TEMPERATURE SYNCHRONIZATION OF NUCLEAR AND CELLULAR DIVISION IN *BACILLUS MEGATERIUM*[1, 2]

M. ELIZABETH HUNTER-SZYBALSKA,[3] WACLAW SZYBALSKI, AND EDWARD D. DELAMATER[4]

Section on Cytology and Cytochemistry, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania; and the Institute of Microbiology, Rutgers University, the State University of New Jersey, New Brunswick, New Jersey

Received for publication April 25, 1955

It was accidentally observed by the senior author that, when agar plates supporting young cultures of *Bacillus megaterium* were stored for a short time in the refrigerator, the cytological picture was strikingly and uniformly changed. The nuclear material of actively growing cells appears both filamentous and granular (figure 1). Stained nuclei of refrigerated cells (figures 2 and 3) became highly condensed masses with two distinct granules, in a configuration suggesting the mitotic figure. Similar action of chemical bacteriostatic agents on divisional stages of the microbial nucleus was recently described by the present authors (DeLamater, 1953; DeLamater *et al.*, 1955). On the other hand, it is well known that low temperatures may affect the course of mitosis or meiosis in cells of many organisms (Belling, 1925; Barber and Callan, 1943, among others). Chilling decreases the rate of chemical reactions associated with nuclear division, but its effect is apparently not uniform throughout the divisional cycle. Certain stages are considerably more affected than others, resulting in the accumulation of a particular nuclear configuration. This phenomenon was utilized by McClintock (1945) for synchronization of the meiotic divisions in Neurospora (*personal communication*) and by Hotchkiss (1954) for synchronization of cell division in pneumococci. For the same purpose, Scherbaum and Zeuthen (1954) used intermittent heat treatment of Tetrahymena cells.

The present paper describes the synchronization of nuclear and cellular division in the bacterial species *Bacillus megaterium*, and is a logical outcome of our preliminary observations (Szybalski and Hunter-Szybalska, 1955) and the studies of other authors, some of which have been discussed.

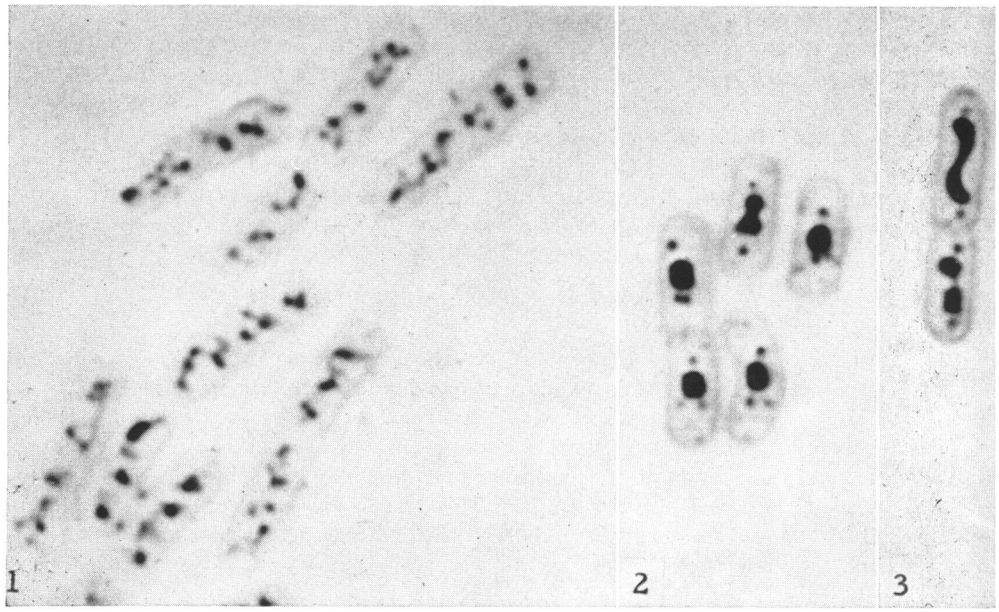
Since the experimental work was completed, a few papers have appeared bearing directly on this subject. These include a description of synchronized cell division in Salmonella induced by intermittent heat and cold treatment (Lark and Maaløe, 1954), the editorial of Adams (1954), and the cytological studies of nuclear division in *B. megaterium* by Fitz-James (1954). In the work of Fitz-James the synchrony of nuclear and cellular division was based on the assumption of simultaneous germination of the spores. This method was previously used by McGregor (1954) in the studies of the nuclear behavior of *Streptomyces sp.* Other methods leading to controlled cell division include differential centrifugation (Yanagita, 1954), fractional filtration (Maruyama and Yanagita, 1955), and classification of the cells according to their form (Knaysi, 1942) or to their length (Dondero and Zelle, *personal communication;* Adolph and Bayne-Jones, 1932). Hegarty and Weeks (1940) showed that loga-

[1] A summary of this work was presented at the 55th meeting of the Society of American Bacteriologists, New York City, May 8–12, 1955 (Szybalski and Hunter-Szybalska, 1955).

