SYNCHRONIZED CELL DIVISION IN *TETRAHYMENA PYRIFORMIS* FOLLOWING INHIBITION WITH VINBLASTINE

G. E. STONE

From the Department of Anatomy, University of Colorado Medical Center, Denver, Colorado 80220

ABSTRACT

The ability of *Tetrahymena pyriformis* to undergo synchronous division following release of inhibition with vinblastine was examined. The degree of synchrony was shown to be correlated with the period of time spent under the influence of vinblastine. Cells were inhibited for different periods of time with vinblastine and then washed free of the inhibitor with fresh medium. The increase in cell number and division index was followed subsequent to release of inhibition. Inhibition for a period of time equal to about two generation times was required to produce a complete doubling of the population during the first division. Inhibition for longer periods of time resulted in the population's increasing by more than a factor of two during the first division burst. The nuclear cytology indicates that the micronucleus is probably blocked in mitosis.

INTRODUCTION

Analysis of the action of reversible cell-division inhibitors can provide an indirect method for determining some of the necessary prerequisites for cell division. Experiments dealing with the stage of the cell cycle that is sensitive to the inhibitor can also indicate when these prerequisites are fulfilled. The additional value of reversible cell-division inhibitors is related to their possible use in producing large populations of cells in similar stages of the cell cycle for biochemical studies.

Vinblastine (VLB) is an inhibitor which has been shown to act as a mitotic poison on mammalian cells in vitro (9) and in vivo (3). It has not been reported that VLB can act as a reversible inhibitor in mammalian cells, but a report which indicates that excessive doses of glutamic acid and other amino acids can partially overcome inhibition in vivo (7) indicates that vinblastine inhibition may be reversible under the proper conditions. Malawista et al. (8) have shown that vinblastine caused reversible attrition of the mitotic spindle in live oocytes of an annelid worm. *Tetrahymena pyriformis* does show reversible inhibition of cell division under the influence of VLB (15). As a first step towards analyzing the mode of action of VLB, we have examined the question as to whether this drug does produce synchronized divisions in a population of cells following release of inhibition. The approach to this question has been to block division for different periods of time and to measure the increase in cell number and division index following release from inhibition in nutrient and nonnutrient medium.

This approach determines whether VLB is specific enough in its inhibition to cause phasing of an entire population which will result in synchronous cell division when released from inhibition. If synchronous cell division does take place, it may also indicate the mechanism whereby inhibition produces phasing of the population. For

example, does VLB inhibit a specific step in the cell cycle, allowing other cells to progress to this point such as is the case with amethopterin or FUdR in Hela cells (12)? This type of inhibition is in contrast to the phasing of a cell culture of *Tetrahymena* by the heat-shock synchronizing method first described by Scherbaum and Zeuthen (14). This type of inhibition is considered by Zeuthen (18) to be operating through a differential set-back mechanism wherein older cells are set back more than young cells, and, as a result of repeated heat shocks, the entire population is brought into phase and undergoes synchronous divisions when heat shocks are discontinued.

MATERIALS AND METHODS

Tetrahymena pyriformis, Strain WH6, Mating Type I, was used in these studies. Cultures were grown in 500-ml flasks with 50 ml of a glucose-tryptone medium (4) adjusted to pH 7.4. The medium was autoclaved for 40 min because it has been shown that VLB will not act as an inhibitor unless the medium is treated in this manner (15). The culture temperature was maintained at 33° ± 0.5C in a constant temperature water bath.

Culture growth was measured as increase in cell concentration with a Coulter electronic particle counter. The cultures were between 12 to 18 hr of age at the beginning of each experiment and contained between 10,000 and 30,000 cells per ml. Control cultures were run during each experiment in order to assure a uniform generation time; this is particularly essential when one experiment run with one batch of medium is compared to another, because of the somewhat lengthy autoclaving.

