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Synchronization of *Leishmania tarentolae* by Hydroxyurea

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SYNOPSIS. *Leishmania tarentolae* cells in brain-heart infusion medium were partially synchronized in terms of DNA synthesis and cell division by a 10 hour period of inhibition in 200 μ g/ml hydroxyurea at 27 C. Nuclear and kinetoplast DNA synthesis commenced immediately upon removal of hydroxyurea, and ki-

netoplast and nuclear division occurred after about 5 hr. The Index of Synchronization (3) varied from 33-41%.

A moderate decay of the synchronicity was noted by the 2nd cell cycle. Hydroxyurea was selectively lethal to S-phase cells.

A synchronized culture of a hemoflagellate would be a good tool to study certain aspects of the problem of mitochondrial biogenesis. The hemoflagellates in general probably contain a single mitochondrion (18), a portion of which contains a large amount of mitochondrial DNA. This portion of the mitochondrion is usually a disc-shaped structure situated next to the basal body. Due to the high concentration of mitochondrial DNA, which represents 18-21% of the total cell DNA in *Leishmania tarentolae* (14), this region of the mitochondrion stains deep purple with Giemsa dyes and was known historically as the "kinetoplast" due to its proximity to the basal body. The mitochondrial DNA that is stained by Giemsa dyes is termed kinetoplast DNA (K-DNA).

It has been reported that the S period of the K-DNA (for *C. luciliae*) is synchronous with the nuclear S period (20), and that the kinetoplast genome physically divides a short time prior to nuclear division (in the case of *Trypanosoma mega*) (19). This is unlike the situation in *Tetrahymena* (10), *Physarum* (4) and HeLa cells (7), where the mitochondrial and nuclear S periods are not synchronous. The intrinsic synchronicity of the mitochondrial and nuclear S periods in the hemoflagellates is an interesting problem in the intracellular regulation of organelle biosynthesis and could best be studied by means of a synchronized population of cells. Also, in analogy with recent results on L cells (11), it is possible that mitochondrial biogenesis occurs at one point in the cell cycle.

We have achieved a partial synchronization of DNA synthesis and cell division in *L. tarentolae* by means of hydroxyurea and have defined several parameters of this system.

MATERIALS AND METHODS*

Chemicals. Hydroxyurea—A grade, CalBiochem, Los Angeles, Calif. Hemin—2 \times crystallized, Mann Research Laboratories,

N.Y., N.Y. Bovine serum albumin—Fraction V, Sigma Chemical Co., St. Louis, Mo. Aminopterin—Lederle Labs., Pearl River, N.Y. Methyl-³H-thymidine—Schwartz Bioresearch, Inc. (13.4 C/mM). L-4 emulsion—Ilford Chemical Co., Essex, England. Bacto-Agar—Difco Laboratories, Detroit, Mich. Permablend—Packard Instrument Co., Downers Grove, Ill. All other chemicals were of Reagent grade.

Media. Brain-Heart Infusion medium—(BHI) Difco Laboratories, Detroit, Mich. Dissolved 37 g in 1000 ml redistilled water and autoclaved at 20 lbs/in² for 15 minutes. Medium C—defined medium for *L. tarentolae* (Trager, 1957); sterilized by filtration thru Millipore HA filter. Hemin—dissolved in 0.05 N NaOH and stored at -20 C in small quantities; added to BHI medium and Medium C to a final concentration of 20 μ g/ml just before inoculation with cells. Solution SBG: 0.15 M NaCl, 0.02 M glucose, 0.02 M PO₄ buffer (pH 7.9). Red blood cell extract obtained as described previously (14). Solution 1A: 0.25 M sucrose-1% BSA-2% Tergitol TP-9 (Union Carbide Chemical Co.) Solution CA: 0.25 M sucrose-1% BSA-3 mM CaCl₂.

Agar plating. The medium consisted of 0.58% BHI agar-1.76% red blood cell extract-20 μ g hemin/ml. Cells were diluted in sterile SBG and 0.05 ml was spread with a glass rod. Plates were taped, inverted and stored at 27 C. Colonies were counted after 2 weeks incubation.

Autoradiography. As previously described (14).

Cells. A strain of *Leishmania tarentolae* (Lt-S) was obtained from Dr. S. Krassner of the Univ. of Calif. at Irvine, and maintained both in BHI medium and Medium C at 27 \pm 0.5 C. Two separate clones (Lt C-1 and Lt C-2) were isolated from colonies on BHI agar and maintained in BHI medium. Experimental cultures were maintained in 18 \times 150 mm screw-cap test tubes with a culture volume of 3-10 ml, or in 150 ml bottles with a culture volume of 20-50 ml. Both tubes and bottles were aerated by continuous rotation at 6 RPM.