[2] The preliminary observation and the cytological studies of the effect of cold on agar-grown bacteria (including figures 1–3) were performed at the University of Pennsylvania, under the support of grants from the Atomic Energy Commission, Contract No. AT-(30-1)-1341, from Eli Lilly and Company, and from the National Institutes of Health, Public Health Service (PHS No. C-2189). Subsequent studies herein reported on synchronized growth in broth with simultaneous nuclear staining and microphotography were executed at Rutgers University by W. S. with the collaboration of the first author.

[3] Present address: Warner-Chilcott Laboratories, Morris Plains, N. J.

[4] Present address: Section on Cytology and Genetics, Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia 4, Pa.



Figures 1–3. Influence of chilling on cells of *Bacillus megaterium* growing on agar (fixed in OsO_4 vapors, hydrolized in 1 N HCl, stained with thionin-SO_2 according to DeLamater (1951), mounted in "Abopon" solution). *1:* Actively growing cells from a 1½-hr-old agar culture (34 C). *2 and 3:* 1½-hr-old cells chilled 1 hr in the refrigerator (3 C).

rithmic multiplication in cultures of *Escherichia coli* was represented by a line with definite though slight undulations "possibly due to simultaneous division of many cells".

In thymine-deficient *Escherichia coli* synchronized by thymine starvation, Barner and Cohen (1955) were able to demonstrate stepwise synthesis of desoxyribonucleic acid (DNA) preceding stepwise duplication of the cells. This disproves the widely held contention that the synthesis of the nuclear material in bacteria proceeds in a continuous fashion (Fitz-James, 1954; Tulasne and Vendrely, 1954).

METHODS

Organism. The strain of *B. megaterium* used in previous studies (DeLamater *et al.*, 1955) served as the test organism. Stock cultures were maintained on nutrient agar slants.

Media. Double strength nutrient broth (16 g per L, Difco) was used in the present studies (Szybalski, 1954). The agar medium contained: beef extract, 0.3 per cent; peptone, 0.5 per cent; yeast extract, 0.5 per cent; agar, 1.5 per cent; and was adjusted to pH 7.0.

Experimental procedures. In the preliminary experiments, bacteria were grown in shaker flasks for 4 hr and a heavy inoculum was spread on the pre-dried surface of an agar plate. After incubation at 34 C for 1 hr, the plates were transferred to the refrigerator at 3 C. After varying periods in the cold, the plates were returned to the 34 C incubator. Cytological preparations were made at various intervals during exposure to shifting temperatures.

It was soon realized that liquid cultures would have to be employed for a critical control of the temperature and time of incubation. Bacteria were grown in a shaker flask partially immersed in a water bath and agitated by means of a Burrell wrist-action shaker. Temperature changes involved the quick substitution of another water bath adjusted to the required temperature. Samples for cytological examination and for enumeration of bacteria were taken at measured intervals by means of a graduated pipette. Dilutions were made in chilled saline containing 0.05 per cent "Triton WR-1339;" 0.1 ml of suitable dilution was spread with a glass rod on the surface of a pre-dried agar plate. All counts were done at least in quadruplicate.

Fixation and staining procedures. Fixation in the vapors of 2 per cent osmium tetroxide is a very successful procedure for bacteria grown on

