THE RELATIONSHIP BETWEEN DEOXYRIBONUCLEIC ACID REPLICATION AND CELL DIVISION IN HEAT-SYNCHRONIZED *TETRAHYMENA*

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ABSTRACT

The effect of supraoptimal temperature on macronuclear DNA synthesis in *Tetrahymena* was studied by radioautography during prolonged heat and heat-shock synchronization treatments. Prolonged heat treatments (34°C) delayed the initiation of S, but did not appreciably delay DNA synthesis in progress. Return to optimal temperature (28°C) 50 or 100 min later resulted in initiation of S, in delayed cells, at a rate greater than in controls. During the synchronization treatment, most cells were unable to enter S during a heat shock, but initiated S with a slight delay during the following intershock period. These cells were not appreciably delayed in completion of S by subsequent heat shocks. Supraoptimal temperature appears to affect the DNA synthetic cycle near the G_1 to S transition. Cells subjected to the heat-shock treatment in early G_1 all participated in one S period, and many underwent a succession of two S periods. DNA synthesis occurred in about 50% of the cells between EST and the first synchronous division, with the likelihood of DNA synthesis becoming greater the longer the interval between these two events. In some cells no detectable DNA synthesis occurred between EST and the *second* synchronous division. It was concluded that a precise temporal alternation of DNA replication and cell division is not obligatory in *Tetrahymena*.

INTRODUCTION

DNA replication in actively dividing eukaryotic cells generally occupies a restricted portion of the interphase, designated the S period, which is temporally separate from nuclear and cell division. The positioning of the S period between two mitotic divisions is usually precisely controlled so that cells which enter DNA synthesis appear to be committed to mitosis before another round of DNA duplication can begin. The ciliates represent a class of eukaryotic cells in which the strict alternation of replication and segregation of the genetic material of the polyploid macronucleus is less rigidly controlled. During the normal cell cycle of *Tetrahymena* (Cleffmann, 1968) and *Euplotes* (Kimball and Prescott, 1962) and after the completion of regeneration in *Urostyla* (Jerka-Dziadosz and Frankel, 1970), two consecutive rounds in macronuclear DNA synthesis can occur without macronuclear division. The converse, two successive macronuclear divisions without intervening macronuclear DNA synthesis, has been observed in *Urostyla* during regeneration (Jerka-Dziadosz

and Frankel, 1970). When cell and nuclear division in *Tetrahymena* have been suppressed by treatment with actinomycin C (Cleffmann, 1966), vinblastine (Sedgley and Stone, 1969), or by a repetitive sequence of temperature shifts (Zeuthen, 1963), macronuclear DNA synthesis continues past a normal doubling. A distinct second S period was observed by Cleffmann with actinomycin. These observations are all strongly suggestive of a temporal dissociation of the processes controlling macronuclear DNA synthesis from those controlling macronuclear and, in some cases, cell division.

The heat-shock synchronization system in *Tetrahymena* is favorable for the investigation of the relation of macronuclear DNA synthesis and cell division. Chemical (Scherbaum et al., 1959), microspectrophotometric (Scherbaum et al., 1959), and radioautographic (Hjelm and Zeuthen, 1967 *a*) evidence indicates that cells in which division has been suppressed by heat shocks accumulate amounts of DNA above the normal G_2 quantity. The object of the present study was to investigate the effects of supraoptimal temperature (34°C) on DNA synthesis, and to explore the occurrence of DNA synthesis during and after synchronization by repetitive heat shocks. The results indicate that the high temperature reversibly suppresses the initiation of DNA synthesis, but does not appreciably impede DNA synthesis in progress. During the intermittent high temperature treatments many cells undergo a succession of two normally timed S periods without an intervening division, while afterwards some cells undergo two successive cell divisions without intervening macronuclear DNA synthesis.

MATERIALS AND METHODS

Tetrahymena pyriformis GL-C, an amicronucleate strain, was grown axenically at 28°C in a tryptone-glucose-vitamins-salts medium (Frankel, 1965). The cells were continuously maintained in the exponential phase of growth by daily transfers into fresh medium. Under these conditions the generation time was about 200 min.

Cells were synchronized for cell division by a slight modification of the method originally used by Scherbaum and Zeuthen (1954), which consisted of six 30-min periods of elevated temperature (34°C) alternating with equal periods of optimal growth temperature (28°C). At approximately 85 min after the sixth and final heat shock, most of the cells were in some stage of cytokinesis. Populations of exponentially growing cells were subjected to the temperature treatment in 1-, 5-, and 10-ml volumes at densities of 20,000–50,000 cells/ml. In some experiments individual cells were isolated into capillary micropipettes (Prescott, 1957) at the end of the heat-shock treatment (EST)[1] from populations synchronized at lower densities (2000 cells/ml) in 150-ml volumes.

