MACRONUCLEAR EVENTS IN SYNCHRONOUSLY DIVIDING TETRAHYMENA PYRIFORMIS

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ABSTRACT

The macronuclei of synchronously dividing mass cultures of Tetrahymena pyriformis (strain WH6) were examined with the electron microscope for changes during two division cycles. Samples were prepared at 30-minute intervals for a period of 8½ hours which included the time required to induce synchrony by five heat shocks (4½ hours). The interphase macronucleus contains peripheral, crescent-shaped nucleoli and evenly distributed chromatin bodies. Centrally located RNA bodies, composed of fibers, appear 1 to 2 hours following the initial heat shock. They are completely destroyed with ribonuclease whereas the nucleoli are only partially so. Following the third heat shock the RNA bodies move to the periphery and disintegrate; the nucleoli aggregate and form blebs which protrude into the cytoplasm where they appear to pinch off and may contribute to the cytoplasmic ribonucleic acid. Cytokinesis does not occur at this time. Instead the nuclear events are repeated during the 4th and 5th hours, even though the heat shocks are terminated at 4½ hours. Cytokinesis takes place at about 6 hours. The second division occurs about 2½ hours later during which all the macronuclear events noted above are repeated.

Most studies of mitosis, with both light and electron microscopes, have been accomplished with randomly dividing cells (6, 10). With the advent of techniques for inducing synchronization of the division cycle in mass cultures of protozoa (13, 16), it is now possible to examine the sequence of events during division more precisely. Among these, Tetrahymena pyriformis has come in for its share of attention, particularly as regards biochemical and physical changes during division. Studies of structure, however, even with the light microscope, have been few (9, 21); and the ultrastructural changes occurring during division have only recently been examined (12).

To follow all the morphological changes that occur from one generation to the next in T. pyriformis would be too monumental a task; hence this investigation has had the limited purpose of studying the macronuclear events throughout two division cycles in a micronucleate strain, following heat-shock treatment, employing both light and electron microscopy. Some mention is made of other structures as they relate to the main purpose of this report.

MATERIALS AND METHODS

The strain of T. pyriformis used in this investigation was WH6 (variety 1, mating type I). The cultures were grown axenically in 500 ml Erlenmeyer's flasks in a 2 per cent proteose-peptone (Difco Laboratories, Detroit) medium supplemented with glucose (0.5 per cent), liver extract, and a mineral salt mixture. One hundred ml of media was used to permit an ample supply of oxygen. They were maintained at 35°C through several transfers prior to use in the experiments. The heat-shock treatment was carried out according to the schedule designed for
this strain by Holz, Scherbaum, and Williams (7). Heat-shock intervals of 30 minutes were continued for 41/2 hours in which the temperatures were alternated between 35° ± 0.5°C and 42.8° ± 0.01°C. Approximately 1 hour following the last heat shock 80 per cent of the cells showed cleavage furrows, although it is highly likely that as far as the nuclei were concerned 100 per cent of the cells were in synchrony (21).

Each experiment was started with cells that had grown 96 hours at 35°C and were therefore in stationary phase of the growth cycle; older or younger cultures gave smaller percentages of synchronized cells. The number of cells transferred to fresh media was adjusted so that the final concentration was approximately 50,000 cells per ml. They were then immediately subjected to the cyclic heat treatment.

Ciliates to be examined in the electron microscope were withdrawn from the flasks at 30-minute intervals (starting at the beginning of the first heat shock and continuing for 81/2 hours), fixed with 2 per cent osmium tetroxide (buffered to pH 7.4 with 0.14 M Veronal acetate buffer) for 20 minutes at room temperature, dehydrated with ethanol using 15-minute changes, and embedded in methacrylate (80 per cent n-butyl and 20 per cent methyl methacrylate with 2 per cent Luperco CDB). The cells were infiltrated for 1 1/2 hours (3 changes) and the blocks polymerized in an oven at 60°C. Thin sections were cut with a Porter-Blum microtome and examined in an RCA EMU 3E electron microscope. Original magnifications ranged from 1,500 to 22,000 times and the micrographs were enlarged photographically as desired.