the surface of agar. However, it has to be substantially modified for liquid suspensions of bacteria. The following problem has to be considered. Cells must be almost instantaneously concentrated to the semi-solid state suitable for vapor fixation, because it was often demonstrated that fixation by immersion in a solution of osmium tetroxide introduces considerable distortion. Filtration on "Millipore" filters (Lovell Chemical Co., Watertown, Mass.) seems to satisfy both the criteria of speed and convenience of handling (Szybalski, *unpublished data*). Small discs of "Millipore" filters (about 1 cm in diameter) were cut out with a cork borer and supported by a sintered glass plate (the lower part of the "Millipore Pyrex filter holder"), covered with a thin rubber membrane having a hole of 8-mm diameter. Applying a suitable vacuum, the filtration of 1 ml of bacterial suspension was accomplished in 2 to 5 sec. The disc was then immediately transferred to the surface of a block of agar resting in an OsO_4 chamber. After fixation for 3 min in the OsO_4 vapors, the heavy bacterial deposit was transferred from the filter disc to cover slips. This was done by lifting the block of agar supporting the filter disc with a spatula serving as an electrical conductor when held by a wet hand, and making an impression smear on the cover glass. Avoiding air drying during transfers, these smears were hydrolized in 1 N HCl at 60 C for 5 min and stained in 0.25 per cent thionin containing 0.1 per cent $SOCl_2$ for 1 hr according to DeLamater (1951). The preparations were mounted in a water-soluble mounting medium composed of 2 g of "Abopon" (Glyco Products, Inc. Brooklyn, N. Y.) in 1 ml water (Lieb, 1947).

Photomicrography. The "B. & L." research microscope used was equipped with an achromatic condenser, a 90× apochromatic objective of 1.4 N.A., and 10× compensating oculars. The light source was a 100-watt ribbon filament lamp or 40W zirconium lamp provided with Wratten filters no. 15 and 58 or appropriate interference filters. The system was adjusted for Köhler illumination. Photographs were taken on "Panatomic X" film at a camera length of 20 in. The final magnification on the prints was 4500×.