Cells in the early G_1 stage of the cell cycle were obtained by selecting dividing cells from a population growing exponentially at a density of 1000–2000 cells/ml (Stone and Cameron, 1964). The selection of a group of 15–20 dividing cells took from 10 to 15 min. Cells in the early stages of cleavage were selected first, while those in the later stages were selected last so that most of the cells divided together within a short interval of time. Those cells which took an excessively long period of time to divide were discarded. All the cells which had completed fission were immediately subjected to a temperature shift to 34°C, or incubated at 28°C as a control. Experiments involving single cells were carried out in micropipettes.

Macronuclear DNA synthesis was detected by incorporation of methyl-labeled tritiated thymidine (15μCi/ml, 6.7 mc/mmole, New England Nuclear Corp., Boston, Mass.) in pulse-fix and continuous labeling experiments. Tritiated thymidine has recently been demonstrated to be incorporated predominantly into the DNA of *Tetrahymena* (Wykes and Prescott, 1968). Continuous labeling experiments with populations of cells were conducted by adding isotope to 5- and 10-ml volumes of cells, and removing 0.5 ml samples at various times afterwards. Pulse-fix labeling experiments were conducted by incubating 1-ml volumes of cells with isotope for 12 min and then fixing for radioautography. Individual cells were labeled by expelling them from micropipettes into depression slides containing 100 μl of culture medium with tritiated thymidine. Cells were either drawn into new micropipettes if incubation with isotope was to be continued, or else prepared for radioautography after a 12 min exposure.

Cells to be prepared for radioautography were rinsed in distilled water, which was found to be a necessary procedure for optimal flattening, dried on slides subbed in gelatin (Caro, 1964), and fixed in 3:1 ethanol-acetic acid. Radioautography was done according to the procedures outlined by Prescott (1964). All slides were extracted for at least 10 min in cold 5% trichloroacetic acid after fixation; some slides were also extracted with 0.5 mg/ml desoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.) in 0.2 M pH 7.5 phosphate buffer containing 0.003 M $MgSO_4$, at 37°C for 2 hr. Slides were exposed in the

[1] *The abbreviations used are:* BH, beginning of a continuous 34°C treatment; BST, beginning of the standard synchronization treatment; EST, end of the synchronization treatment.

dark for 10–14 days. Cells were examined after radioautography at 400 × and scored as either possessing or lacking macronuclear labeling. All cells exposed to desoxyribonuclease had unlabeled macronuclei, while cytoplasmic labeling was reduced but not eliminated. Incorporation of tritiated thymidine into cytoplasmic macromolecules other than DNA or RNA has been reported by Wand et al. (1967).

RESULTS

DNA Synthesis in Exponentially Growing Cells

POPULATION KINETICS: The kinetics of macronuclear DNA synthesis in cells growing exponentially at 28°C was followed by pulse-fix and continuous labeling experiments (Fig. 1 A). The percentage of the population with labeled macronuclei increased in the manner which is characteristic of logarithmic populations entering DNA synthesis asynchronously (Cameron and Nachtwey, 1967; Hjelm and Zeuthen, 1967 *a*). Most of the macronuclei were labeled by about 250 min after the addition of tritiated thymidine; however, 100% labeling was not attained (Fig. 1 A, curve 1). This might be due to the difficulty of detecting light macronuclear labeling in a background of cytoplasmic label, which increased progressively during the time of incubation with tritiated thymidine. This cytoplasmic label was presumably a result of mitochondrial DNA synthesis, which has been reported to occur throughout the entire cell cycle of *Tetrahymena* (Parsons, 1965; Stone and Miller, 1965; Cameron, 1966), although a portion may be attributed to nonspecific cytoplasmic labeling (Wand et al., 1967).

During a 12 min pulse of tritiated thymidine 35–45% of the cells were engaged in macronuclear DNA synthesis (Fig. 1 A, curve 2). This provides a crude estimate of the proportion of the cell population in S, but this type of experiment gives no indication of the position of S in the cell cycle.

