NUCLEOLAR AND BIOCHEMICAL CHANGES DURING UNBALANCED GROWTH OF *TETRAHYMENA PYRIFORMIS*

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ABSTRACT

Numerous nucleoli can be observed in the macronucleus of the logarithmically growing ciliated protozoan *Tetrahymena pyriformis*; at late log phase the nucleoli aggregate and fuse. In stationary phase this fusion process continues, leaving a very few large vacuolated nuclear fusion bodies in the nucleus. When these stationary phase cells are placed into fresh enriched proteose peptone medium, the large fusion bodies begin to disaggregate during the 2.5-hour lag phase before cell division is initiated. By 3 to 6 hours after inoculation the appearance of the nucleoli in many cells returns to what it was in logarithmic cells. In view of the possible role of nucleoli in ribosome synthesis, attempts were made to correlate the morphological changes to changes in RNA and protein metabolism. The beginning of an increased RNA synthesis was concomitant with the beginning of disaggregation of the large fusion bodies into nucleoli, which was noticed in some cells by 1 hour after the return to fresh enriched proteose peptone medium. Increased protein synthesis then followed the increased RNA synthesis by 1 hour. The supply of RNA precursors (essential pyrimidines) were removed from cultures which were grown on a chemically defined synthetic medium, in order to study the relation between nucleolar fusion and synthesis of RNA and protein. Pyrimidine deprivation drastically curtailed RNA and protein synthesis, but did not cause fusion of nucleoli. When pyrimidines were added back to this culture medium, RNA synthesis was immediately stimulated and again preceded an increased protein synthesis by 1 hour. These studies suggest the involvement of unfused nucleoli in RNA and protein synthesis and demonstrate the extreme plasticity of nucleoli with respect to changes in their environment.

Recent studies have shown that nucleoli bear a close link to the major macromolecular synthetic processes of cellular metabolism (see 12), especially the synthesis of ribosomal RNA.

The present study is concerned with morphological changes in the nucleoli of *Tetrahymena pyriformis* and the possible correlation of these changes with the course of DNA, RNA, and protein metabolism.

MATERIALS AND METHODS

CULTURE METHODS: A clone of *T. pyriformis*, strain HSM, was cultured axenically in an enriched proteose peptone medium, 1.5 percent (w/v) proteose peptone medium (Difco Laboratories, Detroit) supplemented with 0.1 per cent liver extract (Nutritional Biochemical Corp., Cleveland). The pH of the medium was adjusted to 7.3 with NaOH. A large surface-to-volume ratio for gas exchange was

maintained by growing the cells in a 2.5-liter low form culture flask with 500 ml of medium. Temperature was maintained at 29°C throughout the experiments.

CELL COUNTS: Cell number was determined by two different methods: (*a*) an electronic counter (Coulter Co., Model B), and (*b*) a capillary culture pipette method (18, 22).

MORPHOLOGICAL METHODS: A phase contrast microscope (Leitz Ortholux), equipped with the Heine condenser and with a Pv Fl oil immersion 70×, 1.15 N.A. objective and with a 6× periplanatic eyepiece, was used throughout the study. A Mikroblitz 200-flash attachment (1/1000 of a second flash) was used for photography with Kodak High Contrast Copy film (M135). For slide preparation, cell samples of about 0.5 to 1.0 ml were concentrated by gently centrifuging them (600 RPM) in a reduced capillary tip (Hopkins vaccine) centrifuge tube. A small drop of the pellet was pipetted onto a slide and a coverslip added and allowed to compress the cells until they were sufficiently immobilized for observation and photography.

For electron microscopy the cells were washed in 0.9 per cent saline, fixed with 2 per cent OsO_4 in Palade's buffer for 30 minutes, washed, dehydrated, embedded in Epon, and sectioned with the Porter-Blum ultramicrotome. A detailed description of the procedures is given elsewhere (13). Electron micrographs were taken with a Siemens Elmiskop I (40 kv) at magnifications from 1,000 to 10,000.

BIOCHEMICAL ANALYSIS: The biochemical data were obtained from duplicate 10-ml samples of saline-washed lag phase cells taken at half-hour intervals after inoculation into fresh proteose peptone culture fluid (after inoculation there were 160,000 cells/ml). The inoculum was taken from a stationary phase culture (5 days old). Throughout this time, cell counts were taken with the electronic counter.

The total protein content of each sample of cells was measured by the spectrophotometric method of Lowry *et al.* (10) using bovine serum albumin as a standard.

Nucleic acids were extracted by a modified Schmidt-Thannhauser procedure as described by Blum and Padilla (2, or see 4). The optical density conversion factors of De Deken-Grenson and De Deken (7) were used to convert the spectrophotometric data to μg RNA/10^6 cells and to μg DNA/10^6 cells.