Giemsa-staining. A sample of the culture was mixed with an equal volume of 0.25 M sucrose-1% bovine serum albumin, cooled on ice, and centrifuged at 1500 g for 10 min. The supernatant solution was aspirated off and a thin smear made with a platinum loop and air-dried rapidly. The smears were fixed for 5 min in

* Abbreviations:

SBG = 0.15 M NaCl-0.02M glucose-0.02 M PO₄ buffer (pH 7.9).

HU = hydroxyurea. BHI = brain-heart infusion medium. K-DNA = kinetoplast DNA. N-DNA = nuclear DNA.

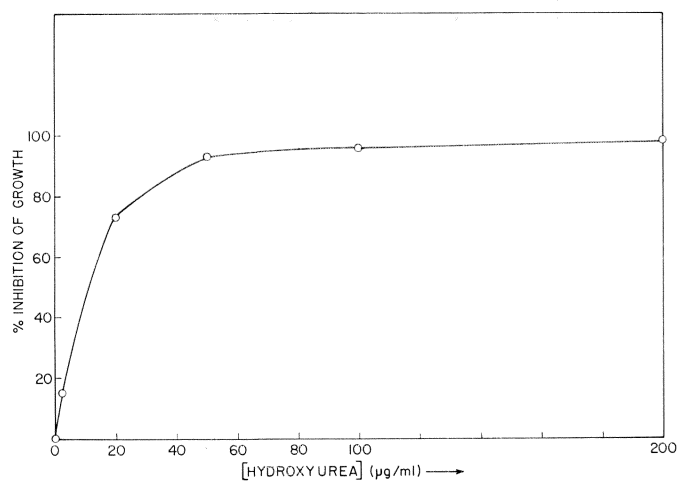


Fig. 1. Effect of hydroxyurea on cell division of *L. tarentolae*. Five identical cultures were given different amounts of hydroxyurea and grown for 4 days at 27°C with rotation. Cell concentrations were measured in a hemocytometer and percent inhibition calculated as compared to the untreated control.

methyl alcohol and stained for 30 min in Giemsa stain diluted 1 to 10 with 0.02 M PO_4 buffer (pH 6.8). Microphotography was done using a green filter and a Zeiss Universal microscope with a 1000 \times apochromatic objective and a 1.4 N.A. condensor.

Measurement of rate of DNA synthesis.

1. Direct counting: Samples of the culture were pulsed for 15 min at 27°C with 50 µg/ml of ^3H -thymidine in Medium C with 1% bovine serum albumin. The pulse was terminated by applying 50 µl to a Whatman 3MM filter disc and dropping it in cold 5% TCA. The discs were extracted 3 \times with 70% ethanol for 30 min, once with 95% ethanol for 5 min, and once with absolute ether for 5 min, and were counted in a fluid composed of 5 g PPO and 0.5 g dimethyl POPOP per liter of toluene in a Beckmann Scintillation Counter. Cell counts were obtained by removing a 10 µl sample from the incubation mixture, mixing this with 10 µl of 10% formalin in SSC, and diluting with 200 µl of SSC; 200-600 cells were counted in a hemocytometer. The uptake of ^3H -thymidine was calculated on a per cell basis.

2. Autoradiography: The cells were pulsed as described above, and then smears were made on presubbed slides, air dried, fixed in methyl alcohol, and extracted with cold 5% TCA, 70% ethanol, and 95% ethanol. After covering with emulsion, exposing and developing, the cells were stained with Giemsa and grains counted at a 2000 \times magnification over the nucleus and kinetoplast. DNase controls were performed as previously described (14).

In some experiments, the pulsed cells were vibrated for 30 sec in solution 1A at 0-4°C to release nuclei, and then the lysis was stopped by the addition of 1 ml of solution CA (13). The nuclei and ghosts were centrifuged down at 10,000 g for 10 min, and autoradiographs were made as described above. This type of preparation gave more accurate kinetoplast grain counts than the intact cell smears.

RESULTS

1. *Selection of the inhibitory agent.* Several known inhibitors of DNA synthesis were examined for use as the blocking agent for the induction of synchronous growth. Thymidine (800 µg/ml), deoxyadenosine (800 µg/ml), and nalidixic acid (800 µg/ml) had no effect on cell division. Aminopterin and hydroxyurea both greatly inhibited cell division at concentrations of 100-200 µg/ml, as shown in Fig. 1 for the latter.