THE NORMAL CELL CYCLE: In order to determine the timing of the S period in the normal cell cycle, 146 individual cells in early G_1 were given a 12 min pulse of tritiated thymidine at various times after division. This procedure permits a reasonably accurate determination of the position of the S period in the cell cycle. The results (Fig. 2 A) indicate that the duration of the G_1 period was about 30–40 min, and that the duration of S varied from 60–70 min to 100–120 min and averaged 90 min. In order to determine the range of division times, 35 G_1 cells were allowed to complete a full cell cycle and were observed for the time of cell separation. The cumulative percentage of divided cells, shown in Fig. 2 A, indicates that 50% of the cells had divided 190 min after the previous cell division. Since 50% of the cells had completed their S period by 130 min, the average duration of G_2 + D is about 60 min. The variation in the duration of the S period in strain GL-C may be a result of differences in the generation times of single cells selected from an exponentially growing population (Prescott, 1959). Selection of dividing cells in micropipettes may also influence the duration of the next generation.

The Effect of Prolonged Periods of 34°C on DNA Synthesis

POPULATION KINETICS: The kinetics of macronuclear DNA synthesis when the culture temperature was rapidly shifted from 28°C to 34°C and maintained at the latter temperature for 250 min was studied in three experiments. The results of a typical experiment are shown in Fig. 1 B. The percentage of macronuclei labeled during a 12 min pulse previous to the temperature shift (BH) was similar to control values (35–45%). This proportion remained constant for about 70 min after BH, at which time the percentage of labeled macronuclei began to decrease, falling from 40% to 25% in the next 80 min (Fig 1 B, curve 4). This decline, with some variation in extent, was consistently observed in three experiments. The results of a continuous labeling experiment (Fig 1 B, curve 3) indicate that very few macronuclei entered a new round of DNA synthesis during the first 140 min after BH. These results also suggest that most macronuclei labeled during pulse experiments in this interval must have initiated DNA replication before BH and continued DNA synthesis despite the elevation in culture temperature. At approximately 140 min after BH (this time varied slightly among different experiments), the slope of the continuous label curve increased, and by about 250 min 77% of the macronuclei had incorporated label. These results and the concomitant increase in the number of macronuclei labeled in a pulse indicate that cells are eventually able to enter S. If a fraction of the population was returned to 28°C after being incubated at 34°C for 50 or 100 min, there was an almost immediate increase in the rate of appearance of labeled macronuclei (Fig 1 B, curves 5 and

