EVIDENCE FOR A TEMPORAL INCOMPATIBILITY BETWEEN DNA REPLICATION AND DIVISION DURING THE CELL CYCLE OF *TETRAHYMENA*

WILLIAM R. JEFFERY

From the Department of Zoology, University of Iowa, Iowa City, Iowa 52240. Dr. Jeffery's present address is McArdle Laboratory for Cancer Research, Medical Center, The University of Wisconsin, Madison, Wisconsin 53706.

ABSTRACT

The mechanism of coordination between DNA replication and cell division was studied in *Tetrahymena pyriformis* GL-C by manipulation of the timing of these events with heat shocks and inhibition of DNA synthesis. Preliminary experiments showed that the inhibitor combination methotrexate and uridine (M + U) was an effective inhibitor of DNA synthesis. Inhibition of the progression of DNA synthesis with M + U in exponentially growing cells, in which one S period usually occurs between two successive divisions, or in heat-shocked cells, when successive S periods are known to occur between divisions, resulted in the complete suppression of the following division. In further experiments in which the division activities were reassociated with the DNA synthetic cycle by premature termination of the heat-shock treatment, it was shown that (a) the completion of one S period during the treatment was sufficient for cell division, (b) the beginning of division events suppressed the initiation of further S periods, and (c) if further S periods were initiated while the heat-shock treatment was continued, division preparations could not begin until the necessary portion of the S period was completed, even though DNA had previously been duplicated. It was concluded that a temporal incompatibility exists between DNA synthesis and division which may reflect a coupling mechanism which insures their coordination during the normal cell cycle.

INTRODUCTION

The orderly progression of cell cycle events involves precise coordination of many cellular activities including replication and segregation of the genetic material. The relationship between nuclear DNA replication and division has been studied by genetic analysis (8, 13, 14, 16, 17, 19, 24, 35), inhibition of DNA synthesis (1, 3, 6, 15, 28, 29, 34), and nuclear transplantation methods (12). The results of most of these studies support the general conclusion that regulation of cell division is intimately linked to DNA metabolism. According to one viewpoint, cell division may be initiated as a terminal step in a linear sequence of biochemical events involving DNA synthesis. Other studies have suggested that the normally rigid coupling between DNA synthesis and division is not obligatory. Inouye (19) has recently described a temperature-sensitive mutant in *Escherichia coli* which is defective in DNA synthesis but can undergo normal cell division at the restrictive temperature. Other investigations (21, 23) have demonstrated that heat-

shocked *Tetrahymena pyriformis* can complete an entire division cycle without participating in macronuclear DNA synthesis These results clearly justify an investigation of the coordination of DNA replication and cell division in a cell system in which these events can be dissociated. The present investigation deals with this problem in heat-shocked *T. pyriformis*, employing methotrexate and uridine (38) to inhibit DNA synthesis

The heat-shock system is particularly suited for studies of this nature because it readily uncouples DNA synthesis and division (37) When *Tetrahymena* cells are subjected to a series of cyclic heat shocks, further progress toward division is prevented, and cells regress to a common state in the division cycle (40). Macronuclear DNA synthesis continues while the division cycle is arrested and new S periods are periodically initiated (2, 18, 21). During the standard six-shock heat treatment used in the present study, many cells can complete two S periods during a single interdivision period lasting about twice the normal generation time (20). After the heat-shock treatment is terminated, cellular morphogenesis, karyokinesis, and cytokinesis are executed in synchrony (40). Since the initiation of these division-related activities can be imposed on the continuing DNA synthetic cycle at any point by merely terminating the heat-shock treatment, this system is also favorable for experimental studies concerned with the reassociation of division with the DNA cycle.

Appropriate manipulation of the timing of DNA synthesis and cell division in *T. pyriformis*, using inhibitors of DNA synthesis and heat shocks, suggested that a temporal incompatibility exists between these events. During the normal cell cycle, preparations for cell division may be suppressed by DNA synthesis in progress. However, once division activities have begun, the initiation of further rounds of DNA replication is blocked until division is completed This coupling mechanism insures a temporal alternation between DNA replication and cell division.

MATERIALS AND METHODS

General Procedures

Axenic stock cultures of *T. pyriformis*, amicronucleate strain GL-C, were maintained at 28°C in slanted culture tubes containing 5 ml of a tryptone-dextrin-vitamins-salts medium (10). During the period in which these experiments were conducted, cells were maintained in continual exponential growth by daily transfers to fresh medium Under these conditions the average generation time was about 190 min.

Groups of cells in the early G_1 stage of the cell cycle (5–10 min after the previous cell separation) were obtained by the method of Stone and Cameron (32) as described previously (21). The basic heat-shock treatment (10) was a slight modification of the procedure originally developed by Scherbaum and Zeuthen (30) and consisted of six 30-min intervals at 34°C, separated by five 30-min intervals at 28°C. DNA synthesis was inhibited by using a combination of methotrexate (amethopterin, American Cyanamid Co., Lederle Laboratories Div , Pearl River, N. Y.) and uridine. In all experiments methotrexate and uridine (M + U) were employed at 0 05 mM and 5.0 mM, respectively, the concentrations originally used by Zeuthen (38). The higher concentrations employed in the more recent investigations of Zeuthen and coworkers (34, 39) always led to lysis of cells cultured in microdrops Protein synthesis was inhibited with cycloheximide (actidione, ICN Nutritional Biochemicals Div , International Chemical and Nuclear Corp , Cleveland, Ohio) at a concentration of 1 µg/ml (11).

