SYNCHRONIZATION OF DIVISION OF A THYMINELESS MUTANT OF *ESCHERICHIA COLI*[1]

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Various aspects of the metabolism of the thymine-requiring mutant of *Escherichia coli* strain 15_{T-} have been studied in this laboratory (Barner and Cohen, 1954). Thymine appears essential only for the synthesis of normal deoxyribonucleic acid (DNA) and, in the absence of thymine, numerous synthetic processes may continue unabated despite the prevention of DNA synthesis and cell division. These continuing synthetic processes result in the production of numerous polymeric substances including ribonucleic acid (RNA) and protein. This growth is also evident in the enlargement of the cells, and in the increase of turbidity of the culture (Cohen and Barner, 1954). A culture of 15_{T-} in a complete medium lacking only thymine will oxidize glucose at an increasing rate and will adapt to and metabolize new substrate such as xylose (Cohen and Barner, 1955). After a period of growth in the absence of division as a result of thymine deficiency, the cells rapidly lose the ability to multiply, as tested in the presence of thymine. Two types of hypotheses may be proposed to explain this consequence of unbalanced growth: *a*. The cytoplasm has developed a structural framework or composition within which a nucleus (and subsequently the entire cell) can no longer divide even when provided with thymine, or *b*. nuclei possessing an incomplete complement of DNA have nonetheless passed through an irreversible change, perhaps division, and can no longer be restored to a normal condition when supplied with thymine. Under either hypothesis the depletion of thymine may be expected to bring all the cells to a critical stage in the process of preparing for division. It was reasoned that, if thymine were added to the deficient culture at precisely the moment when the cells had reached this critical stage, the cells might then enter simultaneously into DNA synthesis and following the formation of these essential polymers, into synchronous cell division.

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MATERIALS AND METHODS

Preparation of bacteria. The various media used and the routine handling of strain 15_{T-} have been described earlier (Barner and Cohen, 1954).

In the estimation of viable count the dilution tubes of mineral medium without energy source were maintained at 15 to 20 C. The number of viable cells was estimated from the number of colonies appearing within 16 hr at 37 C when plated on nutrient broth agar (Difco) to which NaCl, 5 g per liter had been added. Dilutions were estimated so that each plate contained approximately 100 colonies. Each point on the graphs represents the average of duplicate plates. An accuracy approaching ± 10 per cent was routinely attained by these methods.

Phage techniques. Purified stocks of T_{2r}^{+}, prepared as described previously (Cohen and Arbogast, 1950*a*) were used. Broth-grown cells were required for all phage experiments since cells grown in the synthetic medium will not readily adsorb T_2 (Barner and Cohen, 1954). Cultures grown in broth were centrifuged and the cells were washed. For thymineless growth they were resuspended in the synthetic medium supplemented with casamino acids, tryptophan, and glucose. With one exception, phage techniques were those described in earlier papers (Cohen and Arbogast, 1950*a*) the very low adsorption value of T_2 on 15_{T-} obtained with these methods (differential centrifugation) was verified by standard procedures utilizing antiserum to T_2 phage (Adams, 1950). Antiserum was prepared in rabbits (Cohen and Arbogast, 1950*b*) and was standardized according to Adams (1950). Antiserum was also used in the one-step growth experiments to kill free phage and thus allow direct counts of initial infectious centers.

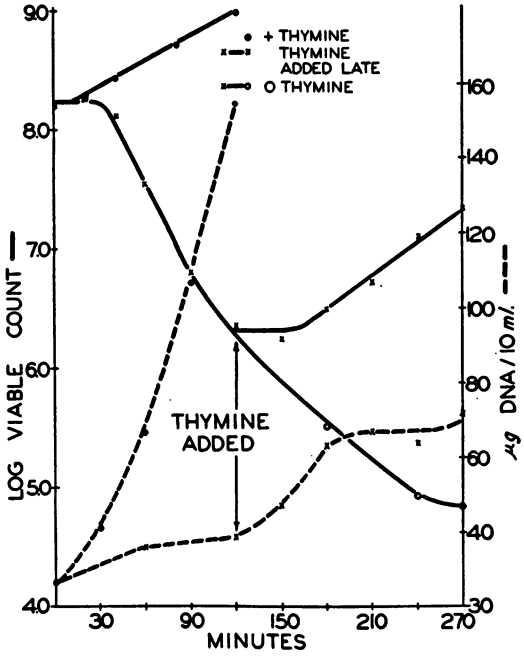

Figure 1. The effect on viable counts and DNA synthesis of delaying the addition of thymine to incubating cultures of 15_{T-}. Control—thymine added at zero time. DNA and viable count were determined periodically on aliquots from the same culture.

