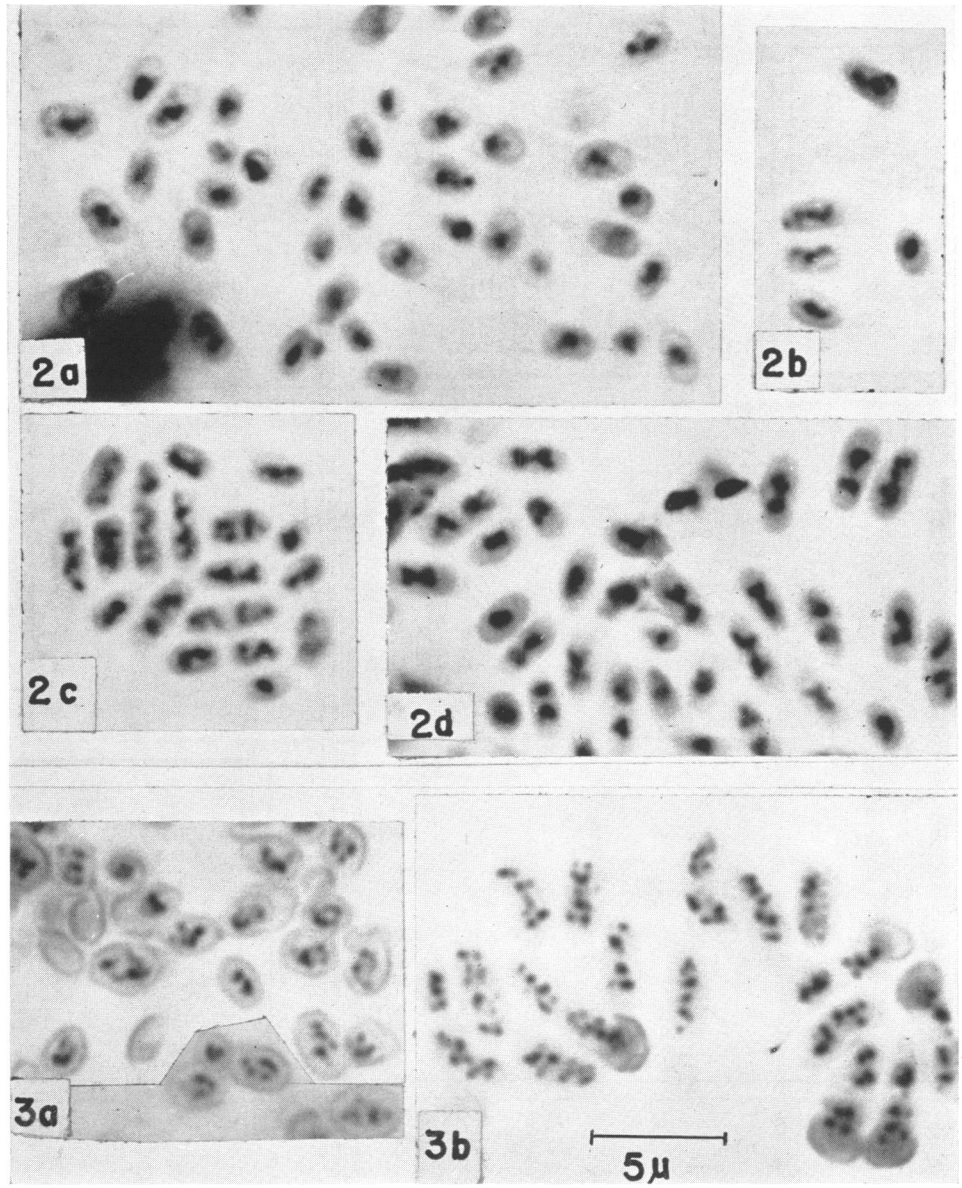


Figure 2. The cytology of rapidly germinating spores of *Bacillus cereus* (R) in semisynthetic medium.

(2a) The chromatin arrangement at 20 min (desoxyribonucleic acid synthesis beginning). Detail in the photomicrograph was partially lost in an attempt to obtain more contrast.

(2b) The chromatin (at 35 min) becoming arranged more longitudinally in the now cylindrical cells.

