

# ANALYSIS OF THE SCHEDULE OF DNA REPLICATION IN HEAT-SYNCHRONIZED *TETRAHYMENA*

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## ABSTRACT

The temporal schedule of DNA replication in heat-synchronized *Tetrahymena* was studied by autoradiographic and cytofluorometric methods. It was shown that some cells, which were synchronized by selection of individual dividing cells or by temporary thymidine starvation, incorporated [<sup>3</sup>H]thymidine into macronuclei in a periodic fashion during the heat-shock treatment. It was concluded that supernumerary S periods occurred while cell division was blocked by high temperature. The proportion of cells which initiated supernumerary S periods was found to be dependent on the duration of the heat-shock treatment and on the cell cycle stage when the first heat shock was applied. Cytofluorometric measurements of Feulgen-stained macronuclei during the heat-shock treatment indicated that the DNA complement of these cells was substantially increased and probably duplicated during the course of each S period. Estimates of DNA content also suggested that the rate of DNA synthesis progressively declined during long heat-shock treatments. These results indicate that the mechanism which brings about heat-induced division synchrony is not an interruption of the process of DNA replication. Further experiments were concerned with the regulation of DNA synthesis during the first synchronized division cycle. It was shown that participation in DNA synthesis at this time increased as more cells were able to conclude the terminal S period during the preceding heat-shock treatment. It is suggested that a discrete period of time is necessary after the completion of DNA synthesis before another round of DNA synthesis can be initiated.

## INTRODUCTION

Despite a number of recent investigations (38, 39), the temporal schedule of macronuclear DNA replication in *Tetrahymena pyriformis* synchronized by multiple heat shocks (31) has remained a controversial issue (3). Zeuthen and co-workers (1, 6, 15) have concluded, primarily on the basis of evidence obtained from autoradiographic and density labeling experiments, that DNA synthesis con-

tinues asynchronously in the form of periodically occurring S periods while cell division is blocked during the heat-shock treatment. This suggestion explained biochemical DNA measurements (16, 22, 29, 30, 40) which revealed varying degrees of DNA accumulation (1.7- to 4-fold) during the treatment, and microspectrophotometric observations (30) that DNA content rose above the normal

G<sub>2</sub> complement in all cells by the end of the treatment (EST).<sup>1</sup> Nonetheless, Byfield and Lee (3) have recently proposed that DNA synthesis proceeds only very slowly during the treatment and that some cells do not complete one S period. Byfield and co-workers (3, 4) have revived the earlier suggestion of Scherbaum et al. (30) that division synchrony is brought about by the selective inhibition of the replication of a particular fraction of the DNA which may code for division-related proteins.

Although there is some disagreement concerning the schedule of DNA synthesis during the multiple heat-shock treatment, it is generally accepted that synchronized division (SD1) eventually results in the phasing of subsequent DNA synthesis (1, 3, 14, 15, 20). However, a considerable proportion of the cell population does not participate in DNA synthesis at any time between the first two synchronized divisions (1, 15, 20, 23). The purpose of the present investigation was to determine the schedule of DNA synthesis during the multiple heat-shock treatment and its relationship to the occurrence of further replication after synchronized division.

Virtually all previous analyses of DNA replication in heat-synchronized *Tetrahymena* have employed mass cultures of cells which were randomly distributed over the normal cell cycle when the heat-shock treatment was initiated (BST). This approach provided sufficient amounts of material for biochemical analysis, but since DNA synthesis remained asynchronous the schedule of macronuclear S periods could not be determined. Our previous communication (20) described an initial attempt to employ small groups of synchronous cells to investigate the timing of DNA synthesis. It was found that some cells (67%) subjected to BST in early G<sub>1</sub> participated in two consecutive S periods without intervening division, but others (33%) engaged in only one S period during the same interval. The present report continues this investigation by characterizing the schedule of DNA synthesis when the heat-shock treatment is initiated at various times during the cell cycle. We have found

that supernumerary S periods are periodically initiated subsequent to subjection to the treatment in G<sub>1</sub>, S, or G<sub>2</sub>. Each of these S periods involve a substantial increase and probably a doubling of the macronuclear DNA complement. Finally, we have established that the occurrence of DNA synthesis between synchronized divisions is dependent on the schedule of supernumerary S periods during the treatment. These results are discussed in relation to the mechanism of heat-synchronization and the regulation of DNA synthesis.

## MATERIALS AND METHODS

### *Cells, Growth Conditions, and Synchronization*

Axenic stock cultures of *Tetrahymena pyriformis* (a-micronucleate strain GL-C) were maintained in slanted test tube cultures at 28°C. The growth medium consisted of a tryptone-dextrin-vitamins-salts mixture (12). These stock cultures were used to inoculate larger flask cultures which contained 150 ml of growth medium.