Chemical estimations. Aliquots for determination of the nucleic acid were precipitated at 0 C with one-tenth volume of 50 per cent trichloroacetic acid (TCA). The precipitate was washed with cold 5 per cent TCA and was twice extracted with 5 per cent TCA at 90 C for 15 min. DNA and RNA in the extract were determined by the diphenylamine reaction and by the orcinol reaction, respectively.

RESULTS

Readdition of thymine to a dying culture. Strain 15_{T-} was grown in the exponential phase to about 2×10^8 bacteria per ml in the glucose-NH_4^+ medium containing 2 μg thymine per ml. The cells were chilled to 10 C, sedimented, washed twice in chilled mineral medium, and resuspended in this medium containing glucose. Thymine was added to or withheld from aliquots of these cells. Exponential increase of cell number and of DNA proceeded in the culture to which thymine had been added. In the medium deficient in thymine, the cells maintained their viable count unchanged for 30 min and then began to "die," or irreversibly lost the power to multiply when plated on broth medium. In the interval before death was observed there was a slight increase in DNA, synthesis of which then ceased. These data are presented in figure 1.

When viable cell counts had fallen in the thymine-depleted culture to *ca.* 1 per cent of the original, thymine was added to the culture. In figure 1 it can be seen that the cell number remained unchanged for about 30 to 45 min although a more than 50 per cent increase in DNA content was observed. After this point the surviving cells began to multiply at a normal rate. At this time the extensive DNA synthesis which had taken place in the dead cells came to a halt, and presumably normal DNA synthesis proceeded in the 1 per cent of surviving multiplying cells. DNA synthesis of this order was

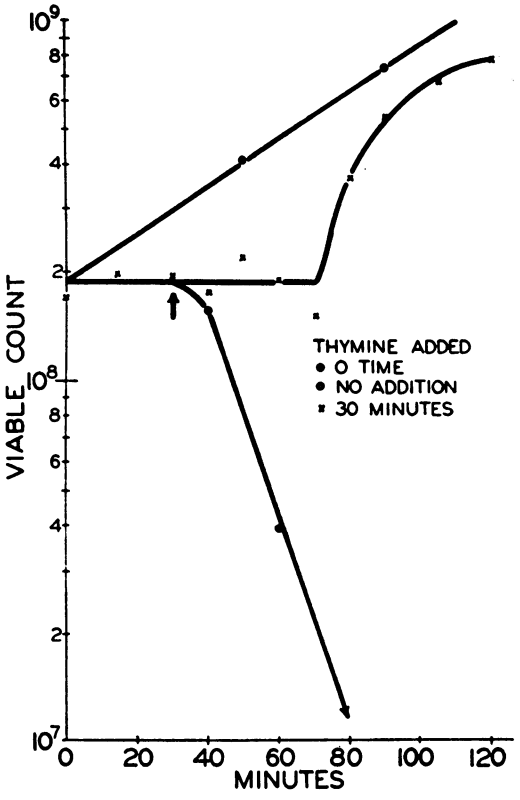

Figure 2. Synchronization of cell division by the delayed addition of thymine. An arrow indicates the addition of thymine at 30 min to the culture represented by the middle curve. No thymine was added to the culture represented by the lower curve. All plating was done on nutrient agar which contains thymine.

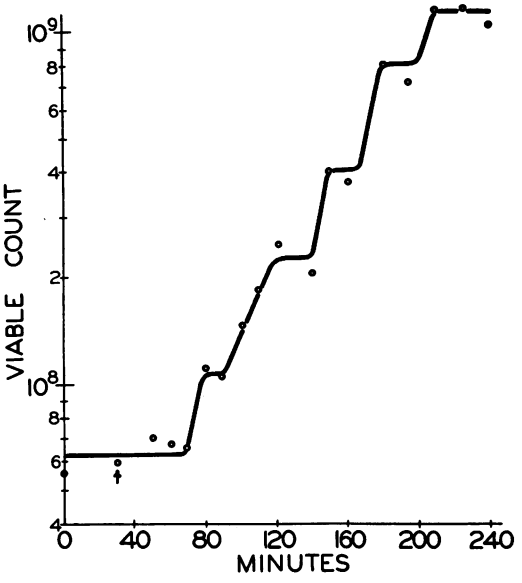

Figure 3. The persistence of synchronous cell division through four cycles. The clarification of synchrony in the later divisions which can be observed in this experiment was also observed in several later comparable experiments. An arrow indicates the addition of thymine at 30 min to a culture incubated in its absence. The post-division drop in viable count which appears in this experiment did not occur regularly.