AUTORADIOGRAPHY: To measure RNA, DNA, and protein synthesis, cells were incubated for 15 minutes in medium containing, respectively, 10 μc/ml H^3-uridine specific activity 3.73 c/mM, 15 μc/ml H^3-thymidine specific activity 6.70 c/mM, 15 μc/ml H^3-leucine specific activity 5.0 c/mM (New England Nuclear Corp., Boston). All cells were air-dried on slides (19, 22). The cells were then fixed for 20 minutes in 3:1 ethanol–acetic acid, given three 20-minute washings in 70 per cent alcohol, and again air-dried. DNA digestion was carried out with a 0.1 per cent DNase solution (Worthington Biochemical Corp., Freehold, New Jersey) made up in a solution of 0.003 M Na_2HPO_4, 0.005 M $MgSO_4$, and 0.007 M KH_2PO_4 (pH 6.8), at 37°C for 2 hours. Autoradiography was done with liquid emulsion (Kodak NTB3) as described by Prescott (19). The autoradiographs were developed after 2 weeks, then stained through the emulsion with toluidine blue.

To determine H^3-uridine and H^3-leucine incorporation, grain counts were made over a unit area of cytoplasm or nucleus with the aid of a Whipple ocular disk. The number of grains per unit area is proportional to the rate of incorporation per unit mass when pulse labeling is employed.

A series of phase contrast observations was also carried out on *Tetrahymena* grown on a chemically defined synthetic medium and then starved of pyrimidine for 72 hours (see reference 4 for details).

RESULTS

GROWTH CURVE: A typical growth curve of a culture of *Tetrahymena pyriformis* grown in enriched proteose peptone medium is shown in Fig. 1. A lag phase of about 2.5 hours is evident from both Figs. 1 and 2, which were obtained from inoculum of 5-day-old stationary phase cells. Phelps (15) and Prescott (17, 18) have shown that the length of the lag period in *Tetrahymena* is independent of the size of the initial inoculum but is dependent upon and, within limits, is proportional to the age of the inoculum. The exponential phase of growth lasted for about 26 hours under our conditions. Increase in cell number then tapers off and the culture enters the maximum stationary phase.

MICROSCOPIC OBSERVATIONS: Phase contrast microscopy on living *Tetrahymena* permitted us to observe certain morphological events and details which have not been reported previously in *Tetrahymena*. Nucleoli about 0.5 to 1.0 μ in diameter are closely associated with and are evenly distributed over the nuclear membrane (Figs. 3 and 4). Observations on unsquashed macronuclei and on sectioned material have assured us of the distribution of nucleoli in relation to the nuclear membrane. In addition, as one focuses deeper into the macronucleus the nucleoli are seen only on the nuclear circumference, but within the macronucleus one can discern smaller bodies apparently corresponding to the chromatin bodies (diameter of about 0.3 μ) seen also in the electron micrographs

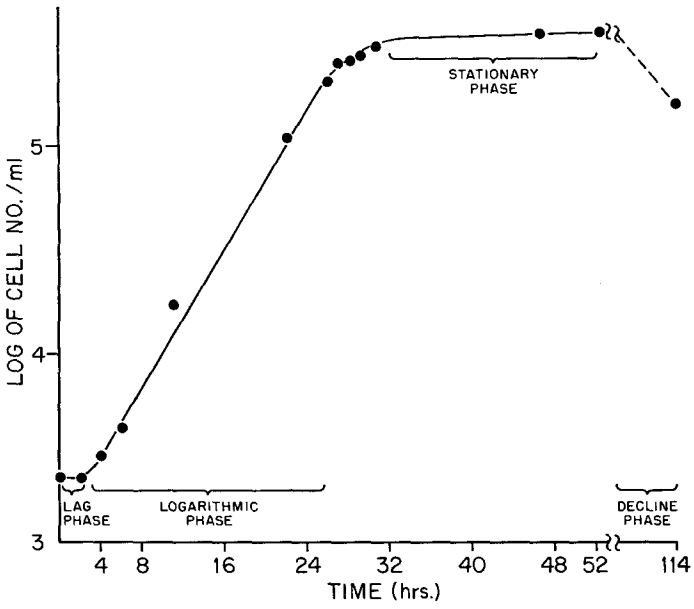


FIGURE 1 Growth curve of *Tetrahymena pyriformis* (strain HSM) grown in enriched proteose peptone medium. The culture was started with an inoculum of stationary phase cells (starting concentration 5×10^3 cells per ml, temperature 29°C). The growth curve, with the four main phases, is typical for cells in mass culture.