Groups of synchronous cells at a particular stage of the cell cycle were obtained by the selection of dividing cells from flask cultures (32) as described previously (20). These cells were subjected to the heat-shock treatment in capillary micropipettes (25) during early G<sub>1</sub> (5–10 min after the previous cell division), early S (50–60 min after the previous cell division), and G<sub>2</sub> (120–140 min after the previous cell division). The timing of cell cycle stages in *T. pyriformis* GL-C was described previously (20).

Synchronization was also achieved by the temporary thymidine starvation method of Villadsen and Zeuthen (34). Methotrexate (Lederle Laboratories, Pearl River, N. Y.) and uridine (M + U), at respective concentrations of 0.05 mM and 5.0 mM (19, 36), were added to flask cultures of exponentially growing cells in order to elicit a thymidine deficiency. Addition of thymidine (5.0 mM) 4 h later resulted in a moderate degree of synchronization. These cultures were subjected to the heat-shock treatment during mid-S (40 min after thymidine rescue) and early G<sub>2</sub> (100 min after thymidine rescue).

The standard multiple heat-shock treatment (31) was administered by an automatic temperature-regulating water bath (24) and consisted of six 30-min periods of elevated temperature (34°C) separated by 30-min intershock periods at optimal growth temperature (28°C). In some cases the standard heat-shock treatment was extended beyond six shocks.

### *Labeling and Autoradiography*

DNA was tritium labeled by expelling cells from their micropipettes into depression slides contain-

<sup>1</sup> Abbreviations used in this paper are:

BST: beginning of the synchronizing (multiple heat shock) treatment.

EST: end of a standard 6-shock heat treatment.

M + U: methotrexate plus uridine.

TR: thymidine rescue.

SD1, SD2: synchronized division 1, 2, etc.

ing [*methyl*-<sup>3</sup>H]thymidine (10  $\mu$ Ci/ml; 6.7 mCi/mM; New England Nuclear, Boston, Mass.) in 100  $\mu$ l of culture medium. Subsequently, the cells were either drawn into sterile micropipettes (with isotope-containing medium) if labeling was to be continued for long periods, or else prepared for autoradiography after a 12-min exposure.

Cells which had been exposed to isotope-containing medium were washed by expelling them from their micropipettes into a depression slide containing 200  $\mu$ l of distilled water. This step was found to provide the necessary degree of cell flattening desired for optimal grain detection (20). After washing, the cells were pipetted onto subbed slides (5) and rapidly dried under a stream of warm air from an electric dryer. The slides were fixed for 30 min in 3:1 ethanol:acetic acid, rinsed in four washes of 95% ethanol, extracted for 30 min in cold trichloroacetic acid, and finally washed in three changes of 70% ethanol. Autoradiography was carried out according to the procedure of Prescott (27) using Kodak NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.) diluted 1:1 with distilled water. The coated slides were stored for 10-14 days at room temperature before developing. Slides were developed as described previously (27). Cells were stained through the emulsion with 0.5% methyl green and examined at 400 $\times$  with phase optics. Previous studies (20) have demonstrated that macronuclear labeling could be almost completely removed by deoxyribonuclease digestion.

#### *Cytophotometric Determination of Macronuclear DNA Quantity*

Macronuclear DNA content was determined by fluorescence cytophotometry (2). The cells were dried on slides, fixed in 3:1 ethanol:acetic acid for 15 min, rinsed with water, dehydrated through an ethanol series, and stored in absolute ethanol at +4°C until stained. A modification of Elftman's (10) Feulgen procedure was used in which the acid hydrolysis was performed in N HCl at 40°C for 40 min, the cells stained for 2 h in Schiff's reagent, and bleached in three changes of SO<sub>2</sub> water for 15 min each. After staining and mounting in Kleermount (Carolina Biological Supply Co., Burlington, N. C.), the relative amount of DNA per cell was measured microfluorometrically. For these measurements the Leitz-MPV-fluorometer (Leitz, Wetzlar, Germany) was used in an optical arrangement that allowed successive and/or simultaneous illumination with transmitted and incident light, and was similar to that described by Ruch (28). The specific optical arrangement in the Leitz-MPV-Fluorometer used for the measurements utilized a high pressure xenon lamp XBO-150W (Osram, Berlin) in combination with an interference heat-protecting filter, a red-absorbing

filter 5-mm BG38, and a Hg546 interference filter to produce a near monochromatic light source at 546 nm. This intense green light was reflected and focused onto the specimen by a dichroic mirror (Leitz) TK580 and a PHACO NPL 100/1.30 objective, respectively. The induced red fluorescence was then collected by the objective lens, passed through the dichroic mirror and a K580 barrier filter (both of which transmit red light), the area of the nucleus optically isolated by the measuring diaphragm of the fluorometer; and the image of the nucleus, projected onto the photomultiplier S-20 type 9558AQ with a quartz window. Only a narrow band of the red spectrum was used for the measurement (2) and was selected by a 60-mm interference wedge filter set at 660 nm. Thus, fluorescence intensities at 660 nm were recorded as photomultiplier voltage output and were proportional to the DNA content (2).