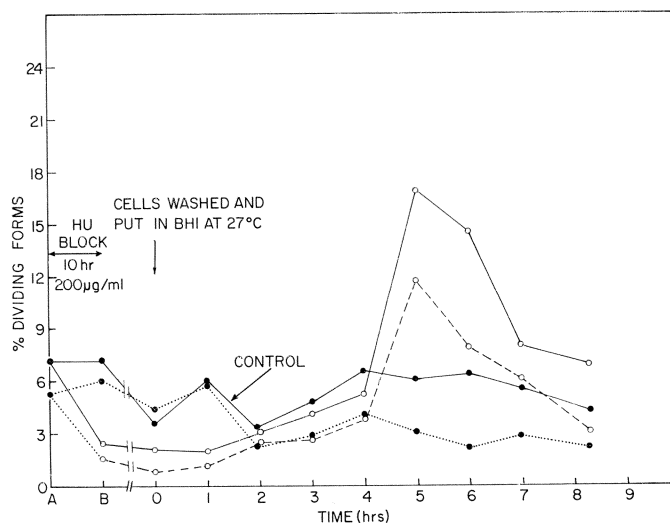


Fig. 2. Percentage of dividing forms as a function of the time after release from the HU block. Cells were treated with HU (200 µg/ml) for 10 hours at 27°C (from T_A to T_B), then washed by centrifugation and resuspended in fresh BHI medium at 27°C at T_0 . The interval T_B - T_0 is the time involved in centrifugation. The control culture was untreated with HU. At least 500 cells were counted for each point. Symbols: ○—○ = experimental, 2K-1N + 2K-2N cells. ○---○ = experimental, 1K-2N + 2K-2N cells. ●—● = control, 2K-1N + 2K-2N cells. ●---● = control, 1K-2N + 2K-2N cells.

Hydroxyurea at a concentration of 200 µg/ml was chosen as the blocking agent.

2. *Extent of synchronization of cell division after treatment with HU.* Early log-phase cells at a concentration of 20×10^6 /ml in BHI medium were inhibited for 10 hours (time A to B) at 27°C with 200 µg HU/ml. Then the cells were resuspended in fresh BHI medium without HU (at time O) and samples were removed for Giemsa-stained smears at hourly intervals. A control culture was untreated with HU, but otherwise handled identically.

The percentages of the various division forms were counted from the Giemsa-stained smears under oil immersion at 1,000 \times . As shown in Fig. 2, the HU-blocked culture had very few division forms for the first 4 hours after release, and then went thru a parasynchronous wave of mitoses between 4-7 hours. The repeatability of the scoring procedure was proved by the small standard deviations obtained in 5 separate counts of a control slide (Table 1). The larger fluctuations ($\pm 2\%$ division forms) observed in the control culture in Fig. 2 may be due to the nutritional step-up involved in the medium change at T_0 , for

TABLE 1. Repeatability of scoring procedure. Five separate counts were made of one slide from an untreated log-phase control culture; 250 cells were scored for each point. The values given are the percentages of the various division forms and the standard deviations.

1N-1K	1N-2K	2N-1K	2N-2K	unusual division forms
91.8 \pm 1.0	1.4 \pm 0.5	1.1 \pm 0.2	4.8 \pm 0.8	1.0 \pm 0.6

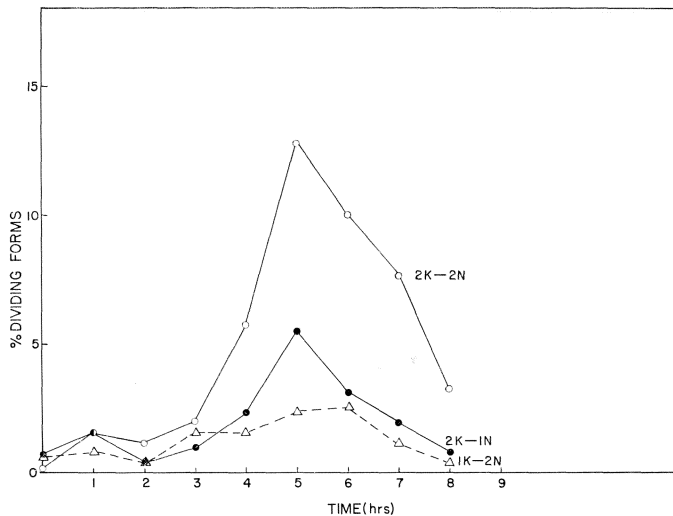


Fig. 3. Percentages of the 3 basic types of dividing forms as a function of the time after release from the HU block. Only the HU-treated culture is shown. Symbols: ○—○ = 2K-2N cells. ●—● = 2K-1N cells. △—△ = 1K-2N cells.

HU-treated experimental cultures had fewer fluctuations during the first 3-4 hours (Figs. 2, 3, 5, 6, 7).

The "Synchronization Index" of Engelberg (3), that is, the ratio of the area between the experimental curve and the control curve to the total area under the experimental curve, was 41.2%, indicating a moderate degree of synchronization.