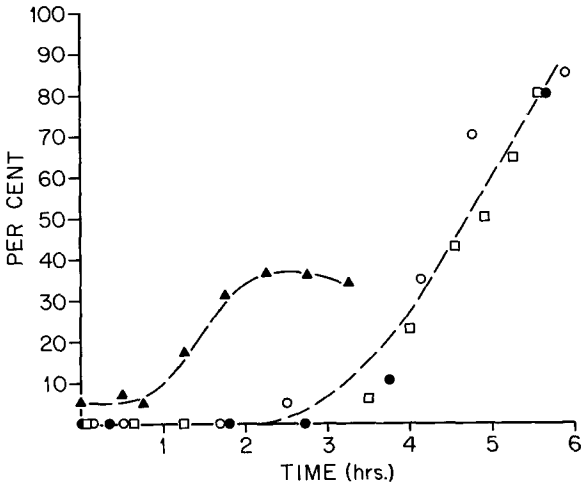


FIGURE 2 The percentage increase in cell number started with a 5-day-old inoculum. Results of three experiments, ●, ○, and □. Percentage of cells incorporating H^3-thymidine into their macronuclei (solid triangles, ▲) plotted against time after inoculation into fresh enriched proteose peptone broth.

(compare Figs. 3 and 5; see also references 9, 20, and 23). There may be as many as 300 nucleoli in each exponential cell, but the number is subject to considerable variability in different cells and apparently in the same cell, depending on the culture condition (as discussed below). The morphological details of the *Tetrahymena* macronucleus and nucleoli have been described by Swift *et al.* (23), Elliott (8), Elliott *et al.* (9), and Roth and Minick (20). The normal electron microscope morphology of