Assay for Cell Division

Cell division was assayed by two methods. The first, employing a model A Coulter Counter (Coulter Electronics, Inc., Industrial Div., Hialeah, Fla) (10), was used to determine cell concentration after addition of inhibitors to mass cultures of cells. The second involved the isolation and observation of cleavage in single cells cultured in microdrops of medium covered with a thin layer of paraffin oil. This microdrop method has been described in detail by Nachtwey and Dickinson (26) and Frankel (11) and has been found to be well suited for inhibitor studies on single cells Groups of synchronous cells were expelled from micropipettes into depression slides at various times after the beginning of the heat-shock treatment (BST). An approximately equal volume of culture medium containing M + U or thymidine-³H at double strength was added to each depression slide. In controls an equal volume of culture medium was added at this time. Individual cells, with a small volume of culture medium, were pipetted from the depression slide to a glass Petri dish. The microdrops, each containing a single cell, were covered by a thin layer of paraffin oil (light weight), and the Petri dishes were either incubated at 28°C or subjected to the remainder of the heat-shock treatment. The microdrops were examined at frequent intervals to determine whether cell division had occurred (presence of two cells per drop).

Detection of Protein Synthesis

The radioassay for protein synthesis was a slight modification of the method described by Byfield and Scherbaum (5). After exponentially growing cells had reached a concentration of about 30,000 cells/ml, a subculture was removed for experimental purposes. Leucine-^{3}H (5 μCi/ml; 29.8 mCi/mм; ICN Corp., Chemical and Radioisotopes Div., Irvine, Calif.) was added at the desired time and the subculture was divided into two equal parts. M + U was added to one part, while the other served as a control. Triplicate 50 μl portions were removed from each part at various times after the initial addition of the leucine-^{3}H. Each portion was pipetted on to a filter paper disc (Whatman 3 MM) and extracted in ice-cold 10% trichloroacetic acid (TCA). After at least 1 hr of extraction, each disc was hydrolyzed in hot 10% TCA (90°C) for 15 min. The discs were then washed twice in ice-cold 10% TCA (10 min for each wash) with vigorous swirling. Lipids were extracted with two washes (5 min each) of 1:1 ethanol:ether. The discs were air dried and transferred to scintillation vials containing 10 ml of 0.4% 2,5-diphenyloxazole and 0.005% p-bis(2[5-phenyloxazolyl])-benzene in toluene. Each vial was counted in a Beckman scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.).

Detection of DNA Synthesis

DNA synthesis in single cells was detected by radioautographic methods (21) after the addition of thymidine-^{3}H (10 μCi/ml; 6.8 mCi/mmole; New England Nuclear Corp., Boston, Mass.).

The effect of M + U on DNA synthesis in mass cultures of cells was determined in the following manner. After cultures of exponentially growing cells had reached a concentration of about 60,000 cells/ml, a subculture was removed for experimental purposes. After the addition of adenosine-^{14}C (1 μCi/ml; 31.3 mCi/mmole, Schwartz Bio Research Inc., Orangeburg, N. Y.) at the desired time, the subculture was divided into three equal parts. M + U was added to one part, M + U and thymidine (T; 5.0 mM) was added to another part, and the third part served as a control. Samples were removed at various times after the addition of adenosine-^{14}C, gently centrifuged, and resuspended in 4 ml of ice-cold 5% TCA. The resulting precipitate was fractionated according to the method of Schmidt and Thannhauser (31). Each sample was brought to a concentration of 1.0 N potassium hydroxide and incubated at 37°C for 20 hr. After base hydrolysis, each sample was brought to a pH between 7.0 and 8.0 by dropwise addition of ice-cold 10% perchloric acid (PCA). The suspension was then refrigerated for 2 hr to allow maximal precipitation, collected on a Millipore filter (Millipore

Corporation, Bedford, Mass.) (0.45 μ), and washed with 20 ml of ice-cold PCA. These filters were processed for liquid scintillation counting as described above.

RESULTS

Extent and Specificity of Inhibition of DNA Synthesis

As shown in Fig. 1 A, addition of M + U to an exponentially growing culture of T. pyriformis caused an immediate suppression of the incorporation of adenosine-^{14}C into the acid-insoluble fraction which remained after base hydrolysis (Fig. 1 A, curve 3). Inhibition was almost complete for 4 hr after inhibitor addition, since the radioactivity in the M + U treated culture (Fig. 1 A, curve 3) was less than 8% of the control level (Fig. 1 A, curve 1). Between 4 and 5 hr after M + U addition, the level of radioactivity increased, indicating that cells were eventually able to recover from the effects of the inhibitor and resume DNA synthesis. Inhibition of DNA synthesis was almost completely prevented if thymidine (T) was added simultaneously with M + U (Fig. 1 A, curve 2). These results support the previous suggestion that M + U affects DNA metabolism by producing a nutritional deficiency for thymidine (34, 38). Furthermore, DNA synthesis can be effectively inhibited, for a period of 4 hr, with a lower concentration of uridine than employed in the recent investigations of Zeuthen and coworkers (34, 39).