The percentage of cells with 2 kinetoplasts (and 1 nucleus) increased before that of cells with 2 nuclei (and 1 kinetoplast) and attained a higher absolute level. This is shown by plotting the data from one of 5 similar experiments in the form of 3 curves, as in Fig. 3. The 2K-2N curve rises first and reaches the highest maximum; the 2K-1N curve follows the kinetics of the 2K-2N curve but attains a lower level, and the 1K-2N curve is lower than the 2K-1N curve and consistently peaks at a somewhat later time. Altho the differences between the 1N-2K and 2N-1K curves were small, the fact that similar results were obtained in 5 separate experiments argues for the authenticity of the differences. These data indicate that (1) the most common mode of cell division involves a fairly simultaneous division of the nucleus and kinetoplast to produce the 2K-2N cell, (2) a less frequent mode of cell division involves a kinetoplast division prior to nuclear division,

TABLE 2. Percentages of various division forms in log-phase control culture untreated with HU, resuspended in fresh BHI at T_0 .

Time (hrs)	Percentage of division forms		
	1N-2K	2N-1K	2N-2K
0	1.4	1.1	4.8
1	1.3	0.33	3.3
2	1.7	0.34	5.8
3	0.78	0	6.6
4	1.6	0.31	4.3
5	2.1	1.2	3.7
6	1.4	0.59	3.2

TABLE 3. Maximum percentage of unusual division forms ($>2K$ and/or $>2N$) occurring in 10 hr-HU blocked cultures and in untreated log-phase control cultures.

Experiment	Time after treatment (hr)	Maximum percentage unusual division forms
1	7	2.1
2	3	1.0
3	6	1.4
4	6	2.3
5	9	1.8
6	1	2.9
		Average $1.9 \pm 0.6\%$
7	7	1.0
8	2	3.7

In Exps. 1-6, 500-600 cells were scored for each value (counting error $\leq 5\%$).

In Exp. 7, 1020 cells were scored, and in Exp. 8, 300 cells were scored.

(3) the least frequent mode involves a nuclear division prior to kinetoplast division.

In the untreated log-phase control culture, the frequency of 2K-1N cells on the average exceeded that of 2N-1K cells and the frequency of the 2K-2N cells exceeded both, indicating that the synchronization process does not grossly perturb the normal mode of cell division (Table 2). It was noted, however, that unusual division forms appeared at a slightly higher frequency after treatment with HU than in untreated cultures. The term "unusual" indicates cells having more than 2 nuclei and/or kinetoplasts. The data given in Table 3 show that the maximum percentage of unusual division forms is not greatly affected by HU treatment and that the rate of production of such forms has no apparent correlation with the parasynchro-

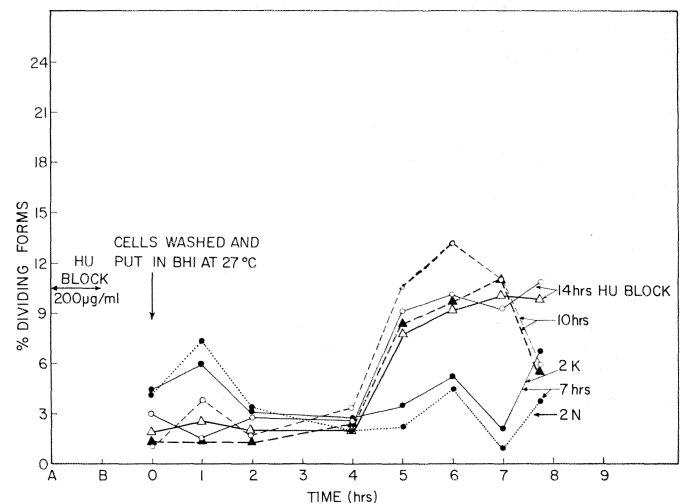


Fig. 4. Effect of variations in length of the HU block on synchronization of cell division. Samples from the same culture were treated for 7, 10 and 14 hours with 200 μ g HU/ml and then washed and put in BHI at 27°C. Symbols: ●—● = 2K-1N + 2K-2N cells. 7 hours. ●—● = 1K-2N + 2K-2N cells. 7 hours. ○—○ = 2K-1N + 2K-2N cells. 10 hours. ▲—▲ = 1K-2N + 2K-2N cells. 10 hours. ○—○ = 2K-1N + 2K-2N cells. 14 hours. △—△ = 1K-2N + 2K-2N cells. 14 hours.