below the level of detectability by our analytical methods. This experiment showed that cells which had lost the ability to multiply were nonetheless capable of extensive DNA synthesis. Such synthesis did not result in a recovery of the ability to multiply. A phasing of division was not detectable in this system under these conditions.

Addition of thymine to a depleted culture. When thymine was added to depleted bacteria prior to the inception of death in the culture, different effects were seen depending on the time of the re-addition of thymine. In the glucose -NH_4^+ medium, death did not usually occur until after 30 min of thymine starvation. When thymine was added at 20 min after depletion there was a lag of 20 min. A slight burst of division (40 to 50 per cent of the cells) occurred in the next 10 min, after which division in the culture proceeded at a rate comparable to that in a control undepleted culture. When thymine was added at 25 min, about 25 to 30 per cent of the bacteria divided between 40 and 45 min. Another lag was then apparent. The remaining cells began to divide at 65 min. After a single division of all the cells, phasing was no longer clearly observable in the culture. When thymine was added at 30 min after incubation in the thymine-free medium, i. e., just before the cells began to die, a lag for all of the cells was regularly extended about 35 min. A rapid division was then obtained, all of the cells dividing once within 10 to 15 min. Following the first cycle, two types of phenomena were then observed. In some instances, as in figure 2, the initial division was immediately followed by a second division.

However, in most experiments a short lag was perceptible after the first division. This result can be seen in figure 3 in an experiment in which the cells were permitted more than four divisions. It appears in this experiment and has been observed in several others that the lag between divisions becomes more pronounced after the second division. We attribute the rapid second division observed in this system to the existence of an already fully prepared cytoplasm, since it will be noted that from the beginning of thymine starvation there has been roughly 70 min of cytoplasmic synthesis without division.

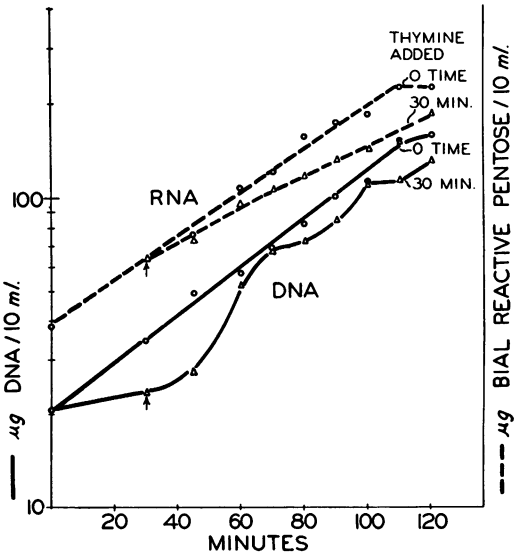

Figure 4. Nucleic acid synthesis in a synchronized culture and in a random culture. Medium—synthetic, containing glucose and NH_4^+. Thymine was added to the random culture at zero time and to the synchronized culture at 30 min.

In figure 3 it can be seen that following this growth of cytoplasmic components, cell number quadrupled in 50 min, a time approximating the normal doubling time of this organism in random culture. Cultures which are phased with respect to the increase in viable count in this manner did not show a phasing in the increase of turbidity in the culture. For a period of three divisions, the turbidity curve was essentially identical with that of the random control culture. Thus the usual turbidity increase was observed during cell growth without division which occurred for the 30-min intervals before and after thymine addition.

The experiments which are presented in figures 2 and 3 were selected from 10 experiments in which synchronous division occurred. Under the conditions described above synchronous division was obtained without exception.

It was observed that bromouracil at 3 μg per ml did not replace thymine in inducing division of a depleted culture. The bacteria remained at constant viable count without division for 15 min and then began to die at a rapid rate. In earlier studies (Cohen and Barner, 1956) it had been observed that bromouracil will support one division when supplied to bacteria before growth in the absence of thymine.