Cells prepared for light microscopy were withdrawn from the flasks at 41/2 hours following the initial heat shock, placed on coverslips, fixed with Carnoy's (formula 2), and allowed to dry in air. Some were stained with azure II (0.1 per cent aqueous) at pH 4.5 and others were subjected to the Feulgen reaction. Cells from the same flasks, which were embedded for thin sectioning, were also used to obtain thick sections for light microscopy. These were sectioned at 1 to 2 microns in thickness, and stained with azure II following the removal of the methacrylate with 10 per cent acetone. Photographs were taken at 1000 magnification on 4 x 5 inch Kodak M plates with a Spencer photomicrographic camera.

Cytochemical studies were made on cells prepared somewhat differently. For electron microscopy, samples were osmium fixed for 2 minutes, washed several times in distilled water to remove the fixative, and then exposed to 0.1 per cent aqueous ribonuclease (RNase) at 35°C for 10, 25, and 35 minutes. Controls without the enzyme were prepared. Following the treatment with RNase, the samples were washed, fixed again in osmium tetroxide for 20 minutes, and prepared for sectioning as described above. For light microscopy the cells fixed in Carnoy's (formula 2) were treated with 0.1 per cent aqueous deoxyribonuclease (DNase) and RNase for 10 minutes at 35°C and then exposed to azure II or the Feulgen reaction as previously described. Controls without enzyme treatment were also examined.

**OBSERVATIONS**

Cells in the stationary phase show mitochondria distributed throughout the cytoplasm, numerous

The time indicated in the figures is taken from the beginning of heat-shock treatment.

**Figure 1**

Longitudinal section of a stationary phase cell grown 4 days at 35°C just prior to the initial heat shock showing mitochondria (M), lipid particles (L), food vacuoles (FV), and the micronucleus (MI) lying within an indentation in the macronucleus (MA). × 4,000.

**Figure 2**

Enlarged macro- and micronucleus (MI) from a stationary phase cell. The micronuclear chromosomes are at interphase. The macronuclear chromatin bodies (C) are evenly distributed and the crescent-shaped nucleoli (N) are peripherally located. The break in the shell is oriented toward the center of the macronucleus. × 6,000.

**Figure 3**

Enlargement of nucleolus showing its close association with the macronuclear membrane (MAM). × 55,000.
lipid bodies, food vacuoles, and the prominent macronucleus with an indentation in which the micronucleus lies (Fig. 1). The diffuse granular nature of the cytoplasm in strain WHs is best seen in preparations stained with phosphotungstic acid (Fig. 24). Membranes of the endoplasmic reticulum are inconspicuous and granules (ribosomes) are evenly dispersed. Improper fixation may account in part for the poor visualization of membranes. However, well formed membranes with adhering granules can be seen by employing the same techniques in some derived strains of variety 9 (unpublished). This may be a strain characteristic.

At this stage of growth most of the cells are in interphase as demonstrated by the position of the micronucleus and the arrangement of the chromosomes within. The macronucleus is conspicuous, showing numerous fibrillar dense granules evenly distributed throughout the nucleoplasm, which are probably chromatin bodies (Fig. 2). Just inside the double nuclear membrane, crescent-shaped bodies with specific morphology are distributed which have been identified as nucleoli by some authors (12, 17). They lie closely associated with the inner macronuclear membrane and consist of a dense outer shell and a less dense inner core (Fig. 3). An enveloping membrane is not present. Most sections show a conspicuous opening or break in the shell which is usually oriented toward the center of the macronucleus (Fig. 2).