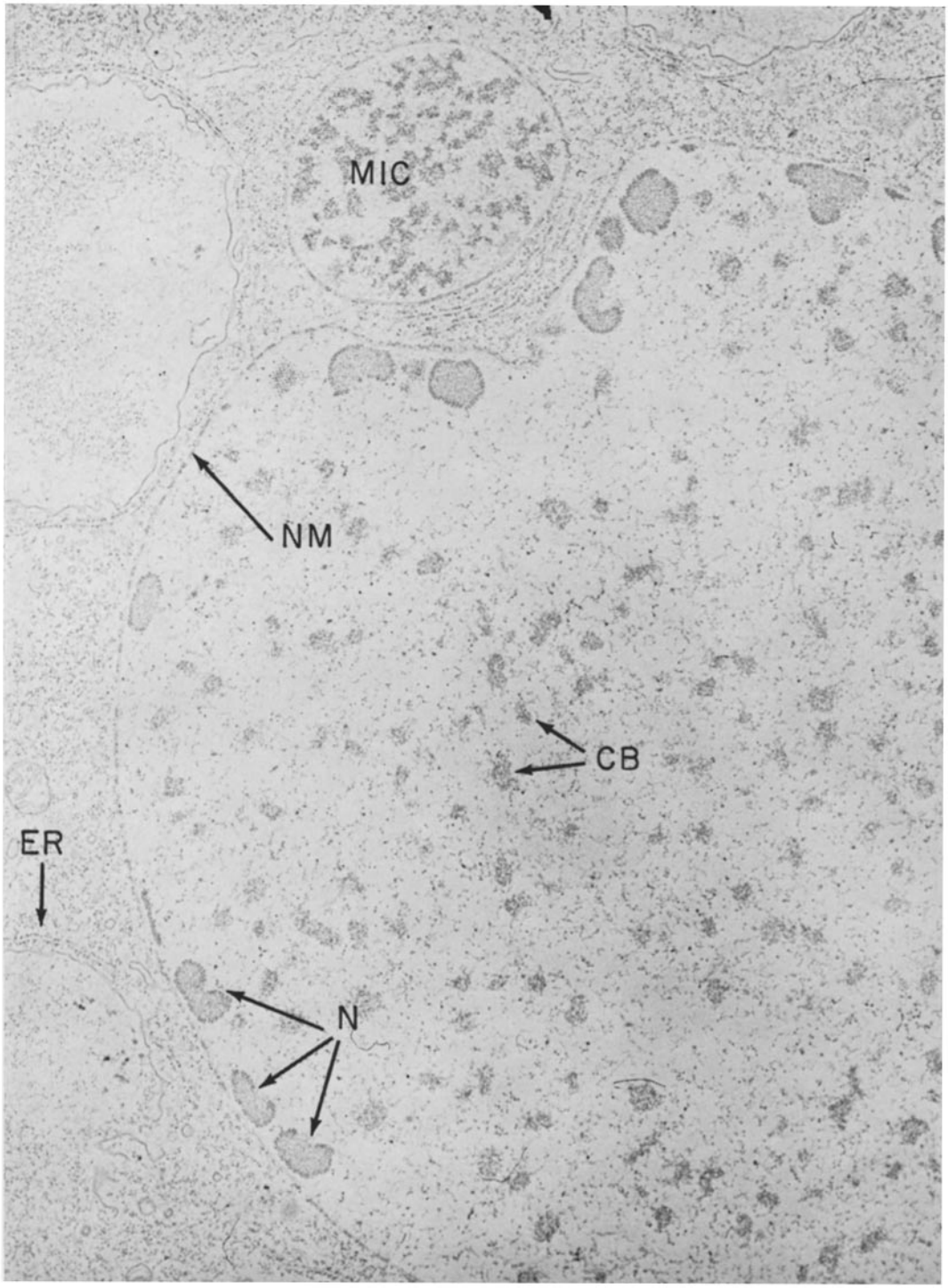


FIGURE 3 Electron micrograph of the macronucleus and micronucleus of exponentially growing *Tetrahymena*. Explanation of symbols used in the figures of this report: *CB*, chromatin bodies; *N*, nucleolus; *FB*, fusion bodies; *MI*, mitochondria; *MIC*, micronucleus; *NM*, nuclear membrane; *ER*, endoplasmic reticulum; *CV*, contractile vacuole. × 16,000.

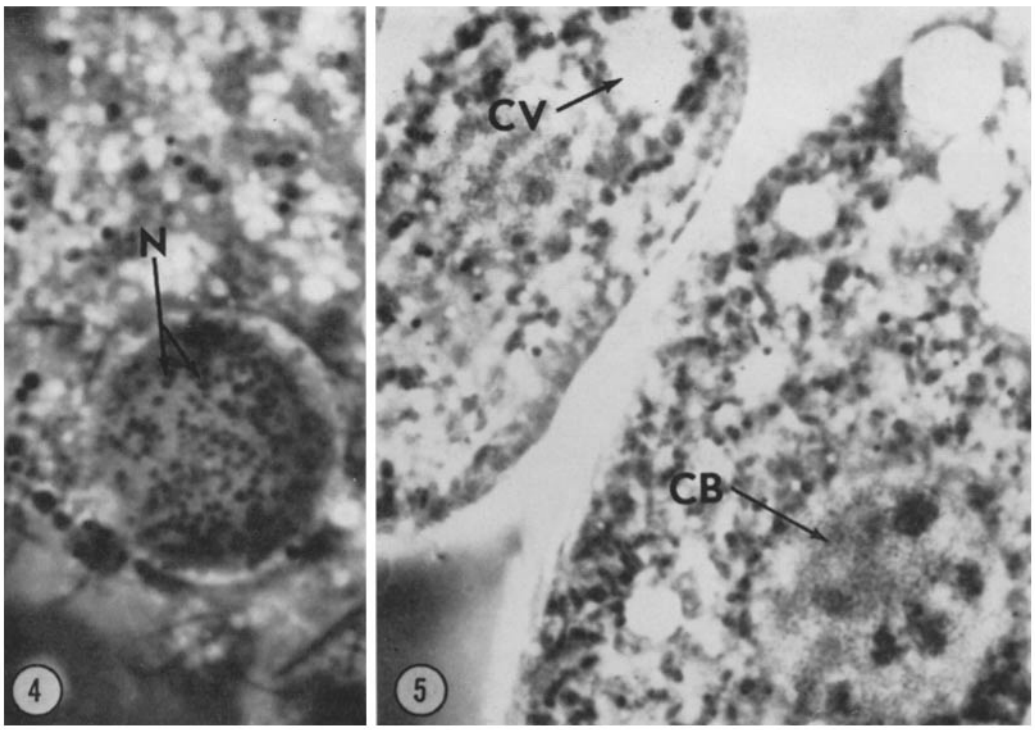


FIGURE 4 Squashed phase contrast preparation of *Tetrahymena* macronucleus. This is a logarithmically growing cell. Note that the nucleoli, *N*, are evenly distributed against the nuclear membrane. Cells which are not squashed show the same even distribution of nucleoli along the nuclear membrane. × 3500.

FIGURE 5 Squashed phase contrast micrograph of *Tetrahymena* taken from a 3.5-day-old culture. Notice the chromatin bodies in the macronucleus. × 3500.

the macronucleus of logarithmically grown cells as seen in this study does not appear to differ from that described by others (8, 20, 23).

Comparisons of the nucleoli as seen under phase optics with nucleoli seen under the electron microscope leave no doubt that they are the same structure. The electron micrographs show the U-shaped nucleoli as they have been described previously (8, 9, 23). They appear to have a cortical granular layer of particles similar in dimensions to free ribosomes (also see reference 23). Inside the cortex is a fiber-like material which appears to be connected by fibrils to other parts of the nucleus. Indeed, in many substructural aspects these nucleoli resemble nucleoli of higher cell types.