The effect of M + U on the incorporation of leucine-^{3}H into acid-insoluble material is presented in Fig. 1 B. The results indicate that treatment of an exponentially growing culture of cells with M + U (Fig. 1 B, curve 2) reduced the level of protein synthesis to about 80% of that observed in the control culture (Fig. 1 B, curve 1). The slight effect of the inhibitor on protein synthesis is probably mediated through effects on RNA synthesis (34). Thus, although M + U is a rapid and potent inhibitor of DNA synthesis, at the concentrations used in this study it also has a moderate effect on protein synthesis. Since translational events are required for cell division in heat-synchronized Tetrahymena (11, 27), even a slight depression in protein synthesis must be considered in attempting to interpret any possible M + U effects on cell division.
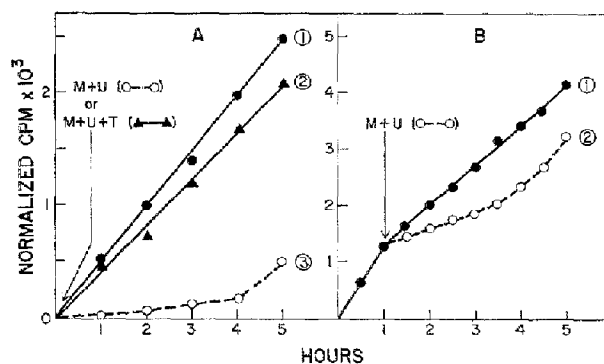
FIGURE 1 The effect of methotrexate and uridine ($M + U$) on the rate of radioisotope incorporation into DNA (A) and protein (B) in exponentially growing *T. pyriformis*. (A) Inhibition of DNA synthesis by $M + U$ and its prevention by thymidine (T). Each curve represents the incorporation of adenosine-[14]C (1 μCi/ml), added at time zero, into alkali-resistant, acid-insoluble material. $M + U$ (curve 3) or $M + U + T$ (curve 2) was also added at time zero (arrow). Curve 1 represents a control culture. Normalized cpm = cpm/unit cell number. (B) The effect of $M + U$ on the incorporation of leucine-[3]H (5 μCi/ml) into acid-insoluble material. $M + U$ was added (curve 2) 1 hr after time zero (arrow). Curve 1 represents a control culture. Leucine-^{3}H was added to both cultures at time zero. Normalized cpm = cpm/unit cell number.

Effect of $M + U$ on Cell Division

The effect of M + U on cell division in *T. pyriformis* was investigated in exponentially growing cells, in which one S period usually occurs between two successive divisions (7), and in heat-shocked cells, in which successive S periods can occur between divisions (2, 18, 21). The general design of these experiments was (a) to inhibit DNA synthesis in mass cultures of exponentially growing cells and subsequently assay for cell concentration, and (b) to inhibit DNA synthesis at known times in relation to the S periods which occur in synchronous cells subjected to the heat-shock treatment in early G_1, and assay for division using the microdrop method.

EXPONENTIALLY GROWING CELLS: The effect of M + U on cell concentration during exponential growth is shown in Fig. 2 A. If M + U was added to a culture of cells (Fig 2 A, curve 2), the cell number increased in the same fashion as a control culture (Fig. 2 A, curve 1) for about 1.5 hr, after which the cell concentration increased only slightly during the remainder of the experiment. The proportion of cells (33%) that were able to divide after the addition of M + U (Fig 2 A, curve 2) was slightly larger than that calculated (25%) from the age-gradient formula (25), if it is assumed that M + U prevented all cells from completing

division except those in G_2 and D. Since M + U was shown to halt rapidly the incorporation of adenosine-^{14}C into DNA (Fig 1 A, curve 3), some late S cells either can divide in the absence of further DNA synthesis or can complete DNA synthesis by using preexisting thymidine reserves (33), before dividing.

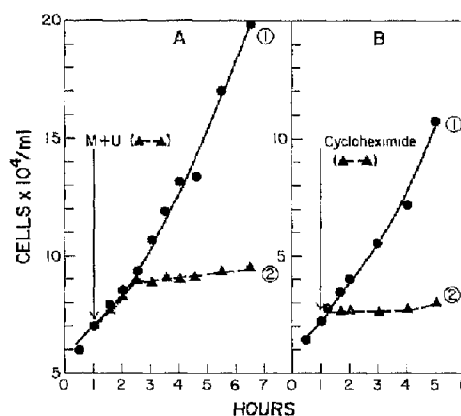The possibility that M + U blocks division

FIGURE 2 The effect of methotrexate and uridine ($M + U$) (A) and cycloheximide (B) on cell number in exponentially growing *T. pyriformis*. In both A and B, curve 1 represents the cell number in controls to which neither inhibitor was added. Curve 2 (A and B) represents the cell number after $M + U$ (A) or cycloheximide (B) was added. Assay for cell number began at time zero, and inhibitors were added 1 hr later (arrows).