For a schematic presentation of the sequence of heat shocks and associated nuclear events see Fig. 29. No obvious changes can be observed following the first heat shock. However, during the second heat shock, oval membraneless bodies appear which are only slightly more osmiophilic than the surrounding nucleoplasm. Within the next hour they become distinct, clearly defined structures (Fig. 4) which completely disappear when the cells are treated with RNase (Figs. 12 to 15). From one to four usually appear in a section and are centrally located. They appear to be fibrous in structure (Fig. 5). Because of their complete destruction with RNase they are referred to as RNA bodies in this report. They differ from the nucleoli in that they are completely destroyed with RNase whereas the nucleoli are only partially destroyed (Figs. 12 to 15). It would appear that they contain RNA with very little protein, whereas the nucleoli are primarily protein. A region of light nucleoplasm free of chromatin material surrounds the entire structure, but fibrils traversing this zone establish connections between the RNA body and the chromatin bodies (Fig. 5).

At approximately 3 hours from the beginning of treatment the RNA bodies move to the periphery of the nucleus and gradually disappear (Fig. 6). Chromatin bodies can be seen within the RNA body before it is completely disorganized. At 3½ hours the RNA bodies are more dispersed and the nucleoli have increased in number and density (Fig. 7). These aggregate into masses interconnected by a network of less dense fibers.

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**Figure 4**
Macronucleus at 2 hours showing well developed RNA bodies (RB). X 8,000.

**Figure 5**
Enlarged RNA body showing the fibers and the fine lines of communication with the chromatin bodies (C). X 34,000.

**Figure 6**
Macronucleus at 3 hours. The RNA bodies (RB) become disorganized and the chromatin bodies (C) have increased in number. Note the chromatin bodies (RBC) within the RNA bodies. X 8,000.

**Figure 7**
Macronucleus at 3½ hours. The RNA bodies are more dispersed and the nucleoli-containing blebs (B) are prominent. The chromatin bodies have doubled in number (compare with Fig. 4). X 7,000.
Blebs containing nucleoli may protrude from the macronucleus at this stage. Normally the cells would undergo cytokinesis at about this time; however, the heat shock prevents division for reasons which have been thoroughly explored (16). Although cytokinesis does not occur, chromatin bodies do appear to double in number. When the number of these bodies in Fig. 4 and Fig. 7 is counted per unit area the ratio is approximately 1:2. Just how doubling is accomplished is not revealed in these studies.

At 4 hours the RNA bodies reappear (Fig. 8) and 1½ hour later blebs form on the macronuclear membrane a second time, sometimes protruding some distance into the cytoplasm (Fig. 9). They contain nucleoli and occasionally RNA bodies. The RNA composition of the RNA bodies can be demonstrated by observing the fine structure of cells containing these bodies (4½ hours) that are prefixed in OsO₄, exposed to RNase for various periods, and then sectioned. Complete destruction of the RNA bodies is observed after 35 minutes of treatment (Figs. 12 to 15).

Light photomicrographs of azure II-stained sections taken from blocks used in preparing the electron micrographs described above also demonstrate that the macronucleus develops blebs or protuberances containing RNA (Fig. 11). Macronuclei of whole mounts of cells showing blebs (4½ hours), when treated with RNase, lose their affinity for azure II (Figs. 16, 17), but retain it after DNase treatment (Fig. 10). Blebs of macro-nuclei are not stained by the Feulgen reaction following treatment with either RNase or DNase (Figs. 19 to 21). Since the blebs contain nucleoli and occasionally RNA bodies, as observed electron microscopically, one can assume from the cytochemical evidence presented here that the blebs contain RNA. As suggested earlier, the nucleoli may be composed, in large part, of protein, and therefore may appear unaffected by RNase.

The two waves of blebs form and disappear in a relatively short time (circa 30 minutes). They begin as slight protrusions of the macronuclear membrane in the vicinity of the nucleoli (Fig. 22). The nucleolus, as well as material which appears to resemble the RNA body, becomes a part of the bleb content. The blebs form long finger-like protuberances extending into the cytoplasm for some distance (Fig. 9). These then apparently pinch off as fragments (Fig. 23) and disappear. What becomes of them is not revealed by these studies.