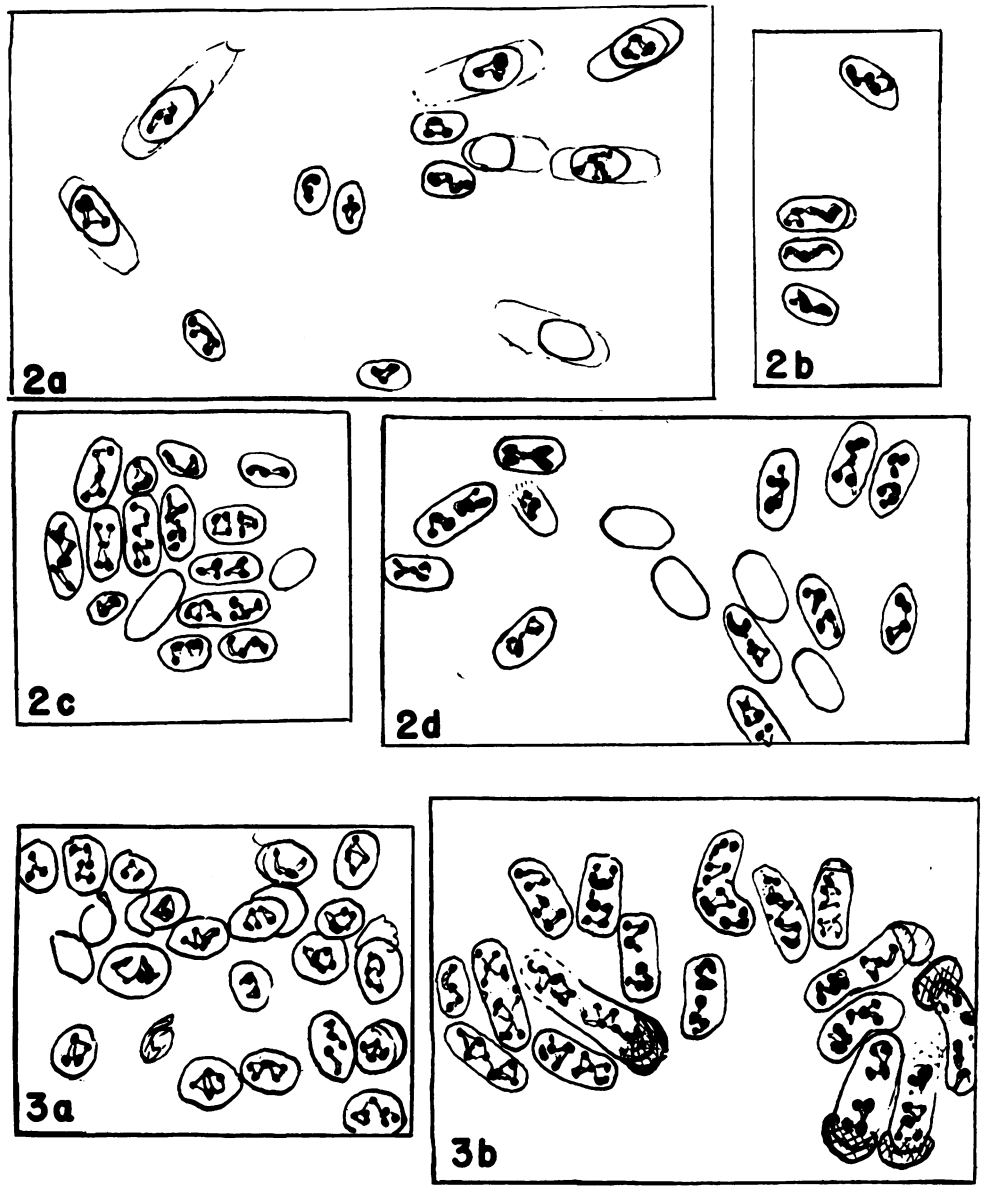
(2c) The separation of the chromatin at 50 min into two daughter triads.

(2d) The appearance of 60 min cells in a separate study stained with unreduced azure A. Detail of the triads and the "butterflies" is mostly obscured in the photomicrographs.