Nucleic acid synthesis in random and synchronized cultures. A liter culture of 15_{T-} in glucose-NH_4^+ medium was prepared by growth in the presence of thymine to 2×10^8 per ml. The bacteria were chilled, sedimented, and washed. The bacteria were resuspended in chilled media free of thymine and added, to a final concentration of 2×10^8 per ml, to two 400-ml aliquots of fresh medium; one aliquot contained 2 μg thymine per ml, the other lacked this substance. The cultures were aerated at 37 C, aliquots being removed periodically for viable counts and nucleic acid analysis. After 30 min, thymine was added at 2 μg per ml to the depleted culture and the experiment was continued. Viable counts revealed a pattern of division comparable to that reproduced in figure 3. In figure 4 the normal pattern of parallel DNA and RNA synthesis in the control (random) culture can be seen. In the culture lacking thymine, RNA synthesis was normal whereas DNA synthesis was very slight. On addition of thymine at 30 min, RNA synthesis continued although at a somewhat lower rate than that in the random culture. DNA synthesis began shortly after addition of thymine and was stepwise, passing through periods of rapid increase, followed by a lag in each case for the two observed cycles of division.

DNA synthesis preceded cell division and a calculation of DNA content per viable cell given in figure 5 revealed that in the synchronized culture, the DNA content slightly more than doubled before division occurred. In the random culture, it can be seen that the DNA content per cell was not appreciably changed. During actual division, DNA synthesis stopped. The failure of the DNA content of the cells to return to the base level after the first division is due to the rapid onset of the second cycle of DNA synthesis following division. However, even in the absence of a clear demarcation for the end of the first cycle, it can be seen that at the end of the second division at 110 min the DNA content per cell had fallen almost to the base line, indicative of a greater degree of synchronization of the cells at the onset of the third division.

The initial lag of division of thymine-depleted cells, the burst of DNA synthesis, and the lag in division following the resupply of thymine at 30 min and the burst of division of all of the cells

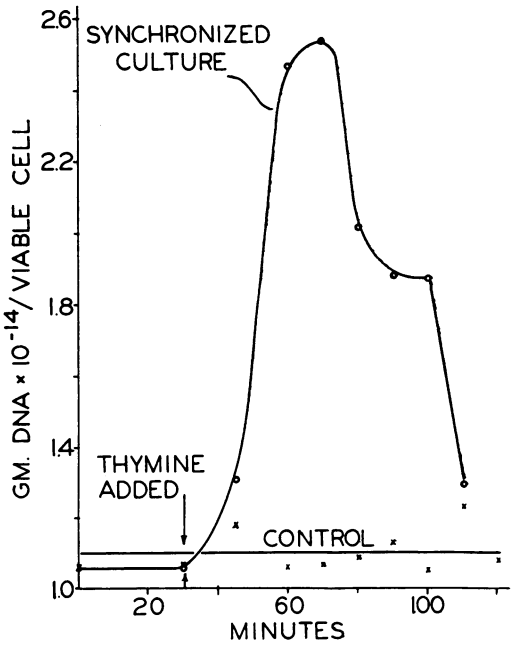

Figure 5. The variation in DNA content per cell in cultures grown and synchronized in a synthetic medium. These calculations are made from the data obtained in the experiment described in figure 4. Viable counts and nucleic acids were determined simultaneously in both random and synchronized cultures.

following this lag have been highly reproducible. Our subsequent experiments, therefore, have concentrated on this first cycle of lag and division, although additional data on the second cycle usually have been obtained as well. For example, with this system it has been possible to perform experiments on the ultracentrifugal properties of extracts of synchronized bacteria at the following points in the history of the culture: *a.* following growth without either division or death under conditions of thymine starvation; *b.* after DNA doubling without division following addition of thymine; *c.* during cell division without DNA synthesis. The detailed results of this investigation will be presented at a later date.

Virus infection of synchronized bacteria. Of the T phages, strain 15_{T-} is sensitive only to T_2. It was not possible to study T_2 infection in strain 15_{T-} grown and synchronized in the glucose-NH_4^+ medium by the addition of thymine. As reported earlier (Barner and Cohen, 1954), strain 15_{T-} grown in the glucose medium elaborates a capsular material which inhibits T_2 adsorption. The bacteria, therefore, were grown in broth, washed, and synchronized in the synthetic amino acid medium used in the earlier study. The division time in this medium is significantly less than in the glucose-NH_4^+ medium. It was found that in the amino acid medium, optimal results were obtained by addition of thymine at 20 min after growth in the absence of the pyrimidine, in contrast to 30 min in the glucose medium. A typical experiment is presented in figure 6. As in the glucose medium, there was a lag before rapid division began. Also DNA synthesis began shortly after addition of thymine and almost doubled before division began.