Approximately 1 hour following the last heat shock, i.e., at about 5½ hours, the macronucleus elongates and the cleavage furrow becomes obvious (Fig. 24). No RNA bodies are visible and the nucleoli cluster at the ends of the elongated macronucleus. The fibers reported by Roth and Minick (12) were not observed, which may be a result of different techniques in handling the cells. Shortly thereafter the daughter macronuclei form (Figs. 25, 26) and almost at once the RNA bodies...
reappear (Figs. 25, 27). The chromatin bodies are fewer in number and appear smaller and less dense during division, shortly thereafter increasing in size. Apparently the increase is due to doubling at this stage. Macronuclear division and cytokinesis are completed by 6½ hours.

The second division takes the same course as the first. The blebs appear (Fig. 28) about 1 hour following the first division (7½ hours) and the cycle is completed 1 hour later (8½ hours).

The sequence of events noted during the two division cycles based on our studies is portrayed schematically in Fig. 29. The time periods at which each stage occurs are approximate, varying as much as 30 minutes.

The nucleus in the stationary phase begins to show internal change during the second heat shock when RNA bodies appear near the center. These become conspicuous, move to the periphery, and disintegrate during and following the third heat-shock. Blebs containing nucleoli form during the fourth heat-shock, and subsequently appear to pinch off. It may be assumed that they contribute to the cytoplasmic RNA, although no isolated nucleoli have been seen in the cytoplasm. Presumably an untreated cell would undergo cytokinesis at this time. Instead the cycle starts over during the fifth heat shock, resulting in cleavage about 1½ hours later, that is, at approximately 6 hours from the beginning of the initial shock. Immediately following cleavage the cycle is repeated and the nucleus again divides about 2½ hours later. Of the three macronuclear cycles, the last two result in cytokinesis. It is probable that the first cycle doubles the number and possibly the amount of chromatin bodies (twice the quantity as in untreated cleaving cells) which is then reduced to one-half during the first cell division. The second cycle seems to include a normal macronuclear division.

**DISCUSSION**

The macronucleus of *T. pyriformis* was first observed with the electron microscope in 1954 (2). Only casual reference has been made to it in later publications in which the macronucleus was not under scrutiny (5, 11, 17). Light microscopic studies of the interphase macronucleus reveal numerous evenly distributed Feulgen-positive bodies which are probably the chromatin bodies seen with the electron microscope. The only other obvious structures in the interphase macronucleus are the peripherally located nucleoli. Their close adherence to the membrane (Fig. 3) leads one to believe that this position is somehow related to function. The position is maintained throughout the division cycle as well as during log growth and starvation (unpublished). Ehret and Powers (4) in their study of conjugating and vegetatively reproducing *Paramecium bursaria* describe nucleoli which are similar in structure to those of *T. pyriformis*. They range in size from 0.2 micron (young) to 0.5 micron (mature) which compares favorably with those of *T. pyriformis* (0.3 micron). While a
size variation of the nucleoli is obvious in the electron micrographs, it is impossible to decide their age because of the difficulty of determining where the cut is taken. In spite of this obstacle, nucleoli of interphase cells seem to be remarkably similar in size (Fig. 2). Wells and Ray (20) describe both centrally and peripherally located RNA-containing bodies in *T. pyriformis* in conjugating and vegetative dividing cells. Since the stage in division was not ascertained the waxing and waning of the blebs as described in this report were not observed.

Summers et al. (19) observed that the macronucleus varies in volume during the growth cycle, being smallest during the stationary phase and largest during the lag period. They suggest that the macronuclear volume may be related to generation time. These observations were confirmed by Williams and Scherbaum (21) who demonstrated, in addition, that the small stationary phase macronuclei show more than a fourfold increase between the third and fourth heat shocks. While there has been no effort to compare nuclear size in these studies (volume cannot be determined from a section), it is interesting to note that the increased size reported by these investigators occurs approximately at the time when blebs are most pronounced, namely, between the third and fourth heat shocks and just prior to synchronous division (Figs. 7, 22, 23). Perhaps if measurements were taken between the second and third and the fourth and fifth heat shocks the volumes would be less. These are the periods just preceding and following bleb formation when the diameter of the macronucleus is smaller.