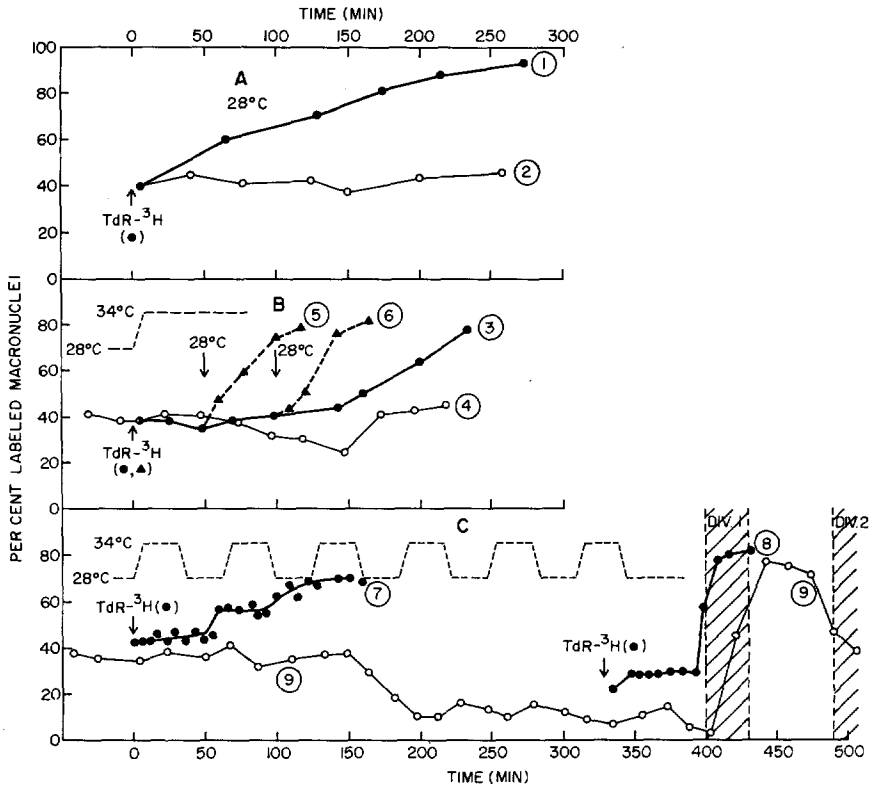

FIGURE 1 The kinetics of macronuclear DNA synthesis in populations of *Tetrahymena pyriformis* GL-C during pulse (open circles) and continuous incubation (closed circles) with TdR-^{3}H. A(top). The percentage of labeled macronuclei in cells growing exponentially at 28°C. Curve 1 represents the percentage of labeled macronuclei during continuous incubation with TdR-^{3}H added at time 0. Curve 2 represents the percentage of macronuclei labeled during 12-min pulses of TdR-^{3}H. Each point on curve 2 and all the pulse-labeling curves which follow is placed at the midpoint of the 12 min exposure time. Curves 1 and 2 represent experiments conducted with the same population of cells. B(middle). The percentage of labeled macronuclei following a rapid temperature shift from 28°C to 34°C at time 0, with subsequent continuous maintenance at 34°C. Curve 3 represents the rate of appearance of labeled macronuclei during continuous incubation with TdR-^{3}H since time 0. Curves 5 and 6 (closed triangles) represents the rate of appearance of labeled macronuclei in fractions of the culture originally incubated with TdR-^{3}H at time 0 which have been returned to 28°C at 50 and 100 min, respectively, after BH. Curve 4 represents the percentage of macronuclei labeled in 12-min pulses of TdR-^{3}H. Curves 3, 4, 5, and 6 represent experiments conducted with the same population of cells. C(bottom). The percentage of macronuclei labeled when cells are subjected to the standard heat-shock synchronization procedure at time 0. Curve 7 represents the rate of appearance of labeled macronuclei during continuous incubation with TdR-^{3}H since time 0. Curve 8 represents the rate of appearance of labeled macronuclei during continuous incubation since EST. Curve 9 represents the percentage of macronuclei labeled during 12-min pulses of TdR-^{3}H. Each curve was drawn from a separate experiment. The heat-shock synchronization regimen is indicated above. The hatched areas represent the interval of cell separation in the first synchronous division, and the beginning of the second synchronous division.

6). The rate of entry into S after reincubation at 28°C was greater than that in exponentially growing controls or during the continuous exposure to 34°C. This is further indication that during incubation at 34°C cells accumulate at a position in the cell cycle just previous to the initiation of DNA replication.

TIMING OF DNA SYNTHESIS: In order to determine the effect of prolonged incubation at 34°C on the timing of the S period, 202 individual