Vinblastine sulfate was obtained from Eli Lilly and Company Inc. (Velban) as a dry preparation. A stock solution of VLB was prepared by diluting the VLB up to 1 mg/ml with glucose-tryptone medium. At the beginning of an experiment VLB was added to a growing culture to give final concentration of 20 μg/ml. An identical amount of diluent was added to the control cultures. Cell concentration was determined immediately following the addition of VLB and every 30–60 min thereafter as a check that culture growth was, in fact, inhibited.

The procedure for releasing inhibition involved removing cultures from the water bath, centrifuging in 50-ml tubes at 800 rpm for 5 min, aspirating the supernatant and resuspending the cells up to the original volume in either prewarmed growth medium lacking VLB or in an inorganic salt solution (5) lacking VLB. This procedure took approximately 10 min, and the cultures were replaced in the water bath as soon as possible. After aspiration, the volume of medium plus cells remaining in the tube was approximately 0.5 ml. The dilution factor of the VLB was between 80- and 100-fold. Control cultures were also treated in this manner so as to assess the effects of centrifugation and fresh medium on the generation time.

Cell counts were made every 15 min following release of inhibition. The division index, which is the percentage of a population in division at a given time, was also determined in some experiments during the recovery period. A drop of culture was placed in a depression slide, 1% formalin in the inorganic medium was added, and the division index was determined immediately. Recovery time was measured from the time that fresh medium lacking VLB was added to the centrifuged cells.

RESULTS

The generation time in control cultures in medium autoclaved for 40 min was between 2.5 and 3 hr. Consequently, the shortest time of inhibition employed was 3 hr, the rationale being that, if cell division synchrony could be induced with VLB, it would require at least one generation time for all the cells to accumulate at a common point.

Recovery in Nutrient Medium

Fig. 1 illustrates the increase in cell number following release of inhibition after different periods of time spent under the influence of VLB. From this figure, it can be seen that no cell divisions occurred during the first 60 min following release of inhibition. This holds true irrespective of the length of time cell division was blocked. The first increase in cell number was detected at 75 min after release of inhibition except for cells blocked for 3 hr in which case it was 90 min before the cell number increased. The differences observed with different durations of blockage concern the percentage of the population that divides during the first division burst and the presence or absence of a definite plateau following this division burst. Only when the population has been blocked for 5 or 6 hr does the population exhibit an exact doubling. Inhibition for 3 hr results in approximately a 65% increase by 120–135 min. Although there is no definite plateau, the rate of cell increase slowed and then began to rise at 150 min. The population inhibited for 4 hr increased by 82% at 120 min, and a clear-cut plateau was observed after this increase. This value ranges between 70 and 80% in other experiments not reported here.