## RESULTS

### *The Schedule of DNA Synthesis*

#### SYNCHRONIZATION BY CELL SELECTION

The first series of experiments was designed to study the timing of DNA synthesis when groups of synchronous cells were subjected to the multiple heat-shock treatment at different times during the cell cycle. The studies reported in the first three sections are *short pulse-fix* experiments in which cells were incubated with [<sup>3</sup>H]thymidine for 12 min and then immediately fixed for autoradiography.

**EARLY G<sub>1</sub> CELLS:** As shown in Fig. 1, cells which were in early G<sub>1</sub> at BST participated in an S period (S<sub>1</sub>)<sup>2</sup> during the first part of the heat-shock treatment. Another S period (S<sub>2</sub>), which involved about 50% of the cells, was initiated during the latter part of the treatment. These results supplement those which were obtained previously (20) and have now been confirmed and extended to

<sup>2</sup> The terms of S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> are operationally defined in the following manner: (a) S<sub>1</sub> is the normal period of DNA replication which follows the last cell division before BST. During S<sub>1</sub> the amount of macronuclear DNA rises from typical G<sub>1</sub> to G<sub>2</sub> values. S<sub>1</sub> may occur wholly during, partly during, or wholly before the heat-shock treatment, depending on whether the treatment is initiated in G<sub>1</sub>, S, or G<sub>2</sub>. (b) S<sub>2</sub> refers to the period(s) of DNA replication which occurs after the completion of S<sub>1</sub>, but before the first synchronized division. During S<sub>2</sub> the macronuclear DNA content rises above the usual G<sub>2</sub> values. (c) S<sub>3</sub>

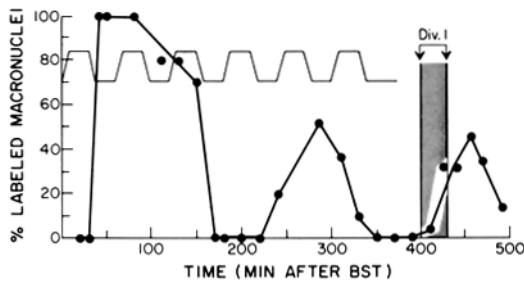


FIGURE 1 The timing of macronuclear DNA synthesis in *T. pyriformis* subjected to the 6-shock heat-synchronization treatment in early  $G_1$ . Synchronous groups of cells were obtained by cell selection methods. Each point represents the percentage of cells with labeled macronuclei after a 12-min pulse of [ $^3\text{H}$ ]thymidine. This graph represents a total of 14 experiments involving 341 cells all in early  $G_1$  at BST (time 0). The heat-shock regimen is indicated above. The vertical arrows represent the range of time in which cell separation occurred during the first synchronized division.

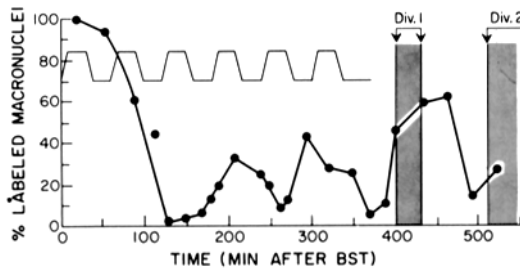


FIGURE 2 The timing of macronuclear DNA synthesis in *T. pyriformis* subjected to the 6-shock heat-synchronization treatment in early S. This graph represents a total of 12 experiments involving 443 cells all in early S at BST (time 0). All other details are similar to Fig. 1.

include the timing of DNA synthesis between SD1 and SD2 (Fig. 1). A third S period ( $S_3$ ) was initiated concomitant with macronuclear division and the beginning of cleavage furrow formation at about 70 min after EST. As shown previously (15, 20),  $S_3$  was initiated fairly synchronously and in the absence of a detectable  $G_1$  period.