All preparations are osmium fixed; and hydrolyzed with N HCl for 9 min (60 C).

2a, 2b, and 2c are stained with SO_2-azure A (after Huebschman, 1952).

Figure 3. The arrangement of chromatin seen in thick cultures of germinating *Bacillus megaterium* spores at 60 min after inoculation (3a) and 120 min (3b). Both preparations fixed in osmium tetroxide vapor, hydrolyzed with N HCl (8 min, 60 C), and stained with SO_2-thionin (after DeLamater, 1951). Line drawings of the chromatin are shown opposite figures 2 and 3.



FIGS. 2-3—Continued

3.3 times that of the spore. The structures typical of this stage of development are shown in figure 3b.

DISCUSSION

Beaded granules of chromatin, in configurations similar to those described here, were observed by Delaporte (1950) in germinating spores and young vegetative cells of *Bacillus mycoides* (her figures 59 to 62). Using the same techniques as were applied here, Robinow (1953, his figure 6) has observed similar structures in germinating and vegetative cells of *B. megaterium*. Murray and Truant (1954), studying the moraxella, likewise observed beaded chromatin in what they called division stages. Granular chromatin has been observed by DeLamater (1952) and interpreted to represent the stages of a typical mitotic process, presumably a process in which separation of the chromatin (chromosomes) follows the

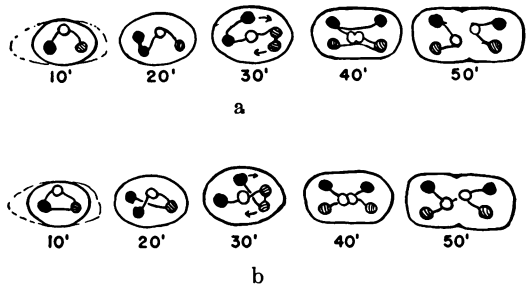


Figure 4. A diagrammatic outline showing two variations of coincidental linear duplication and separation of bacterial chromatin during spore germination. The different shading indicates a supposed genetic distinction between the granules.

(4a) The successive reduplication, rotation, and exchange of end granules starting with an open triad and ending with two similar structures.

(4b) A process similar to that in 5a, starting with a closed but ending with an open structure.

duplication. However, the studies reported here indicate that in germinating spores (at least) the duplication and the separation are going on at the same time and as a continuous process. In fact, many of the "metaphase" structures described by Hunter and DeLamater (1952) correspond to arrangements seen here when the desoxyribonucleic acid had just begun to increase. Since duplication and separation of the chromatin in the germinating spores proceed simultaneously, the process of mitosis as seen in higher forms would hardly apply.

Alternatives to mitosis. The chromatin structures seen in the initial stage of germination, before the desoxyribonucleic acid has increased, appear in stained preparations as a triad of granules often connected on at least two sides by fine strands. In the vegetative cell formed at the end of the germination process, two such triads are present and by then the desoxyribonucleic acid has doubled. Thus, during the germination period the chromatin of the spore was duplicated both in amount and in arrangement. Two mechanisms of coincidental duplication and separation of the chromatin of germinating bacteria which fit the structural and chemical changes observed come to mind as alternatives to a typical mitosis, a linear and a lateral process. In the discussion to follow, an assumption is made that each granule or condensation of the original triad is a distinct genetic structure and that this distinction is carried over into the daughter triads.

In the first of these processes (figure 4), each granule reduplicates itself until a chain of six granules has formed, which then breaks into two new groups of three. In order to distribute the three different granules into each daughter triad, certain restrictions of the linear arrangement become essential. Moreover, the gradual and continuous increase in chromatin is coincident with the steady increase of desoxyribonucleic acid in synchronously germinating cultures, and the Y, X, and double V forms observed all suggest that duplication might be occurring first in one granule, then in the others in succession. In a linear process, an exchange of end granules during division is required in order to distribute the newly formed chromatin equally in the separating structures.

In figure 4, two possible variations of this linear process are outlined. Each granule of the original triad is given a distinctive shading to represent the supposed genetic differences. In the first variation the original triad is assumed to be an open structure, as interpreted from the photomicrographs; and the process of successive linear duplication, rotation, and exchange of end granules necessary to produce two such open daughter triads is outlined. The exchange of granules in this scheme involves a breakage of strands at the completion of separation. If the triad is assumed to be a closed structure, no simple process of linear growth will produce similar closed triads (figure 4b). The limitations of these linear processes indicate that a second mechanism may be more applicable.