During the various growth phases, one can observe reproducible changes in the macronucleus. As the cell culture reaches the deceleratory growth phase (usually 2 to 3 days after inoculation), the nucleoli begin to aggregate and fuse, thus becoming larger and fewer in number (see and compare Figs. 3 to 6; Figs. 10 to 12 are electron micrographs of this same fusion phenomena). We believe that the "RNA nuclear bodies" as seen in the electron micrographs by Elliott and co-workers (8, 9) are fused nucleoli as seen in Figs. 10 to 12. Indeed, heat shocks such as those used in the work of Elliott *et al.* are now known to stimulate fusion of nucleoli (6).

In maximum stationary phase cultures (3 to 6 days old) the fusion process has continued until a very few large nuclear bodies persist (Figs. 7, 12). When stationary phase cells (5 days after inoculation) are placed into fresh enriched proteose peptone medium, they show a lag phase of 2.5 hours before the first cells begin to divide. During the first few hours in the fresh medium the large spherical and sometimes vacuolated nuclear fusion bodies flatten out against the nuclear membrane and begin to disaggregate into numerous smaller bodies about 0.5 μ in diameter (Figs. 8 and 9 are stages in this process). The cells do not synchronously

undergo this disaggregation. Thus, only the general course of temporal events is described here. At first these smaller nuclear bodies, nucleoli, are non-randomly distributed (clumped) on the nuclear membrane, but by 3 to 6 hours after inoculation most cells return to the even distribution characteristic of logarithmic cells. As the first cells divide, all of the large nuclear bodies may not have disaggregated; however, after the culture has reached a 2 to 4-fold increase in cell number, large nuclear bodies are no longer present in any of the cells.

Prior studies using *Tetrahymena* have shown that removal of required pyrimidines from the chemically defined synthetic growth medium leads to decreased RNA and protein synthesis. Replacement of the pyrimidines brings about an immediate increase of RNA synthesis (3, 4). We turned to such an experimental system to study the relation between the availability of RNA precursors (pyrimidines) and nucleolar fusion. Thus in the non-growing cells starved of pyrimidine for 72 hours, phase contrast microscopy reveals the presence of numerous nucleoli with no fusion of nucleoli as is seen in stationary phase cells. Apparently pyrimidine starvation, although it does drastically curtail RNA synthesis, does not cause fusion of nucleoli, and the unfused nucleoli allow an immediate increase in RNA synthesis after pyrimidine replacement.

BIOCHEMICAL DATA: The average cellular content of RNA, DNA, and protein during the lag phase (inoculum from a 5-day-old culture) is shown in Fig. 13. The cellular content of RNA does not appear to show an increase for at least 1 hour, but between the 1st and the 2nd hour there is a dramatic increase in cellular RNA (about a 60 to 75 per cent increase); after 3 hours the increase is about 100 per cent, and this accumulation of cellular RNA continues throughout the period of measurement (4.5 hours). Cellular DNA content is not increased appreciably during the lag phase, though a small increase is not excluded. Cellular protein content shows no marked increase up to 3 hours after inoculation. The increase after 3 hours, although it appears to be real, is not so great as in the case of RNA. It appears that cellular RNA content is increased faster and to a greater extent during the lag phase than is cellular protein content.

AUTORADIOGRAPHIC DATA: At intervals, samples of lag phase cells were given 15-minute pulse exposures either to H^3-uridine to measure RNA synthesis or to H^3-leucine to measure protein synthesis. The rate of isotope incorporation was measured by counting silver grains per unit area over cells in autoradiographs. As H^3-uridine is not a specific precursor of RNA but is also incorporated into DNA, it was necessary to make DNase digestions on our material prior to the autoradiographic procedures. From control autoradiographic experiments it became clear that our DNase digestion procedure would remove essentially all of the radioactivity from cells whose nuclei were labeled previously with H^3-thymidine. Table I and Fig. 14 show that the rate of H^3-uridine incorporation begins to increase shortly after inoculation. This finding is true for both a unit area over the nucleus and a unit area over the cytoplasm. The average coefficient of variability of 30 per cent (Table I) shows that there is a large variability of different cells in their response to re-