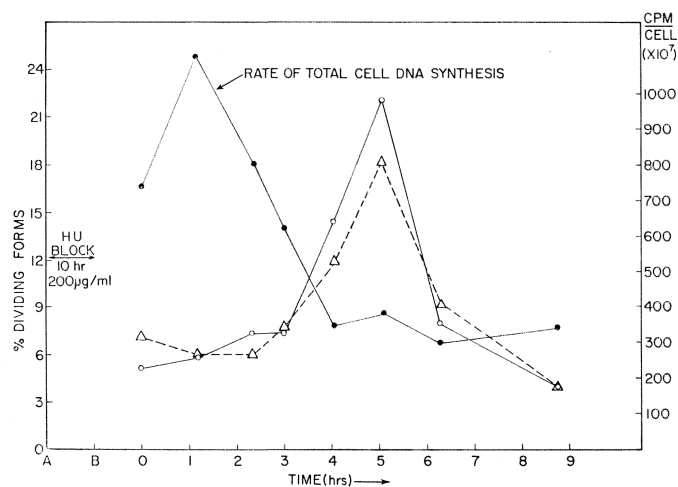


Fig. 5. Percentage of dividing forms and rate of total cell DNA synthesis as a function of the time after release from the HU block. See Fig. 3 for details of scoring dividing forms and see Materials and Methods for technique used to measure rate of DNA synthesis. \circ = 2K-1N + 2K-2N cells. \triangle = 1K-2N + 2K-2N cells. \bullet = cpm/cell ($\times 10^7$) per 15 min pulse.

nous wave of cell divisions. They are probably an abnormal mode of cell division, but the frequency is low enough to be neglected, even in the HU-treated cultures.

The time of treatment with HU was varied to determine the optimal time for producing synchronized cell divisions. A 10-hour treatment yielded the most synchronous growth (Fig. 4). The 7-hour treatment had little effect and the 14-hour treatment spread out the wave of divisions, lowered the peak and moved the peak further on in time.

3. Extent of synchronization of DNA synthesis. The data of Fig. 5 reveal that total cell DNA synthesis begins soon after the removal of HU, reaches a maximum after about 1 hour, and decreases to a low plateau by 4 hours, the time when cell divisions are beginning to increase in frequency. The Synchronization Index for this experiment was 33%. It can be concluded that total cell DNA synthesis is also fairly well synchronized by a 10-hour HU-block, and that the cell cycle after the block comprises an immediate S phase of about 4 hours, a short G_2 phase, a mitotic phase (nuclear and kinetoplasmic) of about 3 hours, and a G_1 .

To determine the length of G_1 , and to ascertain the decay of synchronization with time, a culture was followed for several divisions (Fig. 6). The time between the 1st and 2nd peaks of cell division was 5 hours, as compared to the log-phase doubling time for untreated cells of about 10-12 hours. The extent of synchronicity decreased until the 3rd division was hardly evident above the background. The time from the 1st mitotic peak to the next peak of DNA synthesis was 3 hours; hence G_1 must last less than 3 hours.

We found by light microscope autoradiography that the nuclear and kinetoplast S periods were synchronous (Fig. 7). In this experiment the ratio of kinetoplast grains to

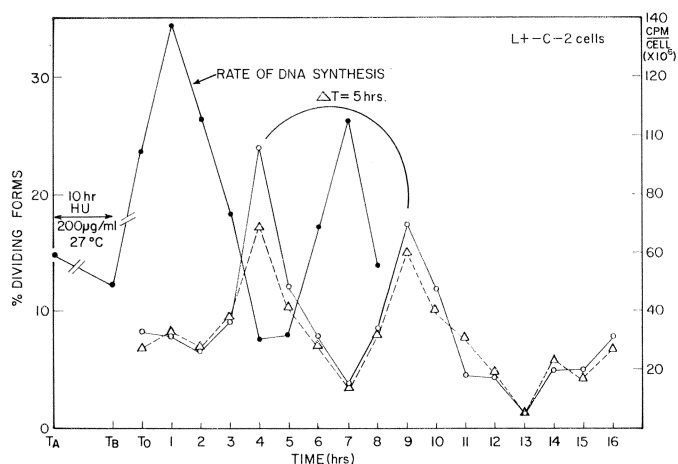


Fig. 6. Decay of synchronization of cell division and DNA synthesis. The percentage of dividing forms and the rate of total cell DNA synthesis were measured as in Fig. 6. \circ = 2K-1N + 2K-2N cells. \triangle = 1K-2N + 2K-2N cells. \bullet = cpm/cell ($\times 10^6$) per 15 min pulse.

nuclear and kinetoplast grains at T_0 was 15%, which is probably somewhat low because of the inaccuracy in scoring 1-grain kinetoplasts due to the closeness of nuclear grains. Another experiment using the detergent lysis method described in Materials and Methods gave a value of 19% at T_0 , which corresponds well with the relative amount of K-DNA in the total cells, as obtained by grain counts of cells totally labeled with ^3H -thymidine (19-21%) and by measuring the areas under the kinetoplast and nuclear bands in preparative and analytic CsCl gradients of total cell DNA (18%) (15).