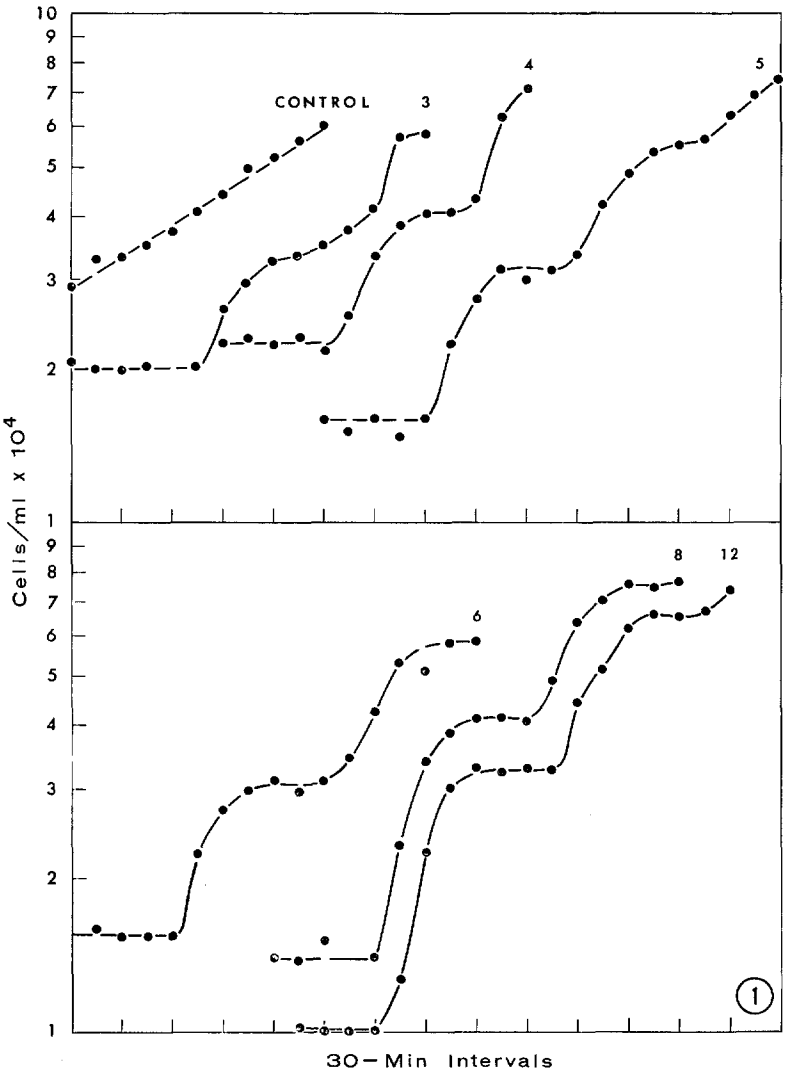


FIGURE 1 Increase in cell number on a logarithmic scale following release from inhibition with VLB for varying periods of time. The time spent (hours) under the influence of VLB is denoted for each curve.

An increase of 194 and 225% was observed for populations inhibited for 8 and 12 hr, respectively. This indicates that 47 and 62.5% of the original cells produced four division products rather than two during the first division burst. Observations on the living culture while the division index was being determined did not reveal cells with multiple division furrows.

The duration of the first and second division bursts is approximately 45 min for the populations studied. The interval between the first and the second division bursts approximates 75 min in those cultures blocked for 6 hr or longer. This can be viewed critically in Figs. 2 and 3 which show the recovery from 6 and 12 hr of blockage, with the division index also included. For cells inhibited for 6 hr (Fig. 2), the first division index peak reaches a first maximum of 60% at 65 min and a second of 38% at 140 min after release of inhibition. Cells blocked for 12 hr (Fig. 3) show a division index maximum of 72% at 85 min and a second peak of 43% at 175 min.

The division index for the first division burst appears to be delayed by about 20 min in the

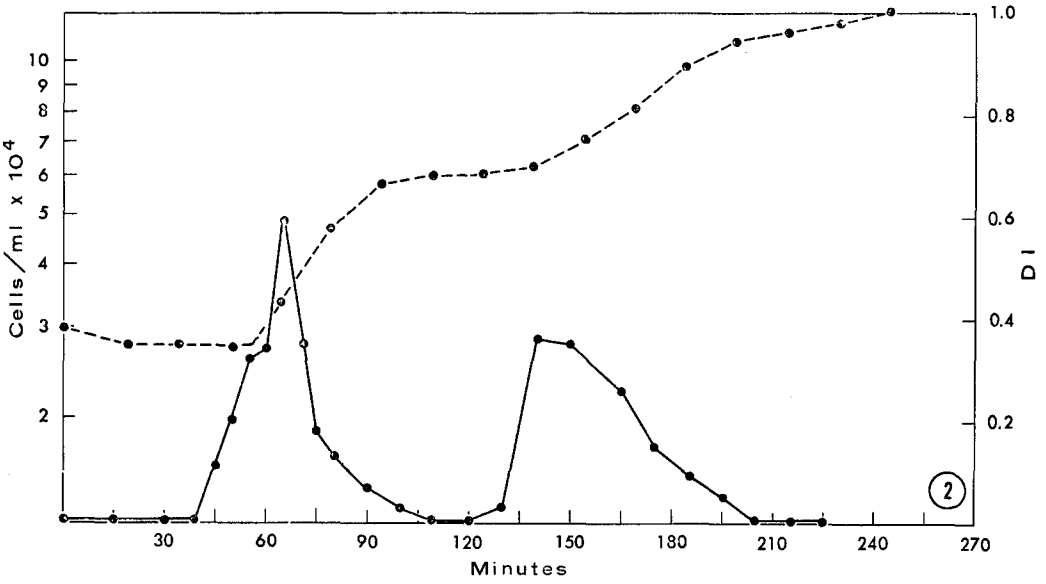


FIGURE 2 Increase in cell number (----------) and division index (———) following release from VLB inhibition for 6 hr. *DI*, division index.

population inhibited for 12 hr as compared to those inhibited for 6 hr. The division index for cells inhibited for 12 hr is not so steep in the right of the peak, which may represent cells going through a second division.