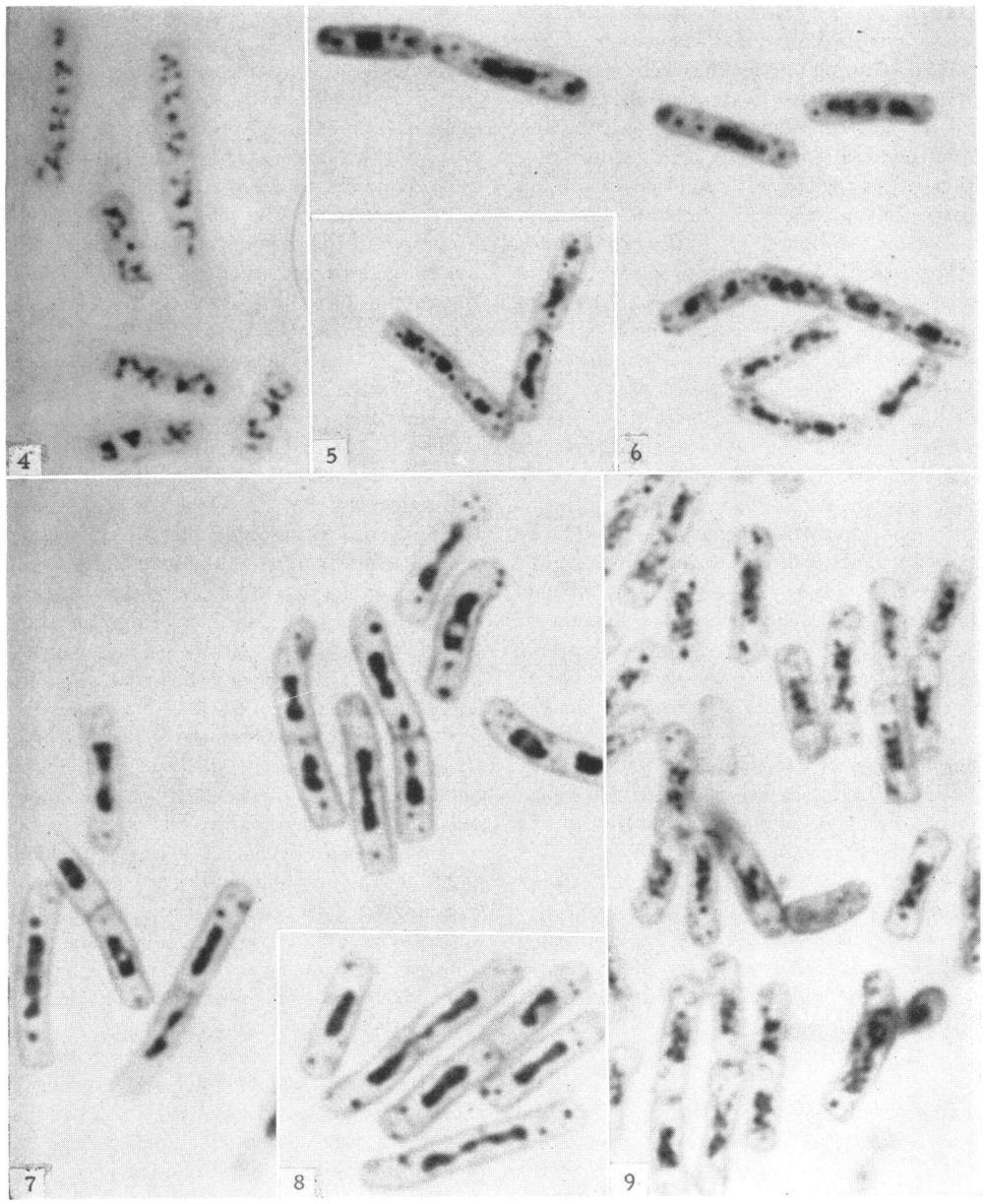
RESULTS

Synchronization of Cellular Division. An active inoculum was prepared by several transfers of barely turbid cultures grown in shaker flasks at 34 C as described in "Methods." In the first experiment, this active inoculum was so adjusted as to obtain a starting concentration of cells of approximately 10^6 cells per ml. The culture was shaken at 34 C and the number of bacteria was assayed every 5 min, starting 30 min after inoculation. The plot of time against the logarithm of the number of cells gave a straight line through the period of over 48-fold increase in the number of cells. The calculated mean generation time amounted to 33.6 min at 34 C.

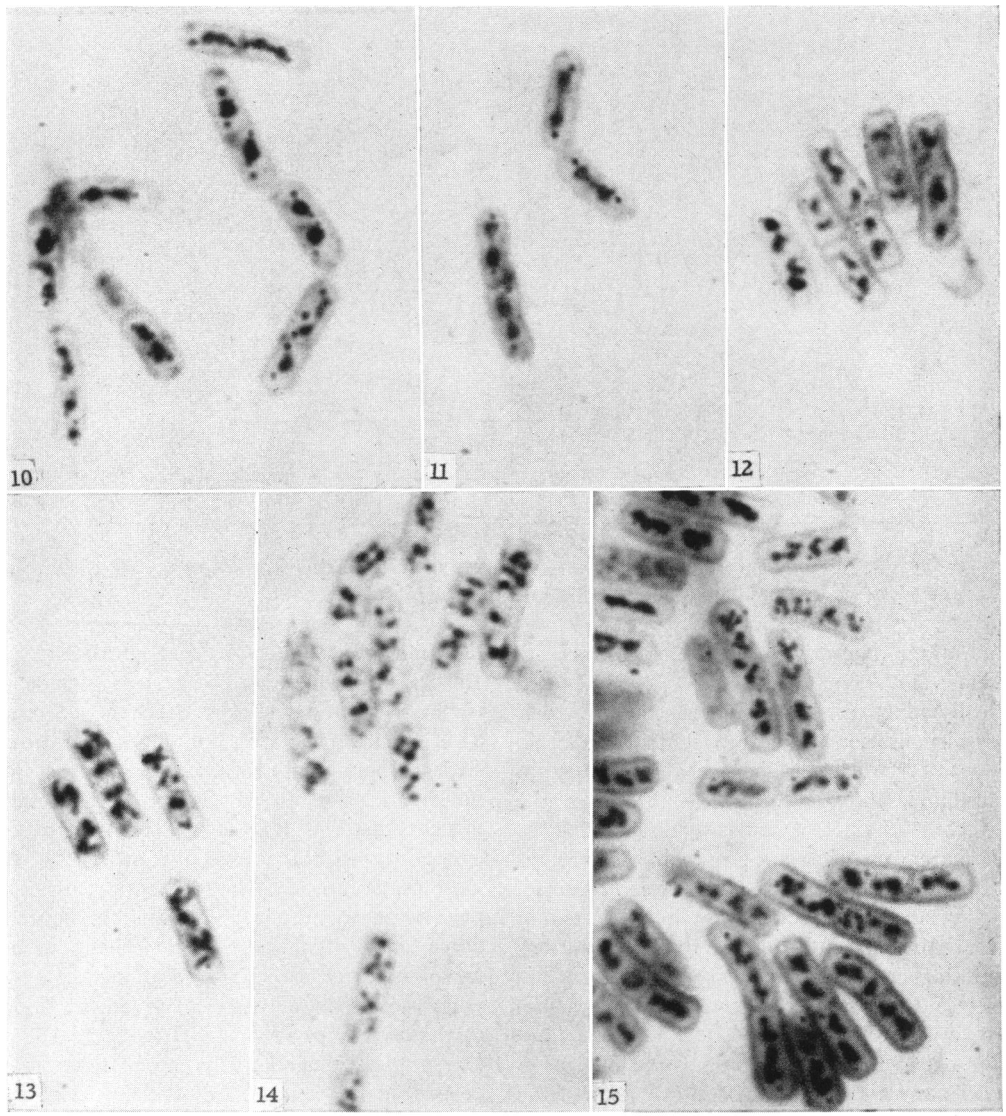
The main experiment, demonstrating the effect of cold on the course of bacterial multiplication, was composed of three parts: incubation at 34 C, chilling to 12 C for 30 min, and reincubation at 34 C. Throughout all these operations, flasks were energetically agitated. When the logarithms of the numbers of cells were plotted against time, the following result was obtained: The part of the curve preceding the chilling represented a straight line as already described. During the chilling period, there was practically no increase in the number of the cells beyond the first 5-min period. After the reincubation at 34 C, a complex curve was obtained. As represented in figure 16, it is composed of relatively stationary periods and burst-like, 2-fold increases in the number of cells. The periods between bursts roughly correspond to the mean generation time as previously determined. The preliminary lag period is about 40 min, but it depends to some degree on the temperature and the length of the chilling period. These data are analogous to the results of Hotchkiss (1954) and indicate a cold-effected synchronization of bacterial division.