**EARLY S CELLS:** Cells subjected to BST in early S continued DNA synthesis until the second intershock period and subsequently initiated  $S_2$  (Fig. 2). Participation in  $S_2$  was observed to occur in a bimodal fashion with peaks of macronuclear labeling occurring at the conclusion of the fourth heat shock and the middle of the fifth intershock period.

refers to the period of DNA replication between the first two synchronized divisions.

This pattern may be explained by variation in the frequency of initiation as well as decay in original synchrony. A bimodal second S period was observed by other investigators when *Tetrahymena* cells were treated with low concentrations of actinomycin during  $G_1$  and failed to divide (8, 17).

At the time of macronuclear fission,  $S_3$  was initiated, and at about 135 min after EST 60% of the cells were engaged in DNA synthesis (Fig. 2). The duration of  $S_3$  was similar to that observed in exponentially growing cells.

**$G_2$  CELLS:** As shown in Fig. 3, very few cells which were subjected to BST at 120–140 min after the previous division became labeled during the first heat shock and the following intershock period. The majority of these cells had completed DNA replication before BST and were in  $G_2$  at the beginning of the initial heat shock. The proportion of labeled macronuclei ( $S_2$ ) began to gradually increase at the beginning of the second heat shock and reached a maximum (60%) during the third heat shock. Participation in  $S_2$  was again bimodal, since a second maximum of labeled macronuclei occurred at the conclusion of the fifth heat shock.

Since cell division is known to occur in some very late  $G_2$  cells during the first part of the heat-shock treatment (33), further experiments were conducted in order to determine whether  $S_2$  may actually have been preceded by a cellular or macronuclear division. A total of 151 cells, each in an individual capillary micropipette, were subjected to BST during  $G_2$  (120–140 min after the previous division). Only 13 (9%) were able to complete cytokinesis during the first 240 min of the heat-shock treatment. In contrast, all control cells treated in a similar fashion, but continuously in-

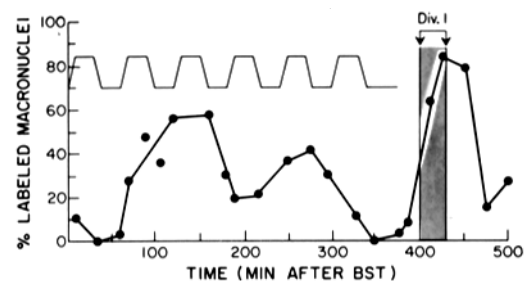


FIGURE 3 The timing of macronuclear DNA synthesis in *T. pyriformis* subjected to the 6-shock heat-synchronization treatment in  $G_2$ . This graph represents a total of nine experiments involving 307 cells all in  $G_2$  at BST (time 0). All other details are similar to Fig. 1.

TABLE I

*The Proportion of Cells Subjected to the Heat-Shock Treatment at a Particular Cell Cycle Stage Which Participate in Each Macronuclear S period*

Cell cycle stage at BST	Macro-nuclear S period	Assay time	Number of experiments	Macronuclear label		Percent macronuclei labeled
				Labeled	Unlabeled	
Early G <sub>1</sub>	S <sub>1</sub>	BST to BST + 150 min	4	73	0	100
	S <sub>2</sub>	BST + 200 min to EST + 50 min	6	61	33	65
	S <sub>3</sub>	EST + 50 min to EST + 150 min	6	28	90	24
Early S	S <sub>1</sub>	Isolation to BST + 120 min	2	61	0	100
	S <sub>2</sub>	BST + 120 min to EST + 50 min	5	177	25	88
	S <sub>3</sub>	EST + 50 min to EST + 150 min	6	148	49	75
G <sub>2</sub>	S <sub>1</sub>	Isolation to BST	2	47	0	100
	S <sub>2</sub>	BST to EST + 50 min	2	47	2	96
	S <sub>3</sub>	EST + 50 min to EST + 150 min	2	43	7	86

BST = beginning of heat shock treatment; EST = end of standard 6-shock treatment.

cubated at 28°C, were observed to divide during the same time interval. In order to determine whether macronuclear division could have occurred in the absence of cytokinesis (11), the cells subjected to the treatment which remained undivided were stained with methyl green. It was found that each had a single macronucleus.<sup>3</sup> These results support the conclusion that the majority of cells which are in G<sub>2</sub> at BST initiate an additional S period during the heat-shock treatment without participating in intervening macronuclear or cellular division.

The results presented in Fig. 3 also show that S<sub>3</sub> was initiated at the time of macronuclear division and a maximum of 85% of the cell population was engaged in DNA synthesis at 100 min after EST.

In summary, the results of the *short pulse-fix* experiments indicate (a) that heat-shocked cells periodically initiate macronuclear S periods in the absence of cell division, (b) that DNA synthesis ceases immediately before synchronized division, and (c) that more cells engage in S<sub>2</sub> and S<sub>3</sub> when BST occurs at progressively later times during the cell cycle.