We attempted to increase the extent of synchronization

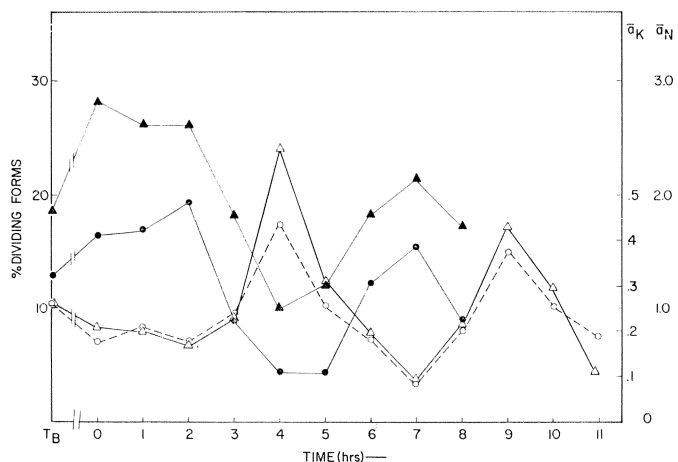


Fig. 7. Synchronization of nuclear and kinetoplast DNA synthesis. The data are from the experiment of Fig. 6. Both nuclear and kinetoplast grain counts were done on intact cells; 200-400 cells were scored for each point. The background was 1.2×10^{-4} grains/area equivalent to the kinetoplast, and 9.4×10^{-4} grains/area equivalent to the nucleus. Hence the grain counts presented in this figure are significant. Symbols: \triangle = 2K-1N + 2K-2N cells. \circ = 1K-2N + 2K-2N cells. \blacktriangle = \bar{a}_N , average number of grains per nucleus per 15 min pulse. \bullet = \bar{a}_K , average number of grains per kinetoplast per 15 min pulse.

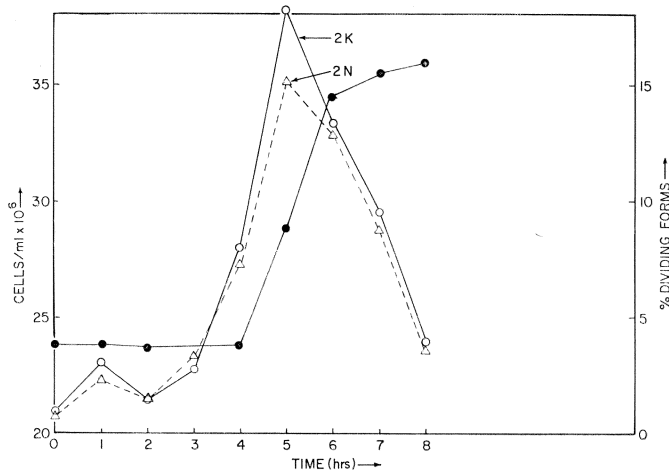


Fig. 8. Number of cells/ml and percentages of dividing forms as a function of time after release from the HU block (10 hours, 200 $\mu\text{g/ml}$). Symbols: ● = number of cells per ml ($\times 10^6$). ○ = 2K-1N + 2K-2N cells. △ --- △ = 1K-2N + 2K-2N cells.

by applying a triple HU block, as described by Galavazi et al. (5) for the synchronization of mammalian cells by high thymidine. The procedure was to let the synchronized cells complete their S phase and then give the cells a second 10-hour HU block. After release from the 2nd block and after completion of the S phase, a 3rd block was applied. The resulting cell growth was completely asynchronous.

4. *Do dividing cells actually complete cell division during synchronous growth?* The increase in the number of cells per ml together with the appearance of the 2 types of division forms is shown in Fig. 8. Fifty-seven percent of the cells divided in 2-4 hours (T_4 - T_8). The increase in cell number occurred in a kinetic sequence to the curve of division forms, implying a causal relationship.