Recovery in Nonnutrient Medium

Fig. 4 illustrates recovery of a population of cells which was inhibited for 6 hr and resuspended in a nonnutritive inorganic medium. The percentage increase in the population during the division burst is 82% in this particular experiment. The division index is delayed by about 20 min (peak at 85 min) compared to cells blocked for 6 hr and resuspended in nutrient medium.

Cells blocked for 6 hr and resuspended in an inorganic medium show only one division burst during a 6-hr period following release of inhibition.

Nuclear Cytology

Feulgen preparations of control cells and cells inhibited for 6 hr (Figs. 5–7) were examined to determine whether differences existed in the nuclear condition of treated and untreated cells. Figs. 5 and 6 were taken at the same magnification (800), and Fig. 7 at about 2500. Two qualitative conclusions can be drawn from these micrographs. The first is that practically all the micronuclei in cells which have been treated with VLB (Figs. 6 and 7) show a strandedness of chromatin material, suggesting that the micronuclei have been suspended in division. Interphase control cells (Fig. 5) demonstrate the small, compact, and homogeneous Feulgen-positive material. The second conclusion is that no differences between the macronuclei are evident except that the VLB-treated macronuclei consistently appear larger.

DISCUSSION

The results presented in this paper demonstrate that cell division can be synchronized by reversing the inhibitory action of VLB in populations of *Tetrahymena*. Consequently, this demonstration suggests that VLB is fairly specific in its inhibitory action although it does not precisely distinguish between a mechanism which may operate like FUdR or amethopterin on Hela cells (12) and a set-back mechanism as described for heat-shock synchronization treatment for *Tetrahymena* (18). Comparison of the length of time inhibited and the subsequent degree of synchrony of cell division indicates that, if cells do move to a common point of inhibition, the cells progress through the cell cycle at a slower rate under the influence of VLB than during logarithmic growth. For example, it might be expected that continuous inhibition for a period of time equal to one generation would produce as high a degree of synchrony as could be