<sup>3</sup> Occasionally multinucleate cells were observed in other experiments. Six binucleate cells and one with an apparently fragmented macronucleus were found among 283 cells that had been subjected to the heat treatment in early G<sub>1</sub> and then Feulgen stained and prepared for cytophotometry.

#### PROPORTION OF CELLS INVOLVED IN EACH S PERIOD

Additional experiments, involving longer labeling periods (*long pulse-fix experiments*), were conducted in order to accurately determine the proportion of cells which participated in each of the S periods. The results indicated that all cells participated in S<sub>1</sub> (Table I). This was found to be true whether S<sub>1</sub> occurred completely before BST (G<sub>2</sub> cells), was initiated before BST and completed during the treatment (early S cells), or took place entirely within the treatment (early G<sub>1</sub> cells). It was also confirmed that the proportion of cells which participated in S<sub>2</sub> and S<sub>3</sub> gradually increased as BST occurred at later times during the cell cycle and the interdivision time was correspondingly extended.

In order to determine whether all cells participated in DNA synthesis during S<sub>2</sub> or S<sub>3</sub>, experiments were conducted in which early G<sub>1</sub> cells were continuously incubated with [<sup>3</sup>H]thymidine from the beginning of the fourth heat shock (first initiation of S<sub>2</sub>) until 150 min after EST (conclusion of S<sub>3</sub>). Six experiments of this type were performed and in each the majority of cells had labeled macronuclei. A total of 223 out of 228, or 98%, of the cells had engaged in DNA synthesis. These data establish that each cell participates in either S<sub>2</sub> or S<sub>3</sub>, but do not exclude the possibility that some cells may participate in both S periods. The latter

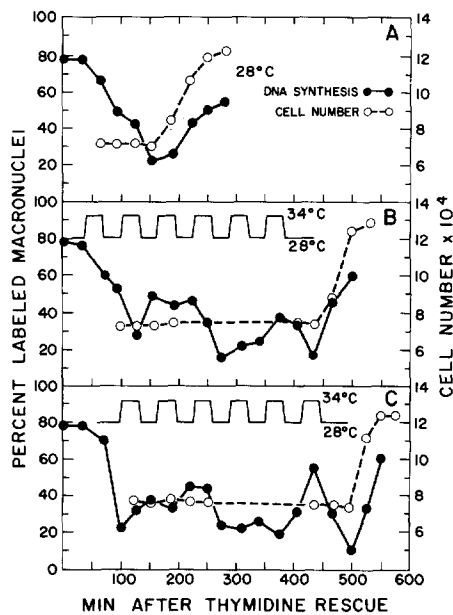


FIGURE 4 The timing of macronuclear DNA synthesis in *T. pyriformis* synchronized by temporary thymidine starvation and afterwards: (A) incubated continuously at 28°C, (B) subjected to the 6-shock heat-synchronization treatment during mid-S (40 min after time 0), (C) subjected to the 6-shock heat-synchronization treatment in early G<sub>2</sub> (100 min after time 0). Time 0 represents the time of reversal of starvation by addition of thymidine to the medium. Each filled point (●—●) represents the percentage of macronuclei labeled (from at least 200 cells) during a 12-min pulse of [<sup>3</sup>H]thymidine. Cell number (○—○) was assayed using a model A Coulter Counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). The heat-shock regimen is indicated in the upper portion of frames B and C.

is obviously true for most cells which were in early S or G<sub>2</sub> at BST (Table I).

#### SYNCHRONIZATION BY THYMIDINE STARVATION

Synchronization of DNA synthesis can also be achieved by treatment of mass cultures of exponentially growing cells with M + U, which block the uptake and synthesis of thymidine (36), followed by addition of excess thymidine (34). This method was utilized in an attempt to determine whether the results obtained by the more precise method of cell selection could be confirmed with mass cultures of cells. Cultures of exponentially growing cells were incubated with M + U for 4 h after which thymidine was added to the medium. As shown in Fig. 4 A, cells treated in this manner,

and labeled during 12-min exposures to [<sup>3</sup>H]thymidine, exhibited a moderate degree of synchronization of DNA synthesis. Nearly 80% of the population exhibited labeled macronuclei immediately after thymidine rescue (TR). This proportion could not be increased using [<sup>3</sup>H]thymidine alone as a rescue agent. DNA synthesis was completed within the next 100 min and cell division occurred within 150–250 min after TR. The temporal pattern of DNA synthesis illustrated in Fig. 4B and C was observed when thymidine-starved cells were subjected to the standard heat-shock treatment after TR. After completing one S period, cultures which consisted of predominantly mid-S and early G<sub>2</sub> cells at BST (40 min and 100 min after TR, respectively) participated in further DNA synthesis in a bimodal fashion during the remainder of the heat-shock treatment. Furthermore, cell numbers did not increase during the treatment (Fig. 4), and it was concluded that supernumerary S periods occurred in the absence of intervening cellular di-