Division time classes (Min after EST)	Number of divided cells observed vs time after BST placed in microdrops			
	40 (End 1st shock)	160 (End 3rd shock)	250 (Beginning 5th shock)	360 (EST + 20)
80-90	4	3	4	4
90-100	4	1	5	6
100-110	4	12	4	6
110-120	10	11	17	9
120-130	7	10	9	14
130-140	8	6	4	5
140-150	2	1	3	2
150 +	0	0	2	1
Totals	39	44	48	47
Average (±SE)	116 4 (±2.8)	115.5 (±2.1)	115.0 (±2.3)	115.0 (±2.4)

The normal interval of division (cell separation) in single cells synchronized in micropipettes was 70–120 min after EST, and average division time was 85.9 (± 1.6).

by affecting protein synthesis was excluded by experiments in which the effect on cell number of cycloheximide, a potent antagonist of protein synthesis in *Tetrahymena* (11), was studied (Fig. 2 B). The results show that cell division ceased almost immediately after the addition of cycloheximide (Fig. 2 B, curve 2), and are consistent with the suggestion that protein synthesis is required for cell division until late in the G_2 period

HEAT-SHOCKED CELLS. Radioautographic studies (21) have shown that all cells subjected to the standard six-shock heat treatment in early G_1 participate in a first postdivision S period (S_1). Furthermore, before the end of the heat shock treatment (EST), 60–70% of these cells participate in a second S period (S_2) without an intervening nuclear or cell division. The following experiments were designed to study the effect of inhibition of DNA synthesis with M + U at various times during S_1 and S_2 on the ability of cells to participate in synchronized division after EST.

Before testing the effects of M + U on synchronized division, preliminary experiments were conducted in order to determine whether the experimental handling, associated with the microdrop method, affected cell division Cells were pipetted into microdrops, without M + U, at various times after the beginning of the heat-shock treatment (BST), and the time of the first synchronized division was recorded As shown in

Fig 3 (curve 1), 90–95% of these cells participated in division after EST, regardless of how long they were cultured in microdrops. However, the average division time of cells cultured in microdrops (about 115 min after EST) was delayed in comparison to the average division times of cells synchronized in micropipettes (about 86 min after EST) (Table I). The observed division delay was not correlated with the duration of time spent in microdrop culture (Table I), and thus was probably the result of a slight shock due to experimental handling, rather than the result of some restriction imposed by the culture method It was concluded that the microdrop method was satisfactory for use in the following experiments.

Fig 3 (curve 2) shows the effect of M + U on synchronized division of cells subjected to the heat-shock treatment in early G_1. Addition of M + U before the terminal portion of S_1 resulted in the complete suppression of the subsequent division. This result agrees with the finding for mass cultures of exponentially growing cells (Fig. 2 A) and suggests that after a normal division progress in the cell cycle at least until late S phase is required for a later division. Addition of M + U between the terminal portions of S_1 and S_2 suppressed cell division in 60–70% of the cells (Fig. 3, curve 2). Since 60–70% of these cells are known to participate in S_2 (21), these results suggest that cells which initiate a second S period during the treatment are re-
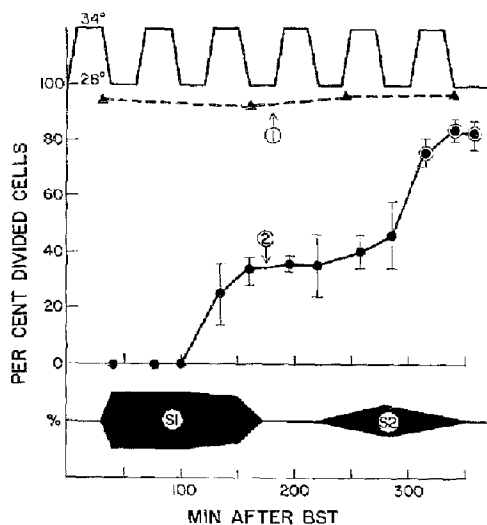
FIGURE 3 The effect of methotrexate and uridine ($M + U$) on heat-synchronized cell division in *T. pyriformis* cells subjected to the heat-shock treatment in early G_1. Curve 1 represents the percentage of divided cells in controls which were placed in microdrop culture without $M + U$ at various times after BST. Each point on curve 1 represents the results of one experiment consisting of 20–40 single cell cultures. Curve 2 represents the percentage of cells dividing once after being placed in microdrops containing $M + U$ at various times after BST. Each point on curve 2 represents the average percentage of divided cells ± the standard deviation of the means (vertical lines) of at least four experiments each consisting of 30–70 single cell cultures. The circled points represent the times of $M + U$ addition after which cells were able to divide twice. Division activity in each microdrop was monitored for 300 min after BST. The timing of the heat-shock treatment in degrees centigrade is shown at the top, and the timing of periods of macronuclear DNA synthesis in the controls (21) is shown at the bottom.