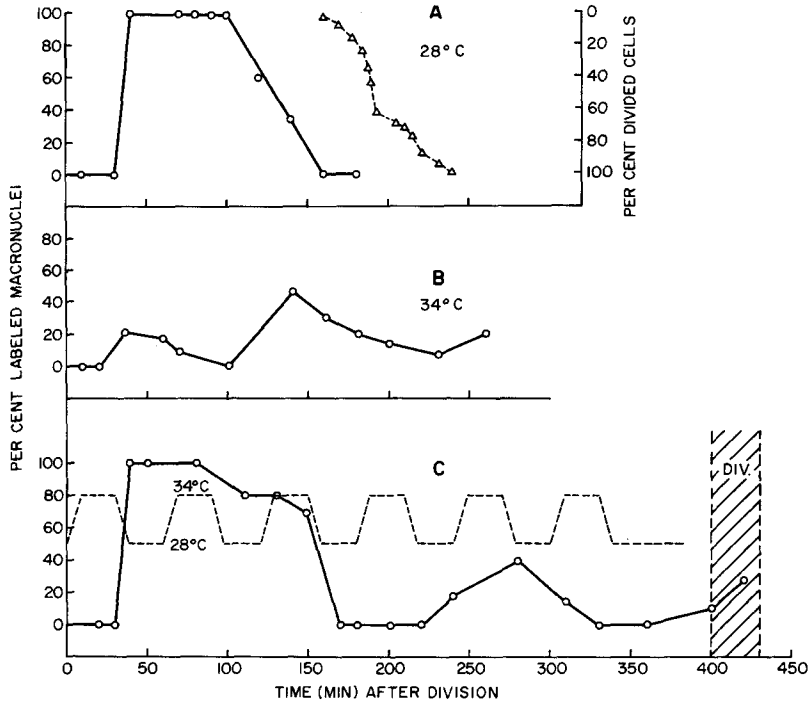

FIGURE 2 The timing of macronuclear DNA synthesis in the cell cycle of *Tetrahymena pyriformis* GL-C at 28°C, 34°C, and during the synchronization treatment. A(top). The timing of macronuclear DNA synthesis (open circles) in cells growing exponentially at 28°C. Each point represents the percentage of cells with labeled macronuclei after a 12 min pulse of TdR-^{3}H. This graph represents a total of six experiments involving 146 single cells, all in the early G_1 stage of the cell cycle at time 0. 8–15 cells are represented for each point. The cumulative percentage of dividing cells (open triangles) was obtained from a record of the time of cell separation for 35 early G_1 cells which had completed one cell cycle. This curve is plotted in an inverted manner as indicated by the right ordinate. B(middle). The timing of macronuclear DNA synthesis in cells at 34°C. Each point represents the percentage of cells with labeled macronuclei after a 12 min pulse of TdR-^{3}H. This graph represents a total of nine experiments involving 202 single cells, all in early G_1 when the temperature was shifted from 28°C to 34°C at time 0. 8–15 cells are represented for each point. C(bottom). The timing of macronuclear DNA synthesis in cells subjected to the standard heat-shock synchronization procedure. Each point represents the percentage of cells with labeled macronuclei after a 12 min pulse with TdR-^{3}H. This graph represents a total of 13 experiments involving 256 single cells, all in early G_1 at BST (time 0). 8–15 cells are represented for each point. The heat-shock synchronization regimen is indicated above. The hatched area represents the range of time of cell separation during the first synchronous division.

cells were given a 12 min pulse of tritiated thymidine at various times after being subjected to a temperature shift in early G_1. A small proportion of cells entered a shortened macronuclear S period "on schedule" (Fig 2 B), but the majority did not incorporate label until about 2 hr after BH. This corresponds to the time when asynchronous populations subjected to the same treatment begin to slowly increase their rate of entry into S (Fig 1 B, curve 3). The effect of incubation at 34°C was to delay the onset of DNA replication and thus to increase the absolute duration of G_1. The extent of this increase in duration of G_1 was not uniform during 34°C, even in cells which were subjected to the temperature shift at the same stage of the cell cycle.

Effect of a Repetitive Sequence of Heat Shocks on Macronuclear DNA Synthesis

POPULATION KINETICS: The kinetics of macronuclear DNA synthesis in populations of cells during the repetitive sequence of six heat

shocks was studied by pulse-fix and continuous labeling. Approximately 70% of the macronuclei synthesized DNA within the first 150 min of the heat shock treatment (Fig 1 C, curve 7), which was similar to the proportion of macronuclei labeled in the same time interval during exponential growth (Fig 1 A, curve 1). However, in the heat-shocked cells entry into S took place almost exclusively during the 28°C intershock periods; very few new cells became labeled during the heat shocks. This observation is consistent with the finding that almost all cells were unable to enter a new round of DNA replication during the first 2 hr at continuous 34°C (Fig 1 B, curve 3).

Pulse-labeling experiments were carried out throughout the heat-shock treatment and during the synchronous divisions that followed. The proportion of cells engaged in DNA synthesis before the beginning of the first heat shock was the normal 35–45%. During the first three heat shocks and the intervening periods of 28°C, the proportion of cells engaged in DNA synthesis (Fig 1 C, curve 9) remained similar to control values (Fig 1 A, curve 2). A striking reduction in the percentage of labeled macronuclei occurred between the third and fourth heat shocks. The percentage of labeled macronuclei decreased from 36% to 12% in about 40 min, and remained relatively constant during the remainder of the heat-shock treatment and during the first 60 min after the end of the synchronization treatment (EST).

If cells were incubated continuously with tritiated thymidine beginning at EST, about 25% of the macronuclei became labeled in the first 25 min. Thereafter, the proportion of labeled macronuclei remained constant until the time of division (Fig. 1 C, curve 8). During the early portion of the first synchronous division, the proportion of cells participating in DNA synthesis decreased virtually to zero. A synchronous burst of DNA synthesis occurred during the period when cell separation was observed (Fig 1 C, curve 9). These results were obtained in four parallel experiments; the results of only one of these experiments is plotted in Fig. 1. They are essentially confirmatory of similar findings by Cerroni and Zeuthen (1962) and Hjelm and Zeuthen (1967 *a*, *b*), with only one difference, namely, that no decrease in macronuclear labeling was observed prior to cell division. Recent studies in Zeuthen's laboratory, in which thymidine-^{14}C was used, have also indicated that there is no decrease in the proportion of labeled macronuclei prior to the first synchronous division (Ikeda et al., 1970).