The relations of DNA content to cell numbers are given in figure 7. The figure also illustrates how the synchronization of DNA synthesis tended to disappear in later divisions. The following test points were nevertheless available in a study of virus infection: *a.* 0 min after thymine addition; *b.* 20 min after thymine addition when DNA synthesis was very active and cell division negligible; *c.* 35 min after thymine addition, when cell division occurred at maximal rate and DNA per cell was falling rapidly. These points were employed in a study of the effect of the stage of the division cycle on the course of T_2 infection.

T_{2r}^+ bacteriophage (5×10^7 per ml) was added under conditions of single infection to bacteria

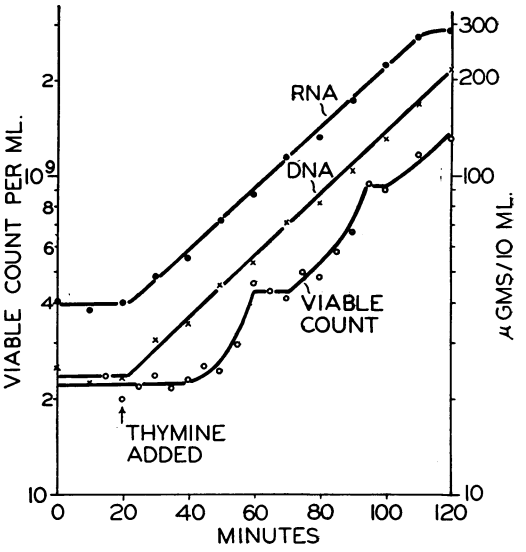

Figure 6. Nucleic acid synthesis and division in broth-grown cells synchronized in a synthetic medium supplemented with amino acids. Washed cells were resuspended in a thymine-free medium. Thymine was added after 20 min incubation.

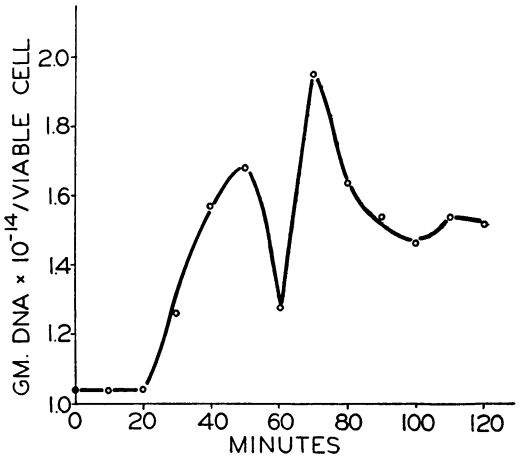

Figure 7. The DNA content per cell in a culture synchronized in a synthetic medium containing amino acids. These calculations are based on data from the experiment shown in figure 6. Each peak represents the end of a lag period in cell division.

(2×10^8 cells per ml) which had been synchronized as described in the preceding paragraph and which had attained the stages of the division cycle described above. Phage was permitted an 8-min adsorption period. The culture was then

diluted 10-fold in the same medium containing antiserum to kill free phage. After 13 min in antiserum, a 25-fold dilution was effected to produce an antiserum concentration which would not inactivate freshly liberated virus. The diluted mixtures were then plated on *E. coli* strain B and the numbers of infectious centers estimated as a function of time. No significant differences in adsorption (about 15 per cent of total virus input) were observed regardless of the division stage of the adsorbing bacteria. In one-step growth experiments carried out simultaneously several comparisons were made of the course of T_2 multiplication in a random culture of bacteria and in one which had been synchronized by addition of thymine after 20 min of thymine starvation. No significant differences in the major parameters, i.e., latent period, rise period, burst size, were observed.

After 20 min of incubation in the absence of thymine, T_2 was added to one aliquot and T_2 + thymine to another. Infection in the absence of thymine in such a culture extended the latent period about 7 min and reduced the burst size to about one-fourth of the usual value.