The second process of side to side reduplication of the original triad which would satisfy the supposed genetic as well as the cytological and biochemical requirements is outlined in figure 5. This reduplication again should be continuous and occur with separation. If the basic nuclear triad is open on one side (as shown in figure 5), then three possible schemes of side to side duplication exist depending upon where the structure first starts to split apart: (1) from both ends towards the center, (2) from one end toward the other through the center, or (3) from the center outwards. In the first variation (figure 5a) one end granule starts to divide slightly ahead

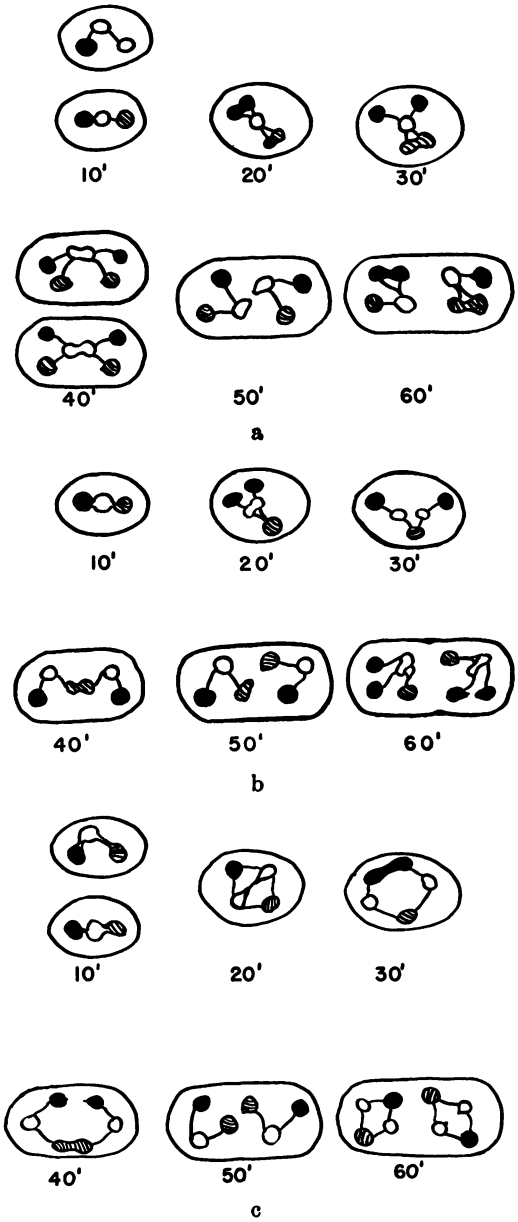


Figure 5. A diagrammatic outline showing 3 variations of coincidental lateral duplication and separation of bacterial chromatin during spore germination (viewed largely from above). The different shading indicates a supposed genetic distinction between the granules.

(5a) The original triad splits from both ends toward the center, one end ahead of the other.

(5b) Splitting from one end, through the center, to the other end.

(5c) Splitting first from the center to one end, then the other.

of the other forming a "Y" pattern. The whole structure rotates within the developing spore at 20 to 40 min and eventually distributes the three supposedly distinct parts of the chromatin into each half of the growing cell. At 40 min the chromatin of the central granule is being duplicated as the still attached parts are pivoting apart. Although drawn in the same plane in figure 5a, many of the dividing triads at this stage appear rotated about the central granule in the long axis of the cell, giving the illusion of axial filaments (see figure 2b). At 50 min the separation and duplication are shown completed, but continued growth of the triads after separation is indicated. Splitting of the triad from one end and the fanning out of the growing chromatin about the other end are shown in figure 5b. Finally the splitting of the triad about the central granule, followed by the separation of first one and then the other of the end granules, is shown in figure 5c. Structures dividing by any of these variations would tend to show similar arrangements in the later stages (40 to 50 min) of separation. If the triad of chromatin is a closed triangular structure, then end and middle granules become indistinguishable and these three variations become the same process. One mechanism of gradual lateral duplication beginning first in one granule, following in the second, and ending in the third would then show the necessary continuous desoxyribonucleic acid rise and chromatin separation.