TIMING OF DNA SYNTHESIS IN AN EXTENDED INTERPHASE: In order to investigate the timing of DNA synthesis when the absolute duration of the interphase has been extended by suppressing division with repetitive heat shocks, it would be desirable to analyze a group of cells in which DNA synthesis is least affected by supra-optimal temperature. For this reason, the timing of DNA synthesis during the extended interphase was determined by giving 233 individual cells a 12 min pulse of tritiated thymidine at various times after being subjected to the first heat shock in early G_1. As shown in Fig 2 C, these phased cells entered S during the beginning of the first intershock period, after a G_1 period of about the same duration as in 28°C controls. The absolute duration of the S phase was not appreciably extended in heat-shocked cells. No labeled macronuclei were observed between 190 and 230 min after the beginning of the synchronizing treatment (BST). Beginning at 230 min and extending to 340 min after BST, labeled macronuclei were again observed but only in a portion of the cells, indicating that some cells reinitiated DNA synthesis. Further experiments were conducted to determine if all phased G_1 cells participated in an S period early in the treatment, and what proportion of these cells entered a second S period later. All of the 77 cells which were maintained in tritiated thymidine between 0 and 180 min after BST had labeled macronuclei, while 48 out of 72 (67%) participated in DNA synthesis during the interval from 200 to 300 min after BST. This evidence strongly suggests that cells entering the heat-shock treatment in early G_1 all participate in a first round of DNA synthesis, and that while division is suppressed a portion of these cells enter a second round of replication. It is interesting to note that the second S period is initiated at approximately 230 min after the previous cell division, which is about equal to a normal generation time plus G_1. The duration of the second S period is probably shorter than that of a normal S period (Fig 2 C). The reduced proportion of cells with labeled macronuclei during the second half of the heat-shock treatment observed in asynchronous populations (Fig 1 C, curve 9) may be explained by re-initiation of DNA synthesis in only a portion of the cell population. After the end of the second S period no labeled macronuclei were observed in phased G_1 cells

TABLE I

The Relationship between the Time of Formation of a Deep Cleavage Furrow of the First Synchronous Division and the Incorporation of Thymidine-^3H into Macronuclei

First division time classes (min after EST)	Number of macronuclei		% Labeled macronuclei
	Labeled	Unlabeled	
55–64	0	2	0
65–69	3	5	37
70–74	5	24	21
75–79	21	36	37
80–84	26	28	48
85–89	16	15	51
90–94	15	8	65
95–99	13	3	81
100–104	7	4	64
105–109	8	2	80
110–119	11	0	100
120–129	4	0	100
Total	129	127	

Cells were isolated from a population (1000–2000 cells/ml) immediately after EST, expelled into 100 μl of culture medium containing tritiated thymidine (15 μCi/ml), and thereafter drawn into capillary micropipettes. The cells were maintained in pipettes until the time of deep furrow formation during the first synchronous division; at this time they were expelled, rinsed, dried on slides, and prepared for radioautography. A total of 25 experiments is represented.

until the synchronous burst of DNA synthesis at the time of cell division.

Relationship of DNA Synthesis after the Final Heat Shock to the Time of Cell Division

The interval from EST to the first synchronous division represents a period when cells are presumably undergoing necessary preparations for macronuclear and cell division. In order to investigate whether there is any relationship of DNA synthesis after EST to the time of cell division, single cells were isolated from the synchronized population immediately after the sixth heat shock, incubated with tritiated thymidine, and fixed for radioautography at the deep cleavage furrow stage of cell division (just previous to cell separation). Of the total of 256 cells treated in this manner, 127, or 49%, possessed labeled macronuclei. These findings are in excellent agreement with the studies on populations by Cerroni and Zeuthen (1962), which also showed that only about 50% of the synchronized population synthesized DNA between EST and cell division. When the results were recorded according to the time of deep furrowing (Table I), they revealed that, for any particular cell, the longer the time interval between EST and cell division, the greater the likelihood of macronuclear labeling. The results support the conclusion that macronuclear DNA synthesis and cell division have been temporally ungeared (Zeuthen, 1963), and further reveal that cells which divide later have a greater chance of initiating DNA synthesis during the interval prior to division. The increase in proportion of labeled macronuclei in late-dividing cells might be related to the sharp increase in percentage of labeled macronuclei observed in populations at about 80 min after EST (Fig 1 C, curve 9; also Hjelm and Zeuthen, 1967 *a*).

In the experiments discussed above, cells were chosen at random from heat-shocked populations,

TABLE II

The Relationship between the Time of Formation of a Deep Cleavage Furrow in Cells Entering the Synchronization Treatment in Early G_1 and the Incorporation of Thymidine-^3H into Macronuclei

First division time classes (min after EST)	Number of macronuclei		% Labeled macronuclei
	Labeled	Unlabeled	
70–74	0	8	0
75–79	2	12	15
80–84	3	6	33
85–89	3	6	33
90–94	4	0	100
95–99	4	0	100
100–104	2	0	100
105–109	4	0	100
110–114	3	0	100
Total	25	32	