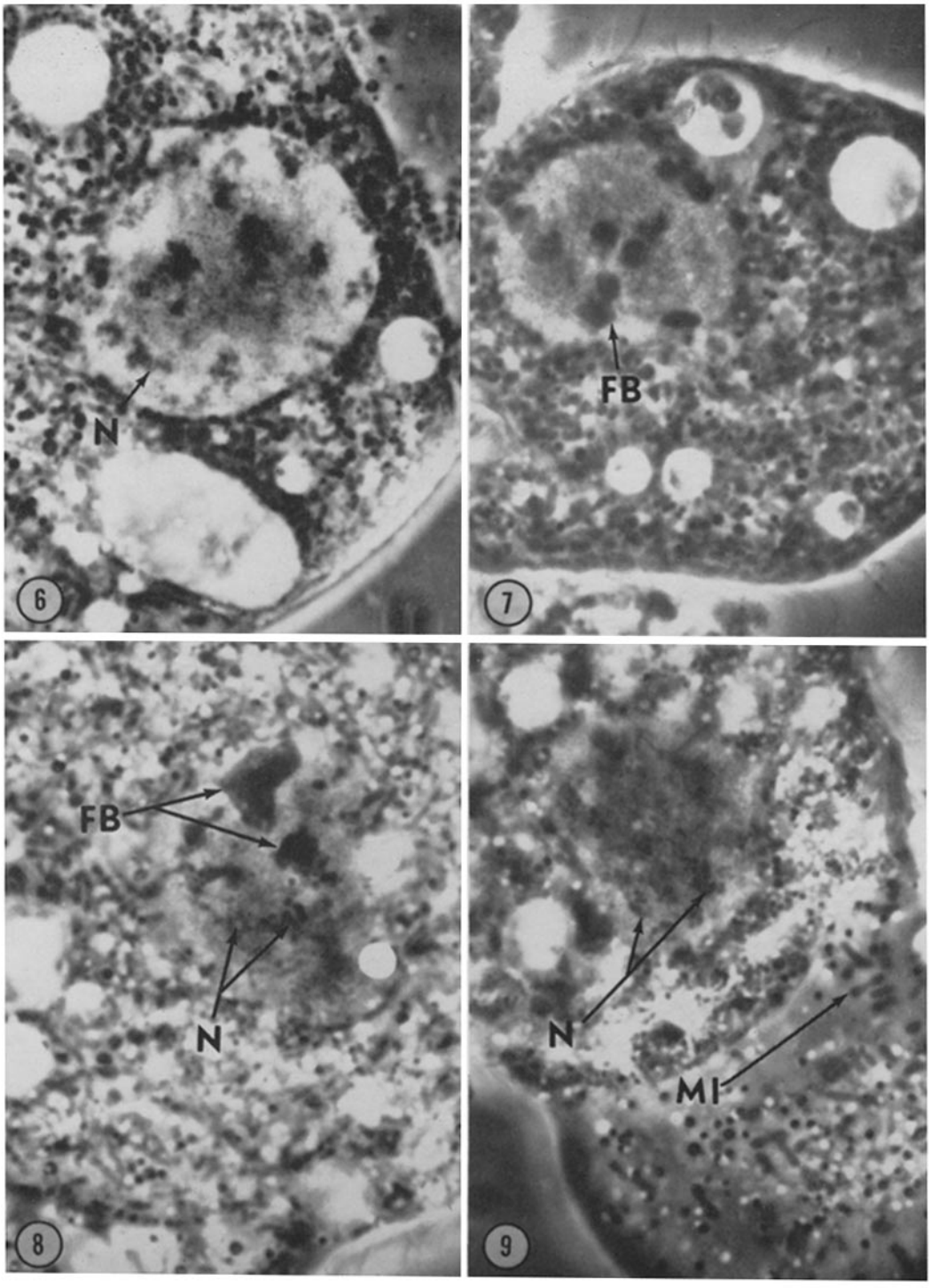
FIGURES 6 TO 9 Series of phase contrast micrographs showing changes in the macronucleus at different times in the growth cycle. All, × 3500.

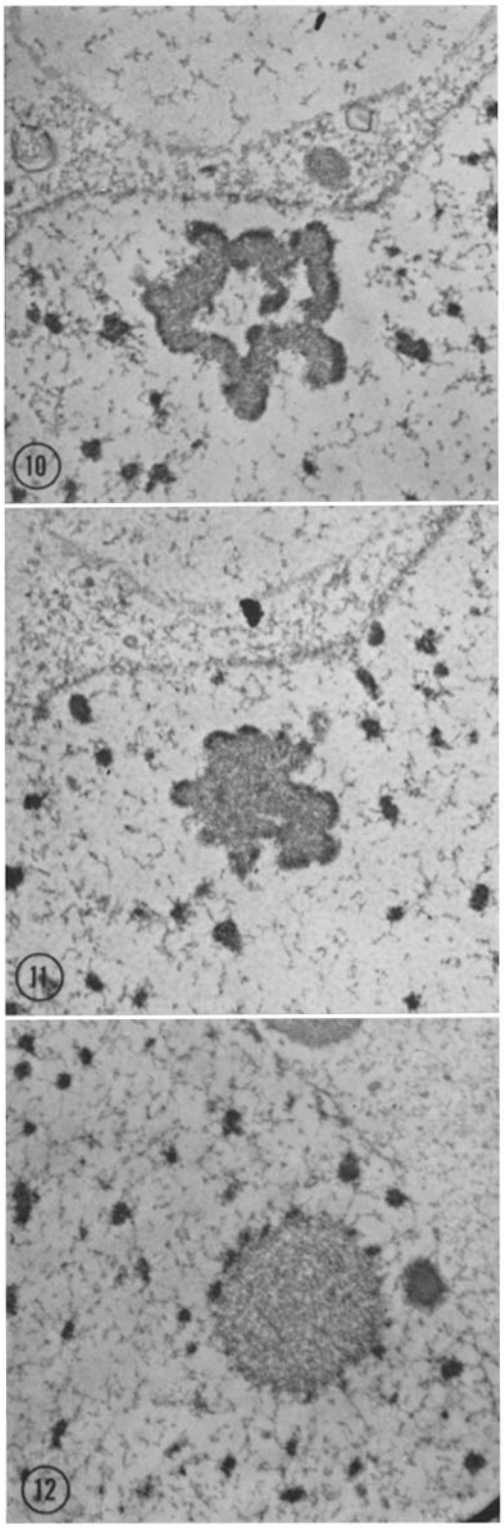
FIGURE 6 Three days post inoculum; the nucleoli are aggregating and fusing into larger nuclear bodies.