In table 1 are presented the parameters of simultaneously performed one-step growth experiments on synchronized bacteria which had attained the stages of division, *a*, *b*, and *c*, as defined above. It can be seen that the patterns of the one-step growth experiments are substantially identical in the three instances, and that T_2 multiplication appears to be essentially independent of the stage of host division.

Nucleic acid synthesis in infected synchronized bacteria. DNA synthesis also was studied in infected synchronized bacteria since the above described one-step growth experiment might not represent a sufficiently sensitive measure of the ability of the cells to elaborate viral components. Burst sizes in the 15_T- $-T_2$ system are low and it was suspected that the prior state of organization of the DNA synthesizing mechanism as reflected in DNA synthesis in the synchronized bacteria might have some effect on DNA synthesis in infected cells. In the additional tests, the first two points selected on the division curve were at 0 and 20 min after thymine addition. The third point was at 45 min after thymine addition when the bacteria had completed one division cycle and had entered a second lag phase. The greater rate of DNA synthesis in this instance may be interpreted in terms of the fact that the number of cells of the culture had doubled. The results of a typical experiment with this system are presented in figure 8. It can be seen that bacteria infected at the moment of addition of thymine are less capable of producing viral DNA than are bacteria engaged in active DNA synthesis.

TABLE 1

Time of Infection After Addition of Thymine	Latent Period	Rise Period	Relative Increase in Infectious Centers
min.	*min.*	*min.*	
0	25	13	43
20	27	12	37
35	26	16	35

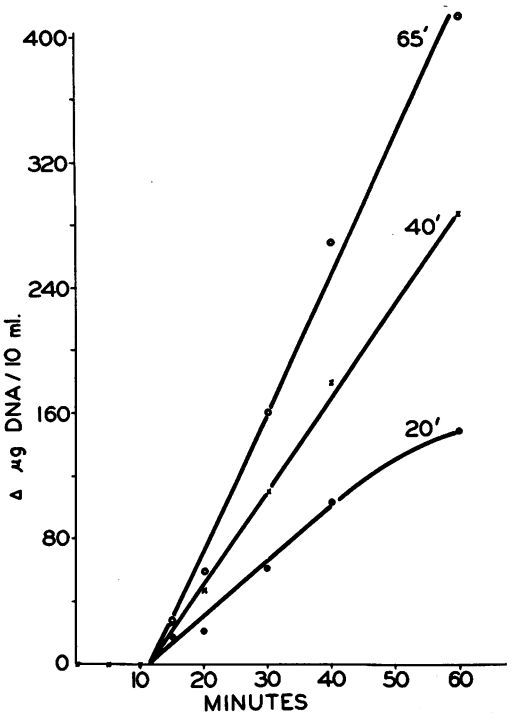

Figure 8. The synthesis of DNA in cultures infected with T_2 at various stages of cell division. Thymine was added to all cultures 20 min after incubation began: 20 min curve—infected simultaneously with the addition of thymine; 40 min curve—infected 20 min after the addition of thymine; 65 min curve—infected 45 min after the addition of thymine. Time on abscissa refers to min after infection. See text for details.

DISCUSSION

Cytogenetic and biochemical developments of the last decade have considerably expanded our

knowledge of nuclear and cytoplasmic differences in structure and function. Many problems can now be clearly posed concerning the division of labor between these cellular organelles in the development and performance of cell function. The recent interest in the synchronization of cell division is obviously a result of the need for experimental material which can assist in the solution of these problems. Until recently such material was limited almost entirely to the use of embryological material in which synchronization of division could be established by the fertilization of the ovum (Mazia and Dan, 1952). A pathological synchronization of bacterial metabolism may similarly be established by viral infection, a process not entirely dissimilar to fertilization.