In figures 4 and 5, the growing structures are drawn in cell outlines, the dimensions of which are the average of some 20 cells. The change in cell outline from a large ovoid at 30 min to a more cylindrical form at 40 min was substantiated by statistical analysis of cell measurements (Fitz-James, 1954 unpublished results). The greater cell width at 30 min might be related to the rotation of the chromatin as outlined in figures 4 and 5.

Three dimensional scale models in colored plasticine (figure 6) when examined from different angles and at various stages of rotation were helpful in explaining many of the arrangements seen in the photomicrographs. The lateral duplication scheme illustrated in figures 5a and 6 would appear to suit best most of the arrangements seen in germinating spores of *B. cereus* and *B. megaterium*.

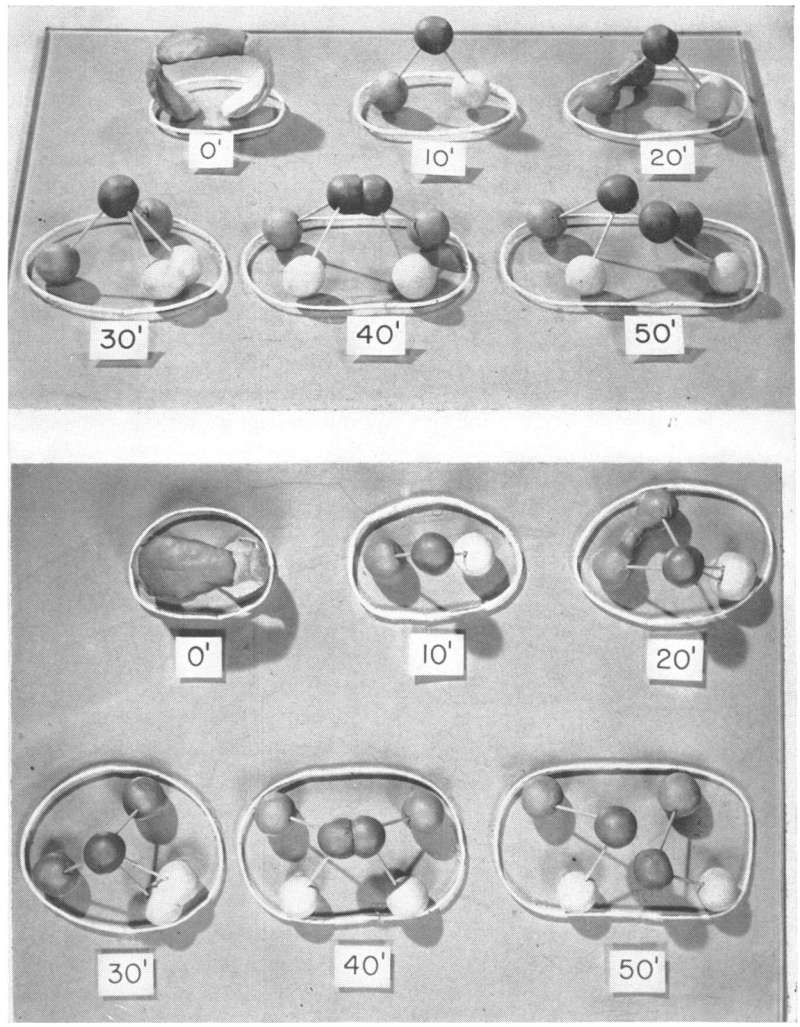


Figure 6. Photographs of colored plasticine models of duplicating chromatin as interpreted in figure 5a viewed from in front and from above. The different colors indicate the supposed genetic distinction of the 3 granules of the basic triad. The shape of the chromatin in the resting spore (0 min) is taken from previous publications (Robinow, 1953; Fitz-James, 1953).

SUMMARY

The continuous increase of desoxyribonucleic acid coincident with the chromatin separation in germinating spores suggests that a typical mitosis is not occurring. Alternative schemes of chromatin duplication and separation are suggested.

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