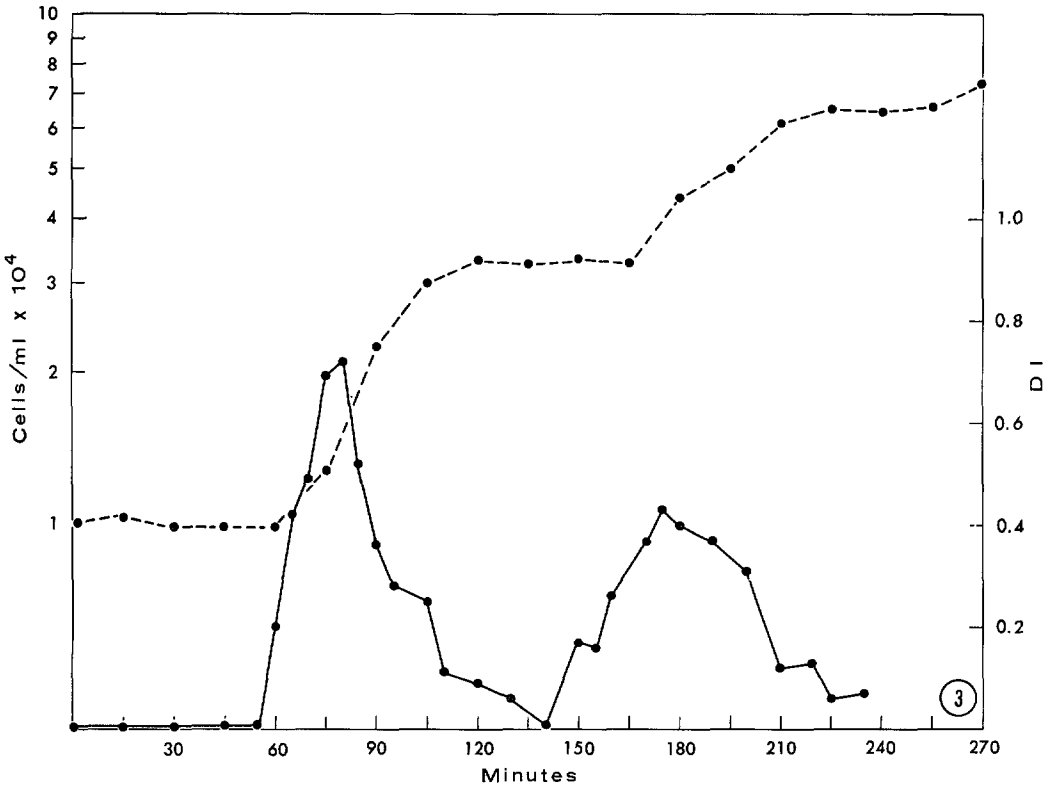


FIGURE 3 Increase in cell number (----·-------) and division index (————) following release from VLB inhibition for 12 hr.

obtained from longer periods of inhibition. The results show that this is not the case; in fact, inhibition equal to about two generation times is required before the cells demonstrate a doubling during the first division burst. Bruchovsky et al. (1) did not detect any retardation on the progress through the cell cycle in L cells under the influence of VLB, but did find that once the cells entered mitosis they were irreversibly damaged.

On the other hand, if VLB inhibition operates through a setback mechanism, it is difficult to explain how one "long" exposure to the inhibitor could be acting to bring about phasing of the population, since it appears that the degree of synchrony produced is directly related to the length of time in the continuous presence of VLB. It would appear, at least superficially, that, if VLB was operating through a setback mechanism, this mechanism is a relatively slow process. It is not inconceivable that VLB is acting through a setback mechanism since the other chemical agents such as *p*-fluorophenylalanine (11) apparently act in just such a manner on *Tetrahymena*. It may also be possible that VLB is acting in both ways. For example, VLB may act to set back the cells at an intermediate point in the cell cycle and at the same time allow other cells to progress near to this point of setback. If VLB is inhibiting a step different but near the point of setback, it could explain why VLB synchronization does not reflect as high a division index during the first division burst as can be obtained with the heat-shock treatment. For example, repetitive exposure to VLB may result in a higher division index than only one exposure period.

One site at which heat treatment and VLB may be acting in common is the subpellicular oral fibers. Williams and Zeuthen (16) have shown that one feature of heat-shock synchronization is the absence of these subpellicular oral fibers in the developing oral primordium during treatment. In view of the interpretation that some subpellicular fibers may be microtubules (10) and in view of the action of VLB on the microtubules of the mitotic

spindle of mammalian cells (9) and the reversible attrition of the mitotic spindle of annelid oocytes (8), it is possible that VLB may be acting like heat-shock to destroy or prevent formation of the fiber system. This particular aspect, oral morphogenesis during VLB synchronization, is now under study and should shed more light on similarities or differences between the two systems.