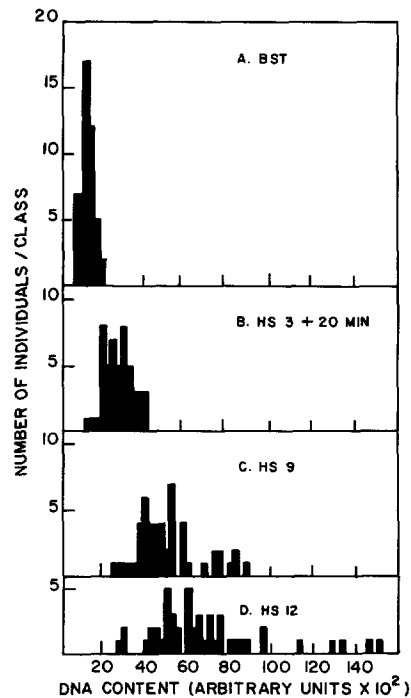


FIGURE 5 Histogram of the values obtained from cytophotometric measurements of the DNA content of asynchronous cells subjected to heat-shock treatments of varying duration. (A) BST; (B) end of heat shock 3 + 20 min; (C) end of heat shock 9; (D) end of heat shock 12.

TABLE II  
Mean Macronuclear DNA Content of Heat-Shocked *Tetrahymena*

Fixation time	Condition at BST									
	Asynchronous cells					Synchronous G <sub>1</sub> cells				
	N	$\bar{X}$	SD ( $\pm\%$ )	SE	F	N	$\bar{X}$	SD ( $\pm\%$ )	SE	F
BST	50	1396	343 (24.6)	48.5	1.0	41	1010	201 ( $\pm 20.0$ )	31.4	1.0
HS 3 + 20 min	50	2864	679 (23.7)	96.0	2.1	95	1818	453 ( $\pm 24.9$ )	46.5	1.8
HS 6	—	—	—	—	—	88	2611	930 ( $\pm 35.6$ )	99.1	2.6
EST + 45 min	50	4093	903 (22.1)	127.7	2.9	26	3162	779 ( $\pm 24.6$ )	152.8	3.1
HS 9	50	4929	1503 (30.5)	212.6	3.5	61	3654	924 ( $\pm 25.3$ )	118.3	3.6
HS 12	50	7021	2869 (40.9)	405.7	5.0	—	—	—	—	—
HS 15	—	—	—	—	—	13	5892	1684 ( $\pm 28.6$ )	467.0	5.8

BST = beginning of heat shock treatment; HS = heat shock; EST = end of standard 6-shock treatment; N = cell number;  $\bar{X}$  = mean DNA content (arbitrary units); SD = standard deviation; SE = standard error; F = DNA content fold difference from mean BST quantity (1.0).

vision. These results indicate that the pattern of DNA synthesis which occurred when cells were synchronized by cell selection, and subjected to the heat-shock treatment in capillary micropipettes, is similar to that which was observed for mass cultures synchronized by thymidine starvation. Thus this pattern actually reflects the alterations in the DNA synthetic schedule produced by subjecting exponentially growing cells to a multiple heat-shock treatment.

#### Changes in Macronuclear DNA Content

Changes in macronuclear DNA content during the multiple heat-shock treatment were measured by fluorescence cytophotometry. DNA content was measured after both asynchronous mass cultures and small groups of synchronous G<sub>1</sub> cells were subjected to the treatment. The evidence obtained from these studies is compatible with our autoradiographic results.

Measurements obtained from originally asynchronous cells showed a progressive increase in DNA content throughout the treatment (Fig. 5). The mean DNA content doubled shortly after the end of the third heat shock and tripled just before synchronized division (Table II). Application of additional heat-shock cycles resulted in a further increase with fourfold DNA values being reached between the end of the ninth and twelfth shocks (Table II). These results indicate that the process of heat-synchronization does not block the continuation of DNA duplication.