quired to complete at least a certain portion of S_2 before being able to divide. If $M + U$ was added after most cells had completed both S periods (Fig. 3, curve 2), the proportion of cells which participated in the following division almost reached control values. Furthermore, some cells were able to participate in two successive cell divisions under the conditions of presumed thymidine starvation (Fig 3, circled points on curve 2) These results suggest that involvement in two periods of DNA replication while cell division was blocked allowed some cells to complete two consecutive cell divisions without inter-

vening DNA synthesis. This supports earlier studies (21) which indicated that some cells did not incorporate labeled thymidine into macronuclear DNA during the interval between these divisions.

Experiments were also conducted to determine the average division time of cells treated with $M + U$. The results indicated that cells cultured in microdrops containing $M + U$ (Table II) divided at the same time as untreated cells (Table I). Thus, the observed division delays were not due to $M + U$ treatment, but also probably due to experimental handling of the cells.

Reassociation of Division with the DNA Cycle

Further experiments, designed to determine when division activities could be recoupled to the continuing DNA synthetic cycle, involved premature termination of the heat-shock treatment. The basic experimental design was to subject synchronous groups of early G_1 cells to the heat-shock treatment, terminate the treatment after a particular heat shock (thus restoring the optimal temperature requirements for division, i e., 28°C), and assay for cell division by the microdrop method. The results of these experiments are summarized in Fig 4, curve 1 Over 80% of the cells divided when the treatment was prematurely terminated after the conclusion of each heat shock (Fig. 4, curve 1) Radioautographic studies, conducted on cells which were labeled with thymidine-^{3}H when the treatment was terminated after the conclusion of the third heat shock, indicated that the subsequent division took place 100–180 min later in the absence of further DNA synthesis (Table III) If the heat-shock treatment had been *continued* many of these cells presumably would have initiated S_2 within the next 180 min (21). These results show that when conditions favorable for division are restored after the completion of S_1, but before the initiation of S_2, subsequent execution of division-related activities blocks the initiation of S_2

In further studies, the same procedure was followed as in the previous experiment but DNA synthesis was blocked with $M + U$ after the termination of the treatment. The results (Fig. 4, curve 2) indicate that the ability to participate in cell division was dependent on whether DNA replication was in progress at the time of $M + U$ addition. Very few cells were able to divide if the

TABLE II

The Time of the First Synchronized Division in Heat-Shocked Cells Cultured in Microdrops Containing M + U Beginning at Various Times during the Heat-Shock Treatment

Division time classes (Min after EST)	Number of divided cells observed vs. time after BST placed in microdrops containing M + U			
	40 (End 1st shock)	160 (End 3rd shock)	250 (Beginning 5th shock)	360 (EST + 20)
80–90	0	9	7	10
90–100	0	6	5	13
100–110	0	5	5	11
110–120	0	6	11	10
120–130	0	5	5	7
130–140	0	4	6	2
140–150	0	3	6	5
150–160	0	2	1	2
160–170	0	1	3	3
170–180	0	2	1	2
180 +	0	1	2	1
Totals	0	44	52	66
Average (±SE)		115.7 (±4.0)	119.8 (±3.4)	114.1 (±3.1)

heat-shock treatment was terminated after the first and second heat shocks when all cells were presumably engaged in S_1 (21). However, almost all the cells divided if the heat treatment was terminated and M + U was added after the conclusion of the third and fourth heat shocks (Fig 4, curve 2). The time period between the end of the third and fourth heat shocks corresponds to the gap between the completion of S_1 and the initiation of S_2, during which very few cells are engaged in DNA synthesis (21). It should be recalled that if M + U was added to cells at a similar position in the DNA synthetic cycle and if division was suppressed by the continuation of the heat-shock treatment, only about 35% of the cells were able to divide (Fig 3, curve 2). These results suggest that the ability to divide, attained after the terminal portion of S_1, was lost when S_2 was initiated. Consistent with this suggestion, the division percentage was depressed to about 50% (Fig 4, curve 2) when the heat-shock treatment was terminated and M + U was added after the fifth shock, when about 50% of the cells are engaged in S_2 (20). Higher percentages of divided cells were observed after M + U addition after the conclusion of the entire treatment (i.e., six heat shocks).

In most of the individual experiments summarized in Fig. 4 (curve 2), division-related activities were reassociated with the DNA cycle after the same heat shock in the whole population

of synchronous cells However, in some experiments division was reassociated after portions of the total cell population had completed various numbers of heat shocks The results of these experiments (Table IV) indicated that the pattern of division percentage shown in Fig. 4 (curve 2) could be observed even within a single experiment.