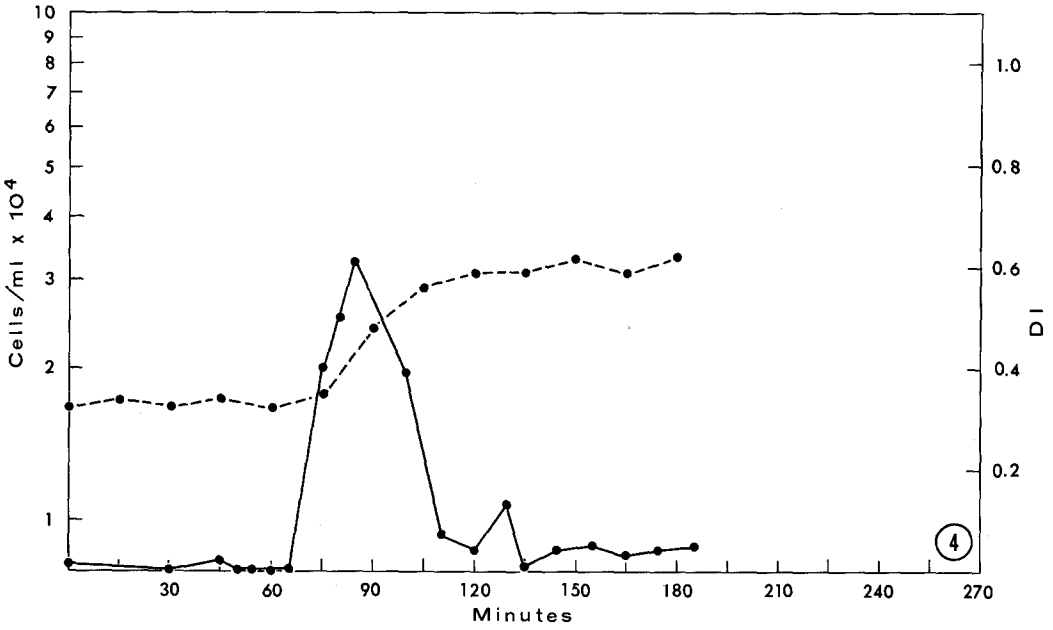
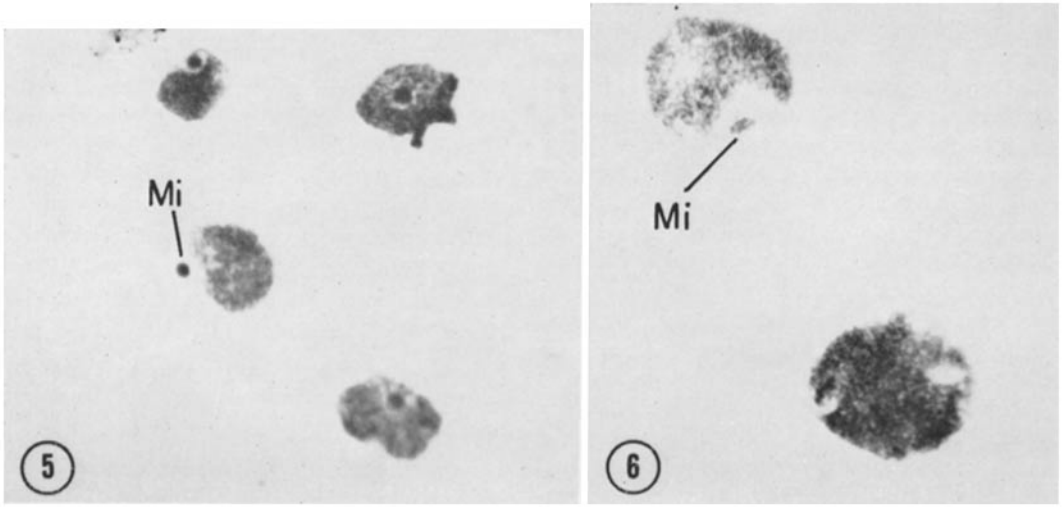


FIGURE 4 Increase in cell number (----------) and division index (————) in an inorganic medium following VLB inhibition for 6 hr.



FIGURES 5 and 6 Photomicrographs of Feulgen-stained nuclei. Fig. 5 shows control cells; Fig. 6 shows cells treated with VLB for 6 hr. Most often the micronucleus is not evident in VLB-treated cells except for areas interpreted as *Mi* in micrograph. × 800.