Similar results were obtained when early G<sub>1</sub> cells were subjected to the treatment (Fig. 6). As

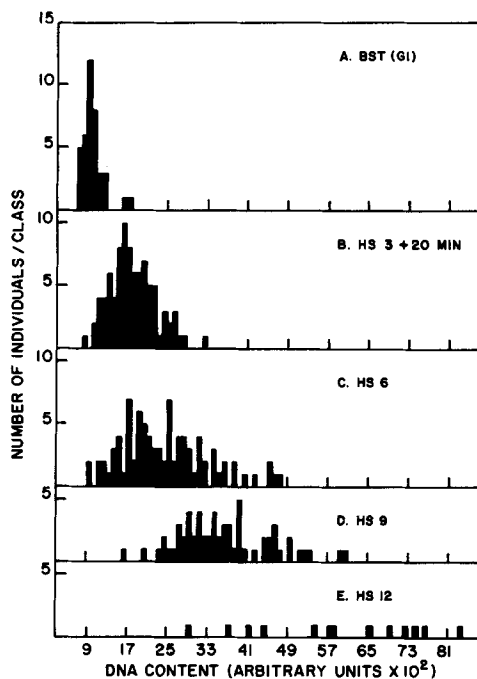


FIGURE 6 Histogram of the values obtained from cytophotometric measurements of the DNA content of synchronous cells subjected to heat-shock treatments of varying duration during early G<sub>1</sub>. (A) BST (early G<sub>1</sub>); (B) end of heat shock 3 + 20 min; (C) end of heat shock 6; (D) end of heat shock 9; (E) end of heat shock 15.

would be expected, the macronuclear DNA contents of these cells corresponded to those of the lower half of the distribution in the asynchronous population at BST (compare Figs. 5 A and 6 A).

The variation observed in measurements of  $G_1$  macronuclei is comparable to that found in *T. pyriformis* HSM by Cleffmann using scanning microspectrophotometry (9), and probably reflects true variation of DNA content in different macronuclei (9). The mean DNA content of the  $G_1$  cells was found to increase 1.8-fold between BST and 20 min after the end of the third heat shock (Table II). This result indicates that at least in most cells the total complement of macronuclear DNA is replicated during the period shown by the autoradiographic experiments (Fig. 1) to encompass  $S_1$ . By the end of the sixth shock, the mean DNA content was intermediate between two- and fourfold values, but variability was very great (Table II, Fig. 6), with the lower part of the distribution coincident with that observed at the end of  $S_1$  (compare distributions B and C in Fig. 6). This result is consistent with the findings of the autoradiographic studies, in which by the end of the sixth shock a new S period ( $S_2$ ) was completed or underway in a portion of the cell population (Fig. 1). Further increases in DNA content to above the fourfold value were noted when the treatment was extended to include fifteen shocks (Fig. 6 D-E; Table II). These results indicate that DNA replication is also complete during  $S_2$ , but the extensive time required for doubling also suggests that the rate of replication is markedly decreased.

### The Regulation of DNA Synthesis

Although DNA synthesis becomes synchronized at the time of  $SD_1$ , a large number of cells do not participate (1, 15, 20, 23). Our autoradiographic experiments indicated that the proportion of cells which engaged in  $S_3$  could be increased by lengthening the preceding interdivision period (i.e., subjecting cells to BST at later times during the cell cycle). Further experiments were conducted to determine if the number of cells engaged in  $S_3$  could be altered by manipulating the length of the heat-shock treatment. Early  $G_1$  cells were subjected to treatments consisting of from three to nine shock cycles. After the conclusion of the terminal shock, the cells were incubated at 28°C, exposed to [ $^3H$ ]thymidine at  $SD_1$  (initiation of  $S_3$ ), and fixed for autoradiography at 160 min after EST (end of  $S_3$ ). The results of these experiments are shown in Fig. 7 (Curve 1). It was found that engagement in  $S_3$  was indeed related to the length of the treatment. Almost all the cells engaged in  $S_3$  when the

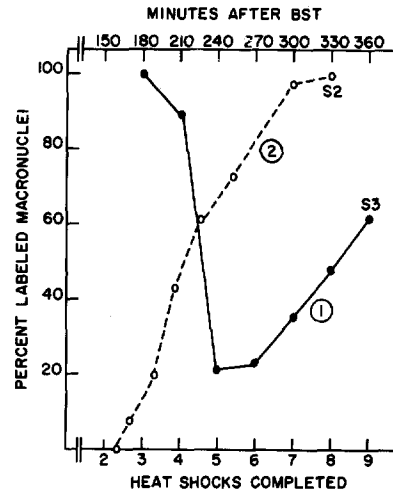


FIGURE 7 Alteration of the percentage of cells which engage in  $S_3$  by variation in the number of heat-shock cycles and its correlation to the timing of  $S_2$ . All cells were in early  $G_1$  at BST (time 0). Curve 1 represents the percentage of labeled macronuclei observed when [ $^3H$ ]thymidine was added for the duration of  $S_3$  to cells that had been subjected to treatments consisting of from three to nine heat-shock cycles (lower abscissa). Curve 2 represents the accumulative percentage of labeled macronuclei observed when [ $^3H$ ]thymidine was added before  $S_2$  (160 min after BST) and a total of eight heat shocks were applied (upper abscissa).