Early G_1 cells were subjected to the heat-synchronization treatment in capillary micropipettes. At EST, cells were expelled into medium containing tritiated thymidine (15 μCi/ml) and then drawn individually into new micropipettes. The cells were maintained in these pipettes until the time of the deep furrow formation in the first synchronous division, and expelled, and prepared for radioautography. A total of four experiments is represented.

so that their position in the cell cycle when first disturbed by the temperature shift was unknown. In order to determine whether the position in the cell cycle at the time of the first temperature shift affects the relationship observed after EST between time of cell division and DNA synthesis, 57 early G_1 cells were subjected to the heat-shock treatment. After EST they were incubated in tritiated thymidine and treated as above. The results, shown in Table II, again suggest that the longer the time interval to cell division, the greater the likelihood of macronuclear DNA synthesis. The position in the cell cycle at the time of the first temperature shift was apparently not correlated with the length of time from EST to cell division; initially phased cells showed as wide a range in division times as asynchronous cells.

DNA Synthesis during the Interval from the Last Heat Shock to the Second Synchronous Division

Zeuthen (1968) has shown that, when DNA synthesis was inhibited by amethopterin and excess uridine supplied at EST, all cells were capable of dividing once, but 80% did not divide a second time. Those cells dividing twice, presumably during a period of thymidine starvation, did so early within the range of the second division maximum. On this basis, we suspected that some cells may complete a shortened cell cycle without participating in DNA synthesis.

In order to test this possibility, single cells were isolated from a heat-synchronized population at EST and were incubated with tritiated thymidine. The cells were fixed for radioautography at the deep furrow stage of the *second* synchronous division, and their individual second division times were recorded. In order to determine the generation time, the time of the first division (cell separation) was recorded in some experiments. The results, shown in Table III, indicate that, of 186 cells incubated with tritiated thymidine between EH and the second synchronous division, 54 (29%) did not imcorporate label into the macronucleus. Unlabeled cells divided almost exclusively during the first half of the second division burst. Labeled and unlabeled cells also differed with regard to the interval between the first and second synchronous division. The range of generation time of 46 labeled cells was 86–161 min and the mean was 115, while in the 16 unlabeled cells the range was 83–123 min and the mean was 103. Thus, although there were some labeled cells which had short generation times, no unlabeled cells had very long generation times. It should be noted that this experiment gives a minimum estimate of the proportion of cells unlabeled between two synchronous divisions, since some of the cells which had labeled macronuclei at division 2 may have synthesized DNA only between EST and division 1.

TABLE III

The Relationship between the Time of Formation of a Deep Cleavage Furrow of the Second Synchronous Division and the Incorporation of Thymidine-^3H into Macronuclei

Second division time classes (min after EST)	Number of macronuclei		% Labeled macronuclei
	Labeled	Unlabeled	
160–169	6	7	46
170–179	7	8	46
180–189	6	18	33
190–199	16	12	57
200–209	27	4	87
210–219	31	5	87
220–229	15	0	100
230–239	9	0	100
240–249	7	0	100
250–259	8	0	100
Total	132	54	

Cells were treated in the same manner as those shown in Table I, except that they were incubated with tritiated thymidine until the formation of the deep furrow of the second synchronous division when they were expelled and treated for radioautography. A total of eight experiments is represented.

DISCUSSION

Previous studies have firmly established that the heat-shock synchronization treatment in *Tetrahymena* synchronizes cell division without phasing macronuclear DNA synthesis (Cerroni and Zeuthen, 1962; Hjelm and Zeuthen, 1967 *a*). The present investigation has, however, shown that the heat-shock temperature (34°C) does affect the cycle of DNA duplication. The evidence obtained, with both asynchronous populations and single-phased cells, suggests that prolonged high temperature treatments (250 min) delay the initiation of DNA synthesis, but do not appreciably interfere with DNA synthesis already in progress. Further-

more, return to optimal temperature (28°C) after a prolonged 34°C treatment resulted in the reversal of the effect of high temperature, as indicated by an almost immediate increase in the rate of initiation of DNA synthesis.

Effects similar to those observed during prolonged heat treatments were observed during the heat-shock synchronization treatment. During a 30 min heat shock most cells were prevented from initiating DNA synthesis, but they entered S with a slight lag during the following 30 min intershock period. Those cells which were "scheduled" to initiate DNA synthesis during an intershock period entered S "on time", and completion of DNA replication was not impeded by subsequent heat shocks. In contrast to the effect of high temperature on cytoplasmic processes associated with cell division, which are subject to age-dependent regression or "set-back" (Thormar, 1959; Zeuthen, 1964), progress through the cycle of DNA synthesis is not subject to regression during a heat shock. Instead, DNA synthesis is halted at a critical point near the G_1 to S transition, and can continue to completion without further interference by high temperature once this point is passed. The lack of age-dependent regression in the DNA cycle is the primary factor responsible for its asynchronous behavior during the heat-shock treatment.