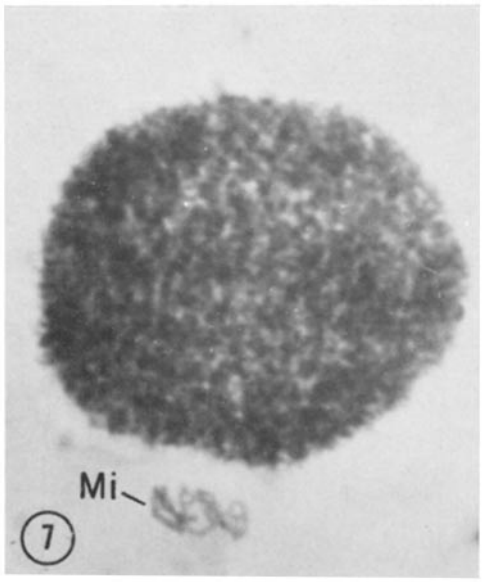


FIGURE 7 Micrograph demonstrating the "stranded" appearance of the micronucleus. × 2,500.

Recovery time, from the end of treatment to the first division burst, is similar in both systems (14), but this similarity may be a fortuitous coincidence rather than indicating a similar mechanism. Recovery from VLB inhibition for a large portion of the population can take place in a nonnutrient medium. This indicates that all the nutrient prerequisites for one cell division are present within the cell after 6 hr of inhibition. It also indicates that recovery from VLB inhibition is not dependent on a nutrient substrate competing with VLB but rather a dilution phenomenon acting to lower the concentration of VLB. This conclusion would depend on the elimination of the salt in the inorganic medium as possible competitors with VLB. If *Tetrahymena* in this type of experiment are compared to heat-synchronized *Tetrahymena* (5), it is seen that they are similar in that both can undergo division, but VLB-synchronized cells divide only once whereas heat-synchronized *Tetrahymena* go through two or more division bursts. This suggests a basic difference in the mechanisms of inhibition between VLB and heat-shock synchronization. The simplest interpretation at this time is that VLB somehow interferes with the accumulation of some critical material needed for more than one cell division whereas heat shock does not.

Another difference between VLB and heat-shock synchronization is the observation that, when *Tetrahymena* is inhibited for 12 hr, some cells go through two successive divisions without a period of growth. This observation is without a corollary in the heat-shock system and may be directly related to a basic difference between the two systems.

The micronucleus appears to be suspended in the process of division, not in an anaphase configuration as in heat-synchronized *Tetrahymena* (6) but rather in a condition that can better be described as disoriented mitotic chromosomes. This agrees with observations on mammalian cells treated with VLB which apparently disorients the mitotic spindle (9). Blockage of mitosis of the micronucleus cannot be considered as *the* site of inhibition. In an earlier study on reversible inhibition with VLB, it was shown that VLB will reversibly inhibit division in an amicronucleate strain of *Tetrahymena* (15). In view of this, it would seem that VLB must also be acting at other sites.

In summary, VLB-inhibited *Tetrahymena* show many similarities to heat-shock–synchronized *Tetrahymena*, but differences do exist and it has yet to be determined whether these differences represent differences in the action of the agents. More information on the effect of VLB on cellular synthetic events is needed to determine other possible sites of inhibition. For example, does VLB inhibit only processes concerned directly with division while cell growth continues? The strongest evidence that the processes of cell growth and division have been separated comes from the observation that some cells result in four division products rather than two during the first division burst. The simplest hypothesis at this time is that some cells go through two successive divisions without a period of growth. There is precedence for the conclusion that cell growth and division are separable events. Studies on heat-shock synchronized *Tetrahymena* (13) show that some parameters of cell growth, i.e. DNA content, mass, cell volume, do continue to increase in the absence of cell division.

The effect of VLB on DNA, RNA, and protein syntheses has been shown to be inhibitory in Ehrlich ascites carcinoma cells by 0.2 μM/ml, an effect which is reversible by the addition of glutamic acid (2). This concentration is well above that required to produce division inhibition, and

so it is questionable whether this activity is directly related to mitotic inhibition. Young and Hodas (17), using a concentration of VLB above that required for mitotic inhibition, found no decrease in thymidine, uridine, or amino acid incorporation in Hela cell monolayers. From those studies, it would appear that mitotic inhibition is not mediated through a general inhibition of DNA, RNA, or protein synthesis.