FIGURE 7 Five days post inoculum the fusion of nucleoli has continued until only a few large fusion bodies remain in the macronucleus.

FIGURE 8 A cell taken 3 hours after inoculating fresh enriched proteose peptone culture medium with the 5-day-old stationary cells (as seen in Fig. 7). The fusion bodies are pressed against the nuclear membrane. Also notice that a few fusion bodies have disaggregated, producing clumps of nucleoli.

FIGURE 9 A cell taken from the same culture, but 4.5 hours after inoculation the disaggregation process has continued until most of the fusion bodies are gone and the nucleoli are becoming evenly distributed on the nuclear membrane.





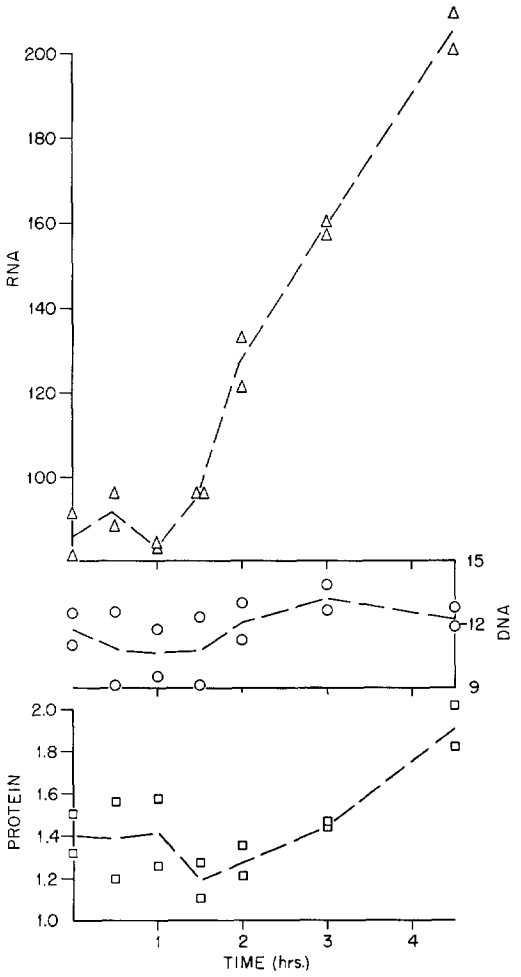


FIGURE 13 Changes in the average cellular content of RNA (top) DNA (middle) expressed in $\mu g/10^6$ cells, and protein (bottom) expressed in $mg \cdot 10^6$ cells and plotted against time (in hours) after inoculation of fresh enriched proteose peptone medium with stationary phase cells (5-day-old culture).

growth conditions. The H^3-leucine autoradiographic grain counts show a longer lag than the H^3-uridine data before the increase in incorporation rate. Here again there is considerable varia-

FIGURES 10 TO 12 Electron micrographs of portions of the macronuclei of stationary phase *Tetrahymena*. Fig. 10 shows one stage in the fusion of nucleoli; Fig. 11 is a later stage in the fusion phenomenon; and Fig. 12 shows a large sphere comparable in size to the large fusion bodies seen in the phase contrast microscope (see Fig. 7). All micrographs, × 16,000.