In summary, the results of the recoupling ex-

TABLE III

Macronuclear DNA Synthesis between the Conclusion of the Third Heat Shock and the Subsequent Cell Division in T. pyriformis

Time of cleavage furrow appearance (Min after heat shock 3)	Total cells	Macronuclear labeling	
		Labeled	Unlabeled
100–120	23	0	23
120–140	15	0	15
140–160	13	2	11
160–180	0	0	9
Totals	60	2	58

Early G_1 cells were subjected to three heat shocks and individually pipetted into microdrops containing thymidine-^{3}H (10 μCi/ml). The Petri dishes containing cells in microdrop cultures were subsequently incubated at 28°C. At the first appearance of a cleavage furrow each cell was washed in distilled water and fixed for radioautography.
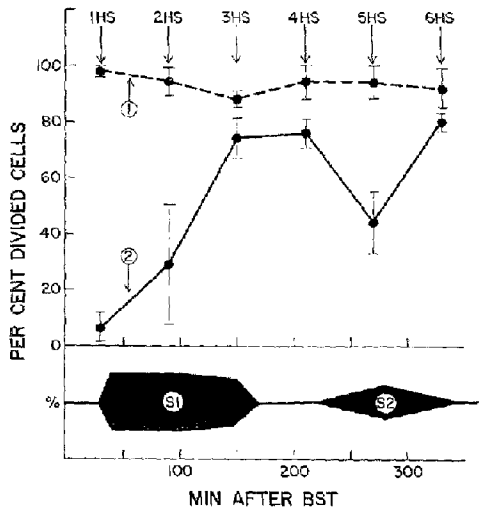
FIGURE 1 Reassociation of cell division with the DNA cycle of heat-shocked *T. pyriformis* after premature termination of the heat-shock treatment and addition of methotrexate and uridine (M + U). Curve 1 represents the percentage of cells divided in controls after termination of the treatment at the end of a particular heat shock. Curve 2 represents the percentage of divided cells observed after termination of the treatment at the end of a particular heat shock and addition of M + U at that time. Each point on curves 1 and 2 represents the average percentage of divided cells ± the standard deviation of the means (vertical lines) of at least four experiments each consisting of 30–70 single cell cultures. Cells were originally subjected to the heat-shock treatment in early G_1 and monitored for division in microdrops for at least 300 min after the end of the particular heat shock. The numbers at the top correspond to the last heat shock (HS) received by the cells represented by points directly below the arrows. The timing of periods of macronuclear DNA synthesis in the controls (21) is shown at the bottom.

periments indicate that cells attain the capacity to divide near the completion of S_1. Commencement of division activities during the gap between S_1 and S_2 prevents the initiation of S_2. However, if division is prevented by further heat shocks and if S_2 is eventually initiated, the capacity to divide in the absence of further DNA synthesis is lost until the necessary portion of S_2 is completed.

DISCUSSION

Recent studies by Villadsen and Zeuthen (34) have shown that M + U, while blocking DNA synthesis, affects both RNA and protein synthe-

sis. However, it is probable that M + U, at the lower concentration used in the present study, did not block division by affecting the synthesis of division-related proteins. This conclusion is supported by two lines of evidence. First, if protein synthesis is required until late G_2 for division, it follows that any severe interference with translational events would be expected to block almost immediately further division during exponential growth. This was observed in the present study when cycloheximide, an effective antagonist of protein synthesis in *Tetrahymena* (11), was added to a population of exponentially growing cells. In contrast to the cycloheximide results, the percentage of the cell population able to divide after M + U addition was consistent with the conclusion that all cells which had completed S could divide. The second and most compelling line of evidence is that some cells, which had presumably completed two S periods during the heat-shock treatment, were able to participate in two successive divisions in the presence of M + U. These results strongly support the argument than when M + U blocks cell division it does so by specifically inhibiting DNA metabolism.

Inhibition of the progress of DNA replication during most of the S period of exponentially growing and heat-shocked *T. pyriformis* resulted in the suppression of the following cell division. However, as reported earlier by Andersen (1), addition of M + U during late S allowed a normal cell division to occur. Since M + U

was shown to stop DNA synthesis very quickly (Fig. 1 A), it is conceivable that the capacity to initiate division activities may be attained shortly before the completion of DNA replication in *Tetrahymena*. However, it is also possible that late-S cells complete DNA synthesis in the presence of M + U, using preexisting thymidine derivatives from the macronuclear pools (33), before dividing.

The experiments in which reassociation of division activities was attempted at various times during the DNA cycle suggest that each S period which is initiated during the heat-shock treatment must be completed before division can occur, even though the normal G_2 complement of DNA was present. This indicates that the attainment of a minimal amount of DNA (i.e., possibly the normal G_2 quantity) may be one condition for division during the normal cell cycle, but a further coupling mechanism may be reflected by a temporal incompatibility between the processes of DNA replication and cell division. This proposal implies that preparations for cell division can only occur during periods of the cell cycle when nuclear DNA is in a nonreplicating state. A similar situation has been recently reported to exist between the initiation of bud formation and the S period in yeast (13).