Cytological Studies. Cytological appearance of control material. Nuclei of actively growing cultures are shown in figures 1 and 4. All divisional stages are represented. Metaphase- and anaphase-like configurations are relatively uncommon, indicating a correspondingly shorter duration of these stages. The number of nuclei varies between 2 and 4 per cell. During the whole pre-chilling period, the picture is essentially the same. The normal course of nuclear division in *B. megaterium* has been described in more detail by DeLamater and Hunter (1951) and DeLamater (1951, 1953).

Effect of chilling. Chilling the actively growing culture of *B. megaterium* to 12 C produced striking changes in the appearance of nuclei in stained



Figures 4–9. Influence of chilling on cells of *Bacillus megaterium* growing in broth. (Instantaneously filtered on "Millipore" pad, fixed in OsO_4 vapors, hydrolized in 1 N HCl, stained with thionin-SO_2, mounted in "Abopon" solution). *4:* Actively growing cells from an aerated broth culture (approximately 10^6 cells per ml; 34 C). *5 and 6:* The same culture after chilling 30 min and 1 hr at 12 C; increased optical density of nuclei, predominance of metaphase-like figures. *7 and 8:* The same culture after chilling 2 and 3 hr at 12 C; elongation of condensed nuclei in elongating cells. *9:* Cells chilled 3 hr and reincubated 10 min; revelation of discrete structure within previously condensed, seemingly undifferentiated nuclei.



Figures 10–15. Recovery from the bacteriostatic effect of cold in a synchronized culture; preparations made in parallel with cell counts diagrammed in figure 16 (staining procedures as in figures 4–9). *10:* Cells chilled 30 min (12 C); effect of cold on the nuclei, as also shown in figure 5. *11:* The same culture reincubated for 5 min at 34 C after chilling 30 min; resumption of divisional activity in the nuclei; separation of still condensed chromosomal masses. *12:* Reincubation for 10 min at 34 C; separation of sister chromosomal masses progressed further; decreased optical density of the nuclei. *13:* Reincubation for 15 min at 34 C; reelongation of chromosomes in progress. *14:* Reincubation for 30 min at 34 C; chromosomes in state of nearly maximum extension; this nuclear configuration is characteristic of subsequent preparations made in parallel with the growth curve (figure 16). *15:* Reincubation for 50 min at 34 C; recurrence of condensed forms of nuclei coinciding with short period immediately following burst-like increase in cell number (figure 16).