The proportion of cells which participated in DNA synthesis during the first 200 min of the synchronization treatment was similar to that observed in controls growing exponentially at 28°C. During this interval most cells were probably able to complete one S period, which may have been subject to alterations in timing due to the effect of high temperature. Subsequently, DNA synthesis continued, but the proportion of the population involved decreased to one-third of the original value. Studies on the timing of DNA synthesis in phased cells, which entered the treatment in early G_1, indicated that some cells participated in no further DNA synthesis after the conclusion of the first S period (S_1). These cells, which presumably contained the normal G_2 quantity of DNA at the time of cell division, were probably partially responsible for the reduced proportion of cells with labeled macronuclei observed in originally asynchronous populations during the latter heat shocks. The remainder of the cells entered a second S period (S_2) late in the synchronization treatment. Examination of the timing of these S periods revealed that the interval from the completion of S_1 to the beginning of S_2 was similar to the sum of the normal G_2, D, and G_1 periods. These results suggest that two normally timed periods of macronuclear DNA synthesis occur even though division is suppressed during the synchronization treatment. This would explain the accumulation of DNA in excess of the normal G_2 quantity observed during this period (Scherbaum et al., 1959). The results of Hjelm and Zeuthen (1967 *a*), who have studied the kinetics of DNA synthesis in asynchronous *Tetrahymena* populations subjected to heat-shock treatments, also suggest that two consecutive S periods of normal duration occur during the treatment. In contrast to their studies, however, we find that the time from one S period to the next is not appreciably extended under these conditions. The occurrence of successive S periods during the normal cell cycle of *Tetrahymena* was also observed by Cleffmann (1968).

The demonstration of temporally distinct periods of DNA synthesis without intervening cell division contributes to the understanding of the relationship between these two events. Since DNA synthesis can be initiated in the absence of a directly preceding division, these is no necessary relationship between the conclusion of cell division and the initiation of DNA synthesis. Multiple S periods also indicate that the completion of DNA replication is not the direct stimulus for cell division. The apparent maintenance of the periodicity of DNA synthesis in the absence of division most clearly shows the independence of the mechanism by which DNA synthesis is regulated.

Although the completion of DNA synthesis is not a direct trigger for cell division, the onset of mitosis in a variety of eukaryotic cells (Rueckert and Mueller, 1960; Taylor et al., 1962; Sachsenmeyer and Rusch, 1964) is dependent on the completion of at least one round of DNA replication. In a population of heat synchronized *Tetrahymena*, some cells, characterized by a somewhat shorter average generation time, did not incorporate tritiated thymidine into macronuclear DNA during the interphase between successive synchronous divisions. Similarly, Zeuthen (1968) has observed cells dividing early within the second synchronous division peak which were able to undergo a complete cell cycle under the conditions of thymidine starvation. These results can be explained in two ways: either heat-synchronized *Tetrahymena* are able to complete two successive cell divisions in the absence of DNA replication, or sufficient intracellular thymidine has accumulated during the long synchronization treatment so that labeled

thymidine supplied in the medium is not being employed as a precursor for DNA synthesis. It appears likely that thymidine does enter cells with unlabeled macronuclei between the first and second synchronous divisions, for the following reasons: Study of food vacuole formation by the uptake of carmine particles indicates that virtually all cells are feeding during this period (Nelsen, 1970); and, furthermore, cells with unlabeled macronuclei do have a light cytoplasmic label. The first of the two explanations, which implies that excess DNA synthesized while division is suppressed fulfils the requirement of genetic duplication in the subsequent division cycle, is the more attractive to the authors. Perhaps the attainment of a critically high amount of macronuclear DNA, as a result of additional rounds of replication, liberates the processes which control cell division from their normal dependence on the completion of DNA replication, resulting in a shortened interdivision period consisting of only the necessary preparations for division and accompanying morphogenesis. Therefore, in contrast to the situation described for most eukaryotic cells, DNA synthesis may not be directly involved in the sequence of events leading to cell division in *Tetrahymena*.

In most actively dividing mitotic cells, with a few notable exceptions (Rasch et al., 1959; Patau and Das, 1961; Goldstein and Prescott, 1967), each division product receives an amount of genetic material equal to the G_1 quantity of the parent cell as a result of the alternate occurrence of DNA replication and chromosome segregation. Such a rigid coupling has been shown not to be mandatory in *Tetrahymena*, since the replication and segregation of the genetic material of the macronucleus are not necessarily alternate events in the cell cycle.

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