The synchronization of single cell types has been accomplished starting with single cells (Tetrahymena) whose progeny will form a culture of synchronized cells (Zeuthen, 1953). Also cells (amebae) in the same stage of division (interphase) may be removed from a culture and pooled to form a culture of cells which enter division approximately synchronously (Mazia and Prescott, 1954). The synchrony of division in these protozoan cultures may also be effected in mass cultures by a procedure involving temperature shifts (Scherbaum and Zeuthen, 1954). A careful control of the temperature has permitted a separation of cell growth and division and by this means a system may be established in which growth is permitted to continue in the absence of cell division. A shift of temperature then permits the cells to divide synchronously. This method of synchronizing division has been applied to a variety of bacterial systems (Hotchkiss, 1954; Lark and Maaloe, 1954; Szybalski and Hunter-Szybalska, 1955). Cultures synchronized in this manner have been used in the study of the effect of cyclical change on various physiological properties, such as the susceptibility of Pneumococci to genetic transformation with DNA, or the efficiency of lysogenization of Salmonella as a function of the number of nuclei per cell. The synchronization of division of the thymineless bacteria by means of withholding and supplying the important DNA constituent, thymine, is the first example of a direct chemical control in the production of synchronized cultures. Although this procedure is limited at present to *E. coli* strain 15_{T-}, methods have been devised for inducing thymine deficiency in normally non-requiring strains (Cohen and Barner 1955) and it appears reasonable to anticipate that division in such cultures may be synchronized after induction of thymine deficiency. Indeed the apparent generality of unbalanced growth, e.g., effects of thymine deficiency, certain antibiotics, and other treatments (Barner and Cohen, 1956) suggests that treatment other than thymine depletion should also provide the preconditions for synchronization of division.

The technical difficulties inherent in effecting sufficiently sharp and rapid temperature changes in large cultures imply that this method will not be satisfactory in synchronizing the large batches desirable for biochemical work. On the other hand a chemical method of control, as in the addition of thymine to a depleted culture is readily applicable to large batches and indeed has been useful in the manner described in this paper.

The major result of the study of synchronized cultures has been the demonstration that DNA synthesis precedes division and that such synthesis stops during division. This result with bacteria is entirely comparable to most of the results obtained with animal and plant cells (Vendrely, 1955) in which it has been observed that DNA synthesis usually occurs in interphase or in very early prophase and that the synthesis ceases during later mitotic stages. It is of interest that in our bacterial system the course of synthesis of the cytoplasmic constituents as indicated by the increase of RNA and of turbidity is not cycled like the nuclear substance, emphasizing the considerable measure of independence of the two types of cell material.

Cells (*Salmonella typhimurium*) synchronized by several methods of temperature shift have been used recently to study the temporal relations of nucleic acid synthesis to nuclear and cell division (Bruce, Lark, and Maaloe, 1955). In a system in which nuclear division was induced by shifting a culture grown at 25 to 37 C, it was found that the temperature shift is followed almost immediately by a rapid synthesis of DNA which precedes a burst of cell division. In this system, RNA and turbidity increase exponentially and the parameters described would seem to follow closely the pattern observed with synchronized strain 15_{T-}. In another Salmonella system synchronized by alternating cycles of incubation at 25 and 37 C more complex results were obtained. The increase in turbidity, DNA, and colony count can be made to occur at approximately the same time at 37 C and essentially

cease at 25 C. The significance of these results is quite obscure at present.

In all of the reported methods for controlling cell division, either by means of temperature or chemicals, it must be recalled that the cell is subjected to stresses resulting in a situation of unbalance approaching pathology to a greater or lesser extent. Synchronization is initiated by a phase of recovery from the imposed stress and unbalance. The relation of such recovery phenomena to phenomena occurring in normal division and interphase can not yet be considered to be unequivocally clear. However, the existence of synchronized cultures in which certain metabolic events may be accentuated or minimized as a function of the phase of the division cycle under examination, presents important experimental material for the study of many critical problems, including the interrelations of diverse cell functions, the identity of early precursors in DNA synthesis, the changes in the polymers during division

SUMMARY

After preliminary growth without division of *Escherichia coli* strain 15_{T-} cells in the absence of thymine in a glucose-NH_4^+ medium, the re-addition of thymine initiated active deoxyribonucleic acid (DNA) synthesis. The DNA content of the cells doubled and the cells then divided synchronously while DNA synthesis stopped. Throughout the period of thymine starvation and the first division cycle in the presence of thymine the turbidity and ribonucleic acid (RNA) increased at rates similar to those observed in non-synchronized cultures.

Cells synchronized in this manner divided rapidly after the first division, the synchrony in the second cycle of division being less marked than that in the first or later division cycles.

Synchronized cultures were infected with T_2 bacteriophage in different states of the first division cycle. No significant differences were detectable in the major determinable parameters of the resulting one-step growth experiments as a function of the differences in division stage and stage of DNA synthesis. However cells infected in the process of DNA synthesis were more active in producing virus DNA than those infected before bacterial DNA synthesis had begun.

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