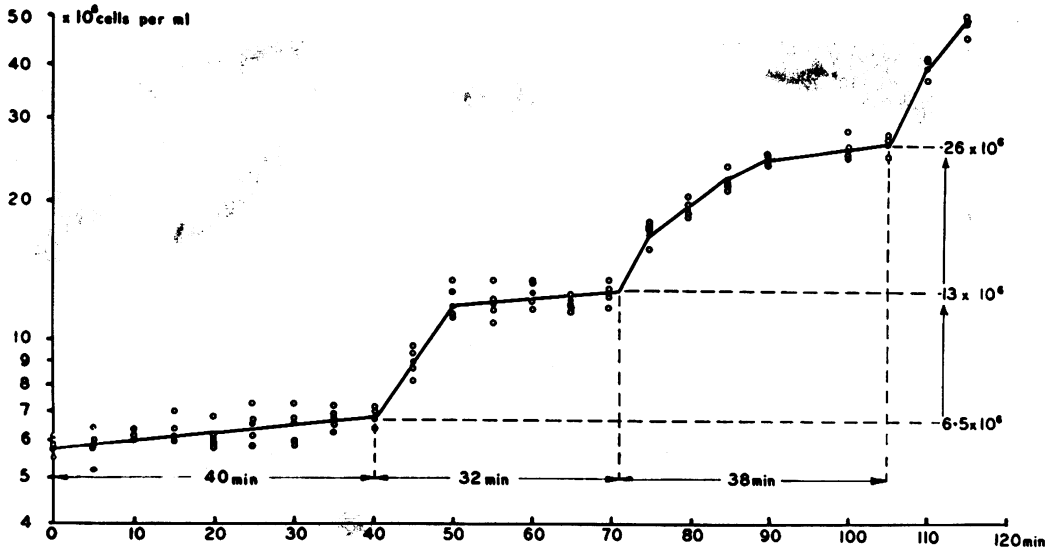


Figure 16. Course of bacterial multiplication immediately following a chilling period of 30 min at 12 C; time shown corresponds to the duration of reincubation at 34 C.

preparations (figures 5 to 8). Three effects were obvious: (1) The stained nuclei were larger and optically much denser. As a consequence, their gross visualization was improved but fine structure was obscured. (2) The size was similarly increased and consequently the visualization of the small but distinct granules corresponding to DeLamater's centrioles, or terminal beads or buds in Knaysi's (1955) nomenclature, was improved. (3) Almost all of the nuclei were in the phases of nuclear division characterized by configurations simulating metaphase and early anaphase. Conversely, nuclei showing filamentous chromosomes were very rare.

Figures 5 to 8 show the progressive changes in the cells transferred from 34 to 12 C. Each of the "haploid" nuclei visible in figure 4 grows into a composite, metaphase-like structure of high optical density. These changes became perceptible after only 10 min of chilling, but were much more striking at 30 min (figure 5). During prolonged chilling of the agitated culture, up to 3 hr (figures 6 to 8), the cells increased in length with elongated dense nuclei lying in their long axes. The multiplication of presumed centrioles proceeded with their consequent accumulation in the polar regions of the cells (figure 7).

Reincubation. When the chilled culture was returned to the 34 C bath, evidence of renewed divisional activity in the nucleus became rapidly apparent. Figures 10 to 15 represent the cycle of nuclear changes parallel to the synchronized multiplication of the cells diagrammed in figure 16. Cultures which had been cooled for 30 min (figure 10) showed changes after only 5 min at 34 C (figure 11). After 10 min, separation of sister chromosomal masses was complete in most nuclei and the optical density of the stained nuclear material was somewhat reduced, concomitantly with the re-elongation of the chromosomes (figure 12). The further progress of this process at 15 min is represented in figure 13. After 20 to 30 min the chromosomes in most nuclei appeared to be in a state of nearly maximum extension (figure 14). Throughout the rest of the growth curve depicted in figure 16, only rare preparations showed accumulations of condensed nuclei (figure 15)—occurring during the periods immediately following the burst-like increases in cell number, at about 50 and 85 min after the transfer to 34 C.

Figure 9 represents a 10-min reincubation at 34 C of a culture chilled for 3 hr (figure 8). The recovery process reveals the complex structure of the previously condensed, elongated nuclei and leads to the establishment of the normal cytological picture in these "transient polyploid" nuclei (or "compound" nuclei, according to Knaysi, 1955).

DISCUSSION

The experimental data clearly document the synchronizing effect of cold on cellular division, as first demonstrated for bacteria by Hotchkiss

(1954). In addition, we have studied the effect of chilling on bacterial nuclei and the correlation between the nuclear and cellular division cycles with two main purposes in mind: the elucidation of the mechanism of synchronization, and further confirmation of the mitotic cycle in bacteria.