5. *Is HU selectively lethal to cells in S-phase?* Early log-phase cells were given a 30 minute pulse of 50 $\mu\text{C/ml}$ ^3H -thymidine at 27 C and processed for autoradiography. After one month, 28% of the cells had more than one grain (DNase-removable) over the nucleus or kinetoplast (background was 9.4×10^{-4} grains/area equivalent to a kinetoplast, and 7.5×10^{-3} grains/area equivalent to a nucleus). These cells had a plating efficiency of 27% (Fig. 9). This low plating efficiency does not mean that only 27% of the cells were viable; viability has been found to be 100% by growth curves. It is comparable to values for plating efficiencies obtained previously with *L. tarentolae* (14) and *Crithidia luciliae* (M. Steinert, 1969. pers. com.), and probably reflects the specialized nutritional and microenvironmental requirements of the hemoflagellates. After a 10-hour HU block, the plating efficiency decreased 20% as compared to the untreated control culture. This implies that most of the cells in the S phase were rendered non-viable by the HU treatment. Further evidence for this selective sensitivity was obtained by treating synchronized cells at different points in the cell cycle for 3 hour periods

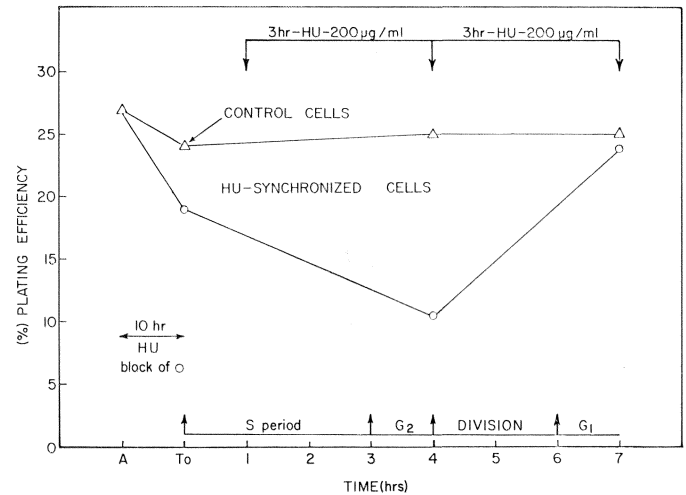


Fig. 9. Effect of HU on plating efficiency of cells at different points in the cell cycle. An early log phase culture was divided (at T_A) into 2 portions and one was treated for 10 hours with 200 μg HU/ml while the other served as an untreated control. At T_0 both cultures were resuspended in BHI medium without HU. Known numbers of cells were plated out on agar at T_A and T_0 . A portion of the synchronized culture was treated for 3 hours during the S phase with 200 μg HU/ml and the cells plated out. Another sample was identically treated during the non-S phase and also plated out. The colonies were counted after 15 days incubation at 27 C and the percent plating efficiency calculated from a knowledge of the number of cells plated.

with 200 $\mu\text{g/ml}$ HU (Fig. 10). The plating efficiency of the S-phase cells decreased to 10.5%, whereas the cells not in S phase were unaffected by the treatment.

6. *Structure of the various division forms.* Light micrographs are presented in Figs. 10 A-I to show the structure of the major division forms that occur in synchronized growth. The rounded appearance of some of the cells is merely due to the thinness of the smears and the rapidity of air-drying.

DISCUSSION

We have approached the synchronization of cell division in a hemoflagellate in culture by interfering with the synthesis of DNA, thereby achieving a state of unbalanced growth with a pile-up of cells at the G_1 -S boundary or in early S phase, and then releasing the cells from inhibition by removal of the blocking agent (cf. 2, 5, 6, 8, 9, 21, 22). Hydroxyurea, a known inhibitor of DNA synthesis in bacteria (12) and animal cells (25) was employed as the blocking agent because it was found to reversibly inhibit cell division. In addition, HU was found to be selectively lethal for S-phase cells of *L. tarentolae*, as has been reported for Chinese hamster cells (16, 17, 26). This property of the drug theoretically increases the synchronization obtained by effectively removing that portion of the population (S-phase cells) which cannot be synchronized by a single blocking treatment (27).

Unlike the situation in HeLa cells (24), the synthesis of mitochondrial DNA as well as nuclear DNA is inhibited by HU in *L. tarentolae*, as shown by the fact that they both commence synchronously upon removal of the drug.