TABLE I

Results of Autoradiographic Grain Count

Cells exposed to	Time after inoculation	Mean and standard error of the mean expressed in grains per unit area above background*	
		Over cell's cytoplasm	Over cell's nucleus
	min.		
H^3-uridine‡	0–15	6.2 ± .58	17.6 ± .45
	30–45	8.1 ± .69	26.5 ± .81
	60–75	11.7 ± 1.8	28.9 ± 1.10
	90–105	17.8 ± .40	48.9 ± .11
	120–135	13.1 ± 2.17	36.2 ± 1.63
	150–165	15.8 ± .37	40.7 ± 2.22
	180–195	24.8 ± .76	61.0 ± 4.31
H^3-leucine	0–15	5.3 ± .38	
	30–45	4.8 ± .36	
	60–75	5.1 ± .43	
	90–105	5.3 ± .34	
	120–135	6.1 ± .45	
	150–165	7.2 ± .65	
	180–195	7.6 ± .67	

* Area over 20 cells was analyzed for each average. Same unit area was used for nucleus and cytoplasm. The average coefficient of variation was 30 per cent in these data.

‡ Cells treated with DNase before autoradiographic coating procedure.

bility from the mean values, suggesting a heterogeneous response between individual cells during the lag phase. Both the H^3-uridine and H^3-leucine data suggest an increased rate of incorporation per unit mass towards the end of the lag period. The autoradiographic grain counts of H^3-uridine and H^3-leucine incorporation appear to increase prior to the cellular content of RNA and protein, respectively. The biochemical data and the autoradiographic data, taken together, show that the rate of RNA synthesis increases after a short adjustment period (about 1 hour) and that this increase in the RNA-synthetic rate precedes an increase in the protein-synthetic rate, which occurs about 2 to 3.5 hours later.

Pulsing of cell samples with H^3-thymidine at intervals in the lag phase and in the stationary phase (Fig. 2) shows that 5 days after inoculation some cells are still involved in DNA synthesis. There appears to be a lag of about an hour before the per cent of cells with labeled macronuclei begins to increase from about 6 per cent to about 36 per cent at 2.25 hours.

DISCUSSION

The function of the nucleolus is a subject of long-standing interest to cell biologists. McConkey and Hopkins have recently listed the experimental evidence of several workers which links the synthesis of ribosomal RNA with the nucleolus (12). They further demonstrate that there is a nucleolus-associated chromatin (DNA) which is complementary to ribosomal RNA. In view of the possible role of nucleoli in RNA (ribosomal) synthesis, there are at least two testable interpretations of our nucleolar fusion data: (*a*) that the intranuclear environment slows down ribosome production by causing nucleoli, which are involved in the synthesis of ribosomal RNA, to aggregate; (*b*) alternatively, that the nucleolus-associated production of ribosomes slows down, causing the nucleoli to fuse.

Experiments can be designed to test these two interpretations. For instance, the pyrimidine starvation experiment was an attempt to see if limiting the precursors of RNA (ribosome) synthesis would eventually lead to nucleolar fusion. That nucleolar fusion did not occur during pyrimidine starvation, therefore, favors the first interpretation; however, more and different experiments must be carried out before the question can be resolved.

In general, our study shows that the time of nucleolar disaggregation is concomitant with the beginning of an increased RNA production, and,

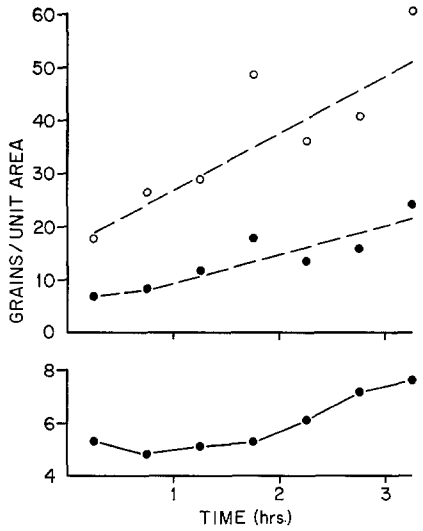


FIGURE 14 Results of autoradiographic grain counts. The average number of grains above background per unit area of cell cytoplasm or cell nucleus (15-minute exposure) is plotted against time after inoculation of stationary phase cells into the fresh enriched proteose peptone medium. The H^3-uridine–treated cells (top two curves) were digested with DNase prior to autoradiographic dipping procedure. Open circles, ○, nucleus; closed circles, ●, cytoplasm (same area was used for cytoplasm and nucleus). The H^3-leucine curve is on the bottom (see text and Table I for details).

therefore, strengthens the relationship between RNA synthesis and nucleolar function. Due to the heterogeneous responses of the cells during the lag phase, it is impossible to say whether the increased RNA synthesis precedes or follows the initial stages of fusion body disaggregation. Clearly, however, the increase in RNA synthesis preceded protein synthesis and cell division. Indeed, other studies suggest the importance of this particular sequence of events in the regulation of cellular growth and division rates whenever the cells' environment is changed (1, 3, 4, 11, 14, 16, 21). Furthermore, our experiments lead us to the conclusion that nucleoli must be in the non-aggregated condition for the cell to support increased RNA and protein synthesis.

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