treatment lasted for only three or four shocks. However, a dramatic decline in participation was observed in treatments consisting of five and six shocks. Treatments consisting of from seven to nine shocks were followed by a gradual increase in engagement in  $S_3$ . The abrupt decrease in  $S_3$  participation after five and six shock treatments is correlated to the timing of initiation of  $S_2$  (Fig. 7, Curve 2). It is suggested that the initiation and continuation of a new S period late during a particular heat-shock treatment abolishes the ability to initiate another S period as a response to the synchronized division that occurs shortly thereafter. This would imply that a discrete time period must lapse from the termination of one replication round to the beginning of another.

### DISCUSSION

The evidence presented indicates that *Tetrahymena* cells subjected to a multiple heat-shock treatment participate in supernumerary S periods without intervening cell division. It is also shown that during these S periods the macronuclear DNA complement is substantially increased and probably com-



pletely replicated. These findings do not support the proposal that division synchrony is induced by a selective inhibition of a particular DNA fraction (3, 4). A convincing argument against this hypothesis is the observation that cells that are in the G<sub>2</sub> period at BST subsequently participate in further DNA synthesis but do not divide. The hypothesis predicts that these cells would divide during the treatment since they had completed a normal DNA duplication before BST (9). It is still possible that the hypothetical DNA fraction could be a G<sub>2</sub>-replicating DNA species, such as nucleolar DNA (7), which would still be partially unreplicated at BST. However, the demonstration that cells are capable of undergoing division if the heat-shock treatment is terminated and further DNA synthesis is blocked with M + U treatment after S<sub>1</sub> (19) makes this possibility unlikely. Thus it appears that the original conclusion of Zeuthen (35), that division synchrony is based on the disruption of thermo-labile processes directly related to cytokinesis, rather than to DNA synthesis, remains the most tenable hypothesis.

A modification in both the frequency of initiation of DNA synthesis and the rate of DNA replication in progress during the heat-shock treatment was observed in this study. The consequence of initiation modification was to alter the timing of S<sub>2</sub> so that it occurred in a bimodal fashion. McDonald (23a) and Prescott (26) first demonstrated the existence of a variation in the individual generation times of *T. pyriformis*, and recent studies by Jauker and Cleffmann (18) have established that this variation may be described by regular oscillations of long and short generation times. It appears that the bimodality of S<sub>2</sub> during the treatment may be explained by a retention of this oscillatory pattern, probably in a somewhat exaggerated form. The modification in rate of DNA replication was found to be extensive only late in the heat-shock treatment and resulted in a substantial extension in the duration of later S periods.

One of the most interesting questions related to the timing of DNA synthesis in heat-shocked *Tetrahymena* is why only a part of the population undergoes replication between SD1 and SD2. Hjelm and Zeuthen (15) suggested that engagement in S<sub>3</sub> may be related to the time of completion of S<sub>2</sub>. Subsequent experiments showed that cells involved in DNA synthesis late in the treatment failed to take part in S<sub>3</sub> (37). The results of our experiments in-

dicating that cells which engage in S<sub>3</sub> have either not participated in S<sub>2</sub> or have concluded S<sub>2</sub> before the beginning of a critical time late in the treatment. During this time period certain preparations necessary for the initiation of DNA synthesis, such as relevant RNA and protein synthesis<sup>4</sup> (3), could be completed. These events apparently cannot be executed while DNA synthesis is in progress. The above hypothesis emphasizes a fundamental difference which distinguishes *Tetrahymena*, and possibly other eukaryotes, from prokaryotes in which, under conditions of rapid growth and proliferation, additional S periods (replication forks) are initiated before the preceding replication round is completed (13). The only indication of overlapping S periods in a eukaryote is the rare observation of a double set of replication bands in the ciliate *Euplotes eurystomus* (21; J. Ruffolo, personal communication). The rarity of the phenomenon, however, makes thorough investigation and interpretation of this curious exception very difficult.

Since it has been shown that cells which do not participate in S<sub>3</sub> between SD1 and SD2 next engage in DNA synthesis between SD2 and SD3 (1), the signal to respond to macronuclear division by initiating DNA synthesis must be short-lived. Cells that do not immediately respond are required to wait one complete cell cycle to eventually initiate replication.

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