The large accumulation of nuclei in configurations simulating the mitotic figure indicates that the chilling process selectively inhibits the reactions connected with the separation of sister chromosomes. Synthesis of nuclear material does not seem to be predominantly affected as inferred from the increase in the stainability and size of the nuclei during the low temperature incubation. Similarly, the growth, augmentation of stainability, and even multiplication of the small centriole-like granules proceeds during this period in the cold. Each composite, metaphase-like structure (figure 5) or elongated complex nucleus (figures 6 to 8) originates from a single nuclear complement of the logarithmically growing cells (figure 4). All these observations lead to the conclusion that the chemical or physical reaction responsible for the final step in the separation of sister chromosomes, i. e., the progression from metaphase to telophase, must be highly sensitive to the decrease in temperature. This high temperature coefficient suggests a high energy of activation. As inferred from the continuous increase in the size and density of nuclei exposed to the cold, synthesis of DNA or other stainable material does not seem to constitute the essence of this reaction. Chemical work as to the nature of this reaction is in progress.

There is striking similarity between the action of cold and the influence of several bacteriostatic agents on the bacterial nucleus (Johnson and Gray, 1949, DeLamater *et al.*, 1955, among others). However, the experiments on the synchronization of bacterial division with bacteriostatic chemicals have not yet been completed.

The studies of time correlation between cellular multiplication and nuclear division demonstrate that both processes proceed in parallel, with nuclear duplication almost a whole cycle ahead. This work is now being conducted independently at the University of Pennsylvania and Rutgers University. The distinct cyclical behavior of nuclear division is in disagreement with the theory of continuous growth of nuclear material as described by Fitz-James (1954). It is difficult, however, to trace the source of this discrepancy, since some experimental details of synchronization and DNA analysis are not supplied by that author.

The simplest conclusion compatible with the experimental data would assume that the process of nuclear division in *B. megaterium* is analogous to that in higher organisms as postulated by DeLamater (1951). The compactness of the nuclei during the chilling process makes it difficult to study their fine structure. The complexity of the nuclear masses, however, is visualized during the recovery period. Correlation between the nuclear and cellular cycles was reported also by Lark and Maaløe (1954) even though the smaller size of Salmonella cells and the method of OsO_4 fixation may have interfered with cytological observations.

Experiments on synchronized cells have yielded information about the nuclear cycle in bacteria, as predicted in the editorial of Adams (1954). Selective low-temperature inhibition of the reaction responsible for the separation of sister chromosomes causes an accumulation of metaphase-like configurations, with consequent synchronization of nuclear division once this temperature block is removed. Cellular divisions and their synchronization appear to be a direct consequence of the nuclear behavior.

ACKNOWLEDGMENTS

The active interest, encouragement, and helpful suggestions of Drs. S. A. Waksman and Stuart Mudd are acknowledged with gratitude by the Szybalskis.

SUMMARY

In actively growing cells of *Bacillus megaterium* (34 C), the nuclei become highly stainable masses made up of contracted chromosomes and associated with distinct centriole-like granules, as a result of a short exposure to the suboptimal temperature of 10 to 15 C. Practically all observed nuclei appear in this metaphase-like configuration. Reincubation at 34 C results in resumed multiplication, synchronous for all the cells and nuclei as ascertained by viable count and cytological studies. Following a 40-min lag period, the number of bacteria increases in stepwise fashion. Each cycle corresponds to one bacterial generation and is composed of a burst-like duplication and a relatively stationary period. Nuclear reduplication precedes cellular division. Sister chromosomes separate within 5 to 15 min after transfer to 34 C. The nuclear components become progressively more filamentous and dis-

persed. It was possible, however, to observe accumulations of condensed metaphase-like nuclei in the very short intervals coinciding with the onset of the stationary phase of each cellular duplication cycle.

The use of dilute, liquid cultures necessitated a process for rapid concentration of the cells. Almost instantaneous filtration on "Millipore" filters was followed by fixation in OsO_4 vapors, HCl hydrolysis, and staining with thionin.

The conclusion may be reached that the most obvious effect of decrease in temperature is on the rate of some chemical or physical reaction causing the final separation of sister chromosomes (the transition from metaphase to telophase). Accumulation of the metaphase-like structures is a natural consequence of this block. Simultaneous onset of further multiplication results in synchronization of nuclear division. Thus the transfer to normal temperature would result in simultaneous divisions of nuclei and consequent synchronization of duplication on the cellular level. Nuclear division in cells thus phased resembles the mitotic cycle in higher organisms.