At present the mechanism through which replicating DNA excludes the execution of division activities remains unknown, but it can be envisioned that such control could arise actively, by the production of specific division repressor substances during the S phase, or passively, by cessation of production of necessary RNA transcripts or competition for an energy source while DNA synthesis is in progress

The demonstration that completion of one S period (S_1) during the heat-shock treatment is a sufficient condition for cell division provides further support for the suggestion that the total macronuclear DNA content is replicated at least once before EST. The existence of DNA in amounts above the normal G_2 quantity in heat-shocked *Tetrahymena* has been previously demonstrated by chemical and microspectrophotometric methods (40). However, Byfield and Lee (4) have recently proposed that the replication of a particular fraction of DNA, which is presumed to code for division related proteins, may be selectively inhibited by the treatment. The results of the present investigation do not support

this proposal. Recently, Andersen et al., (2) have been able to conclusively demonstrate, using density labeling techniques, that all DNA is replicated once during the heat-shock treatment.

As pointed out earlier, experiments concerned with the reassociation of division activities with the DNA synthetic cycle have shown that preparations for division cannot proceed while the DNA remains in a replicating state. However, if division activities were reassociated after DNA replication has ceased (i.e., during the gap between S_1 and S_2), the initiation of further S periods was suppressed until the next interdivision period. Similar results have been obtained previously with populations of heat-shocked *Tetrahymena* (18, 20) during the interval between EST and the first synchronized division. The period of time under consideration (end of a particular heat shock or EST to division) represents an interval when progress in the division cycle is reinitiated synchronously. It includes the reactivation of the development of the cortical structures of the cell (9, 36), and the occurrence of transcriptional (22, 26) and translational (11, 27) events linked to the ultimate commencement of division. In exponentially growing cells cortical development (9) occurs primarily during the latter half of the cell cycle after DNA replication has almost been completed (21). The present study has also shown that division-related protein synthesis is required until late in the G_2 period of exponentially growing cells. On this basis, it is tempting to speculate that some of the underlying processes which are primarily linked with the G_2 events necessary to prepare a cell for division may exclude the initiation of further periods of DNA replication and thereby reflect another part of the coupling mechanism which insures temporal alteration between DNA synthesis and division During the heat-shock treatment this mechanism would be nonfunctional, resulting in the periodic initiation of additional periods of macronuclear DNA replication.

REFERENCES

1 ANDERSEN, H. A. 1971. DNA replication and cellular division in heat synchronized *Tetrahymena* cells. *Exp. Cell Res.* 67:311.

2. ANDERSEN, H. A., C. F. BRUNK, and E. ZEUTHEN 1970. Studies on the DNA replication in heat synchronized *Tetrahymena pyriformis* C. R. Trav Lab. *Carlsberg.* 38:123.

3. BOSTOCK, C. J. 1970. The effect of 2-phenyl ethanol on the DNA synthesis cycle of *Saccharomyces pombe. J. Cell Sci.* 7:523.

4. BYFIELD, J. E., and Y. C LEE. 1970. The effect of synchronizing temperature shifts on the synthesis and translation of replication-supporting messengers in *Tetrahymena pyriformis. Exp. Cell Res.* 61:42.

5. BYFIELD, J. E., and O. H. SCHERBAUM. 1966. Rapid radioassay technique for cellular suspensions. *Anal. Biochem.* 17:434.

6. CLARK, D. J. 1968. The regulation of DNA replication and cell division in *E. coli* B/r. *Cold Spring Harbor. Symp. Quant. Biol.* 33:823.

7. CLEFFMANN, G. 1968. Regulierung der DNS Menge in Makronucleus von *Tetrahymena. Exp. Cell Res.* 50:193

8. FANGMAN, W L., and A. NOVICK. 1968. Characterization of two bacterial mutants with temperature sensitive synthesis of DNA. *Genetics.* 60:1.

9. FRANKEL, J. 1962. The effects of heat, cold, and p-fluoro-phenylalanine on morphogenesis in synchronized *Tetrahymena pyriformis* GL. C. R Trav. Lab. *Carlsberg.* 33:1.

10. FRANKEL, J. 1965. The effect of nucleic acid antagonists of cell division and oral organelle development in *Tetrahymena pyriformis. J. Exp. Zool.* 159:113.

11. FRANKEL, J. 1969. The relationship of protein synthesis to cell division and oral development in synchronized *Tetrahymena pyriformis* GL-C an analysis employing cycloheximide. *J Cell Physiol.* 74:135.

12. GUTTES, E., and S. GUTTES. 1963. Initiation of mitosis in post-mitotic nuclei of *Physarum polycephalum Experientia (Basel).* 19:13.

13. HARTWELL, L. H. 1971 Genetic control of the cell cycle in yeast. II. Genes controlling DNA replication and its initiation. *J. Mol. Biol.* 59:183.

14. HARTWELL, L. H. 1971. Genetic control of the cell cycle in yeast IV. Genes controlling bud emergence and cytokinesis *Exp. Cell Res.* 69:265.

15. HELMSTETTER, C. E., and O. PEIRUCCI 1968. Cell division during inhibition of deoxyribonucleic acid synthesis in *Escherichia coli. J. Bacteriol.* 95:1627.