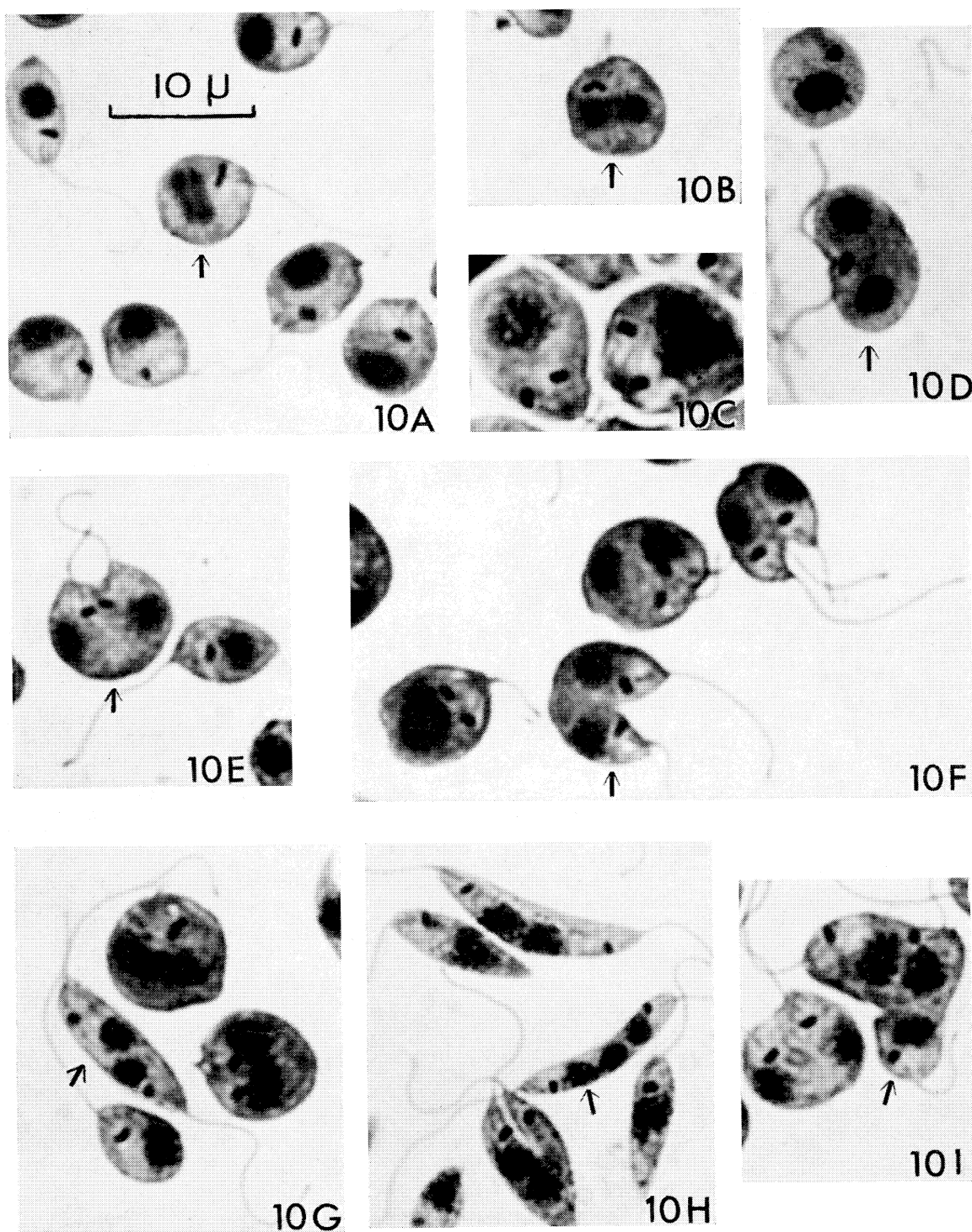


Fig. 10. Successive division stages of parasynchronized *L. tarentolae* cells. The culture was given the standard synchronization procedure. A. Kinetoplast lengthens and nucleus enlarges (1K-1N). B. Kinetoplast begins to divide (1K-1N). C. Kinetoplast

divides before nucleus (2K-1N). D. Early 2K-2N cell. E. and F. Intermediate 2K-2N cell. G. Late 2K-2N cell. H. Unusual division form (2K-3N). I. Unusual division form (3K-3N).

This probably has to do with the normal intrinsic synchronicity of N and K-DNA synthesis in exponentially growing cells (20).

It is of some interest that, in our parasynchronous cultures, the actual division of the nucleus and kinetoplast

also occurred fairly synchronously, with the division of the kinetoplast often preceding that of the nucleus by a short time, as had been found in asynchronous cultures of *T. mega* (19) and *C. luciliae* (20).

The synchronization of DNA synthesis and cell division

lasted for 2 cell cycles only (Fig. 6). This could be due to: (1) An innate variability in the length of S periods among individual cells (1); (2) the low Index of Synchronicity obtained by this procedure.

The decrease in the interval between successive cell divisions in the parasynchronized culture as compared to asynchronous cells (Fig. 6) is similar to the situation found in human tissue culture cells synchronized by excess thymidine, in which G₁ and G₂ phases were shortened after the treatment (6). This shortening of certain phases of the cell cycle has no ready explanation.

Even tho the extent of synchronization of cell division in *L. tarentolae* by hydroxyurea is not as high as would be desired, the coordinated synchronization of nuclear and kinetoplastic DNA synthesis is sufficient for a study of the regulation of the synthesis of K-DNA.

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