REFERENCES

ADAMS, M. H. 1954 Synchronization of cell division in microorganisms. Science, **120**, 793.

ADOLPH, E. F. AND BAYNE-JONES, S. 1932 Growth in size of micro-organisms measured from motion pictures. II. *Bacillus megatherium*. J. Cellular Comp. Physiol., **1**, 409–427.

BARBER, H. N. AND CALLAN, H. G. 1943 The effect of cold and colchicine on mitosis in the newt. Proc. Roy. Soc. (London) Ser. B, **131**, 258–271.

BARNER, H. D. AND COHEN, S. S. 1955 Synchronization of division of a thymineless mutant of *Escherichia coli*. Federation Proc., **14**, 177.

BELLING, J. 1925 Production of triploid and tetraploid plants. J. Heredity, **16**, 463–464.

DELAMATER, E. D. 1951 A new cytological basis for bacterial genetics. Cold Spring Harbor Symposia Quant. Biol., **16**, 381–412.

DELAMATER, E. D. 1953 Structure and division of the bacterial nucleus. VI. Int. Congr. Microbiol. Symp. Bacteriol. Cytol., Rome, 108–135.

DELAMATER, E. D. AND HUNTER, M. E. 1951 Preliminary report of true mitosis in *Bacillus megaterium*. Am. J. Botany, **38**, 659–662.

DELAMATER, E. D., HUNTER, M. E., SZYBALSKI, W., AND BRYSON, V. 1955 Chemically induced aberrations of mitosis in bacteria. J. Gen. Microbiol., **12**, 203–212.

FITZ-JAMES, P. C. 1954 The duplication of bacterial chromatin. J. Bacteriol., **68**, 464–473.

HEGARTY, C. P. AND WEEKS, O. B. 1940 Sensitivity of *Escherichia coli* to cold-shock during the logarithmic growth phase. J. Bacteriol., **39**, 475–484.

HOTCHKISS, R. D. 1954 Cyclical behavior in pneumococcal growth and transformability occasioned by environmental changes. Proc. Natl. Acad. Sci. U. S., **40**, 49–55.

JOHNSON, F. H. AND GRAY, D. H. 1949 Nuclei and large bodies of luminous bacteria in relation to salt concentration, osmotic pressure, temperature and urethane. J. Bacteriol., **58**, 675–688.

KNAYSI, G. 1942 The demonstration of a nucleus in the cell of staphylococcus. J. Bacteriol., **43**, 365–384.

KNAYSI, G. 1955 The structure, composition and behavior of the nucleus in *Bacillus cereus*. J. Bacteriol., **69**, 117–129.

LARK, K. G. AND MAALØE, O. 1954 The induction of cellular and nuclear division in *Salmonella typhimurium* by means of temperature shifts. Biochim. et Biophys. Acta, **15**, 345–356.

LIEB, E. 1947 Permanent stain for amyloid. Am. J. Clin. Pathol., **17**, 413–414.

MCCLINTOCK, B. 1945 Neurospora. I. Preliminary observations of the chromosomes of *Neurospora crassa*. Am. J. Botany, **32**, 671–678.

MCGREGOR, J. F. 1954 Nuclear division and the life cycle in a *Streptomyces sp.* J. Gen. Microbiol., **11**, 52–56.

MARUYAMA, Y. AND YANAGITA, T. 1955 Synchronous culture of *Escherichia coli*. Bacteriol. Proc., **1955**, 37.

SCHERBAUM, O. AND ZEUTHEN, E. 1954 Induction of synchronous cell division in mass cultures of *Tetrahymena pyriformis*. Exptl. Cell Research, **6**, 221–227.

SZYBALSKI, W. 1954 Genetic studies of microbial cross resistance to toxic agents. IV. Cross resistance of *Bacillus megaterium* to forty-four antimicrobial drugs. Appl. Microbiol., **2**, 57-63.

SZYBALSKI, W. AND HUNTER-SZYBALSKA, M. E. 1955 Synchronization of nuclear and cellular division in *Bacillus megaterium*. Bacteriol. Proc., **1955**, 36–37.

TULASNE, R. AND VENDRELY, C. 1954 Conception nouvelle sur la division de l'appareil nucléare chez les bactéries. Schweiz. Z. allgem. Pathol. Bakteriol., **17**, 649–658.

YANAGITA, T. 1954 Single step growth method for *Escherichia coli* B. Bacteriol. Proc., **1954**, 29–30.