16. HIROTA, Y., F. JACOB, A. RYTER, G. BUTTIN, and T NAKAI 1968. On the process of cellular division in *Escherichia coli.* I. Asymmetrical cell division and the production of DNA-less bacteria. *J. Mol. Biol.* 35:175.

17. HIROTA, Y., J. MORDOH, and F. JACOB. 1970. On the process of cellular division in *Escherichia coli* III. Thermosensitive mutants of *Escherichia coli* altered in the process of DNA initiation. *J. Mol. Biol.* 53:369.

18 HJELM, K. K., and E. ZEUTHEN. 1967. Synchronous DNA synthesis induced by synchronous cell division in *Tetrahymena* C. R Trav. Lab. *Carlsberg.* 36:127.

19. INOUYE, M. 1969. Unlinking of cell division from DNA replication in a temperature-sensitive DNA synthesis mutant from *E. coli. J. Bacteriol.* 99:842.

20. JEFFERY, W. R. 1971. Studies on the macronuclear DNA cycle of heat synchronized *Tetrahymena pyriformis* Ph.D. Thesis. University of Iowa, Iowa City.

21. JEFFERY, W. R., K. D. STUART, and J. FRANKEL 1970 The relationship between deoxyribonucleic acid replication and cell division in heat-synchronized *Tetrahymena. J. Cell Biol.* 46:533.

22. LAZARUS, L. H., M. R. LEVY, and O. H. SCHERBAUM 1964. Inhibition of synchronized cell division in *Tetrahymena* by actinomycin D. *Exp Cell Res.* 36:672.

23. LOWY, B. A., and V. LEICK. 1969. The synthesis of DNA in synchronized cultures of *Tetrahymena pyriformis* GL. *Exp. Cell Res.* 57:277.

24. MENDELSON, N. H., and J. D. GROSS. 1967. Characterization of a temperature sensitive mutant of *Bacillus subtilus* defective in deoxyribonucleic acid replication. *J. Bacteriol.* 94:1603.

25. NACHTWEY, D. S., and I. L. CAMERON. 1968. Cell cycle analysis. *Methods Cell Physiol.* 3:213.

26 NACHTWEY, D S., and W. J. DICKINSON. 1967. Actinomycin D: blockage of cell division of synchronized *Tetrahymena pyriformis. Exp. Cell Res* 47:581.

27. RASMUSSEN, L., and E. ZEUTHEN. 1962. Cell division and protein synthesis in *Tetrahymena* as studied with p-fluorophenylalanine. C. R. Trav. Lab. *Carlsberg.* 32:333.

28 RUECKERT, R. R., and G. C MUELLER. 1960. Studies on unbalanced growth in tissue culture. I. Induction and consequences of thymidine deficiency. *Cancer Res* 20:1584

29. SACHSENMAIER, W., and H. P. RUSCH. 1964. The

effect of 5-fluoro-2-deoxyuridine on synchronous mitosis in *Physarum polycephalum. Exp Cell Res.* 36:124.

30. SCHERBAUM, O. H., and E. ZEUTHEN. 1954. Induction of synchronous division in mass cultures of *Tetrahymena pyriformis. Exp. Cell Res.* 18:150.

31. SCHMIDT, G., and S. J. THANNHAUSER. 1945. A method for the determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. Biol. Chem.* 161:83.

32. STONE, G. E , and I. L. CAMERON. 1964. Methods for using *Tetrahymena* in studies of the normal cell cycle. *Methods Cell Physiol.* 1:27.

33. STONE, G. E., O. L. MILLER, and D M. PRESCOTT. 1965. ^{3}H-Thymidine derivative pools in relation to macronuclear DNA synthesis in *Tetrahymena pyriformis. J. Cell Biol.* 25:171.

34. VILLADSEN, I. S., and E. ZEUTHEN. 1970. Synchronization of DNA synthesis in *Tetrahymena* populations by temporary limitation of access to thymine compounds. *Exp. Cell Res.* 61:302.

35. WALKER, J. R., and A B. PARDEE. 1968. Evidence for a relationship between deoxyribonucleic acid metabolism and septum formation in *Escherichia coli. J. Bacteriol.* 95:123

36. WILLIAMS, N. E., and O. H SCHERBAUM. 1959. Morphogenetic events in normal and synchronously dividing *Tetrahymena. J. Embryol. Exp. Morphol.* 7:241.

37. ZEUTHEN, E. 1963. Independent cycles of cell division and DNA synthesis in *Tetrahymena. In* Cell Growth and Cell Division. R. C. J. Harris, editor. Academic Press Inc., New York. 1.

38 ZEUTHEN, E. 1968. Thymine starvation by inhibition of uptake and synthesis of thymine-compounds in *Tetrahymena. Exp. Cell Res.* 50:37.

39. ZEUTHEN, E. 1970. Independent synchronization of DNA synthesis and of cell division in the same culture of *Tetrahymena* cells. *Exp. Cell Res.* 61:311.

40. ZEUTHEN, E., and L. RASMUSSEN. 1972. Synchronized cell division in protozoa. *In* Research in Protozoology. T. T. Chen, editor. Pergamon Press, Inc., New York. In press