This research was supported by the National Institutes of Health Grant GM-13752 and American Cancer Society Grant E-417.

Received for publication 9 May 1968, and in revised form 1 August 1968.

REFERENCES

1. BRUCHOVSKY, N., A. A. OWEN, and A. J. BECKER. 1965. Effects of vinblastine on the proliferative capacity of L cells and their progress through the division cycle. *Cancer Res.* **25**:1232.
2. CREASY, W. A., and M. E. MARKIW. 1965. Biochemical effects of the vinca alkaloids. III. The synthesis of ribonucleic acid and the incorporation of amino acids in Ehrlich ascites cells *in vitro*. *Biochim. Biophys. Acta* **103**:635.
3. CUTTS, J. H. 1961. The effect of vincaleukoblastine on dividing cells *in vivo*. *Cancer Res.* **21**:168.
4. FRANKEL, J. 1965. The effect of nucleic acid antagonists on cell division and oral organelle development in *Tetrahymena pyriformis*. *J. Exptl. Zool.* **159**:113.
5. HAMBURGER, K., and E. ZEUTHEN. 1957. Synchronous divisions in *Tetrahymena pyriformis* as studied in an inorganic medium. *Exptl. Cell Res.* **13**:443.
6. HOLZ, G. G., O. H. SCHERBAUM, and N. WILLIAMS. 1957. The arrest of mitosis and stomatogenesis during temperature-induction of synchronous division in *Tetrahymena pyriformis*, mating type I, variety 1. *Exptl. Cell Res.* **13**:618.
7. JOHNSON, I. S., H. F. WRIGHT, G. H. SVOBODA, and J. VLANTIS. 1960. Antitumor principle derived from *Vinca rosea* Linn. I. Vincaleukoblastine and Leurosine. *Cancer Res.* **20**:1016.
8. MALAWISTA, S. E., H. SATO, and K. G. BENSCH. 1968. Vinblastine and griseofulvin reversibly disrupt the living mitotic spindle. *Science.* **160**:770.
9. PALMER, C. G., D. LIVENGOOD, A. K. WARREN, P. J. SIMPSON, and I. S. JOHNSON. 1960. The action of vincaleukoblastine on mitosis *in vitro*. *Exptl. Cell Res.* **20**:192.
10. PITELKA, D. R. 1961. Fine structure of the silverline and fibrillar systems of three tetrahymenid ciliates. *J. Protozool.* **8**:75.
11. RASMUSSEN, L., and E. ZEUTHEN. 1962. Cell division and protein synthesis in *Tetrahymena* as studied with *p*-fluorophenylalanine. *Compt. Rend. Trav. Lab. Carlsberg.* **32**:333.
12. RUEKERT, 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.
13. SCHERBAUM, O. H., A. L. LAUDERBACK, and T. L. JAHN. 1959. DNA synthesis, phosphate content and growth in mass and volume in synchronously dividing cells. *Exptl. Cell Res.* **18**: 150.
14. SCHERBAUM, O., and E. ZEUTHEN. 1954. Induction of synchronous cell division in mass cultures of *Tetrahymena pyriformis*. *Exptl. Cell Res.* **6**:221.
15. STONE, G. E. 1968. Method for reversible inhibition of cell division in *Tetrahymena pyriformis* using Vinblastine sulfate. *In* Methods in Cell Physiology. edited by D. M. Prescott, editor. Academic Press Inc., New York.
16. WILLIAMS, N. E., and E. ZEUTHEN. 1966. The development of oral fibers in relation to oral morphogenesis and induced division synchrony in *Tetrahymena*. *Compt. Rend. Trav. Lab. Carlsberg.* **35**:101.
17. YOUNG, C. W., and S. HODAS. 1965. Acute effects of cytotoxic compounds on incorporation of precursor in DNA, RNA, and protein of Hela monolayers. *Biochem. Pharmacol.* **14**:205.
18. ZEUTHEN, E. 1964. The temperature-induced division synchrony in *Tetrahymena*. *In* Synchrony in cell division and growth. E. Zeuthen, editor. Interscience Publishers, New York.