Roth and Minick (12), in comparing the ultrastructure of micronucleate (HAM 3) and amicronucleate (W) strains of *T. pyriformis*, describe macronuclear changes occurring during vegetative division that differ between the two. Since the present report covers a sexually active macronucleate strain, comparisons can only be made to their studies of HAM 3. These authors studied single cells from log growth cultures at two stages, namely, interphase and at the time of the appearance of the cleavage furrow. At comparative

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**Figures 16 to 21**

Photomicrographs at 4½ hours when blebs are most conspicuous. X 2,000.

**Figure 16**

Azure II-stained cell showing blebs (B) containing nucleoli.

**Figure 17**

Azure II-stained cell following RNase treatment. The blebs are completely destroyed indicating that they contain RNA. Compare with Fig. 16.

**Figure 18**

Azure II-stained cell following DNase treatment. The macronucleus loses its density but the blebs and a centrally located RNA body (RB) remain.

**Figure 19**

Feulgen-treated cell showing densely staining macronucleus with non-staining blebs (B).

**Figure 20**

Feulgen-treated cell following RNase treatment. The macronucleus is stained whereas the blebs are absent.

**Figure 21**

Feulgen-treated cell following DNase treatment. The macronuclear DNA has been removed. Outline of blebs (B) is visible.
stages, HAM 3 and WH6 seem to show similar morphology. However, the fibrillar elements noted during elongation of the macronucleus were not seen in this study (Fig. 24). Since no other stages were reported, other comparisons are impossible.

It has been demonstrated by a number of investigators that RNA moves from the nucleus into the cytoplasm in several organisms including protozoa (18). The evidence has been based primarily on tracer techniques. Morphological evidence among the ciliates is confined to observations on macronuclear chromatin elimination during normal and synchronous fission which implies that only deoxyribonucleic acid (DNA) is extruded (3, 9). These “subnuclear aggregates” form during fission and/or by extrusion from the macronucleus (14). Since they are Feulgen positive it would appear that they cannot be confused with the RNA-containing fragments seen in this study. Whereas we have seen subnuclear aggregates in some clones, the strain used in this investigation (WH6) seems to show none.

Cameron and Prescott (1) in their studies of cell and macronuclear volumes during normal fission of single cells (T. pyriformis, strain HSM) suggest that RNA is continuously released from the macronucleus during interphase. Our observations confirm such a release but suggest that the RNA may be extruded rhythmically at a precise brief period in the division cycle. It must be remembered, however, that we are dealing with heat-treated cells which may alter the sequence. In untreated log growth cultures one occasionally sees dividing cells which show bleb formation resembling those observed in synchronized cells. Therefore this appears to be a normal process in division and is probably only accentuated by the heat treatment. Ehret and Powers (4) also report nucleolar extrusion during the generation and disintegration of the macronuclei during conjugation and vegetative fission in Paramecium bursaria. They further suggest that mitochondria have their origin within the macronucleus. A serious effort was made to check this point in T. pyriformis but no evidence for mitochondrial extrusion was found.

Scherbaum, Louderback and Jahn (15) report that during heat treatment the DNA content did not keep pace with the macronuclear volume. The evidence presented here indicates that the rapid increase in nuclear volume may be due in a large measure to the increase in RNA. These authors also report that DNA synthesis during the treatment appears to be almost linear, increasing 1.5 to 1.8 times in the average cells. In an earlier report, Iverson and Giese (8) had likewise shown that the DNA essentially doubled (1.82 to 2.35 times) during 8 hours of heat-shock treatment. Our results tend to confirm these observations. The chromatin bodies appear to increase in number during the period between the third and fourth heat shock. By comparing Figs. 4 and 7 in which these bodies can be counted, the number is approximately doubled. The puzzling question is that this seems to be the only DNA increase during and following the treatment. One might assume that during the next wave of nuclear

![Figure 22](image)

**Figure 22**

Macronucleus at 4½ to 5 hours showing bleb (B) formation. Mitochondria (M) are frequently seen closely associated with the blebs. X 16,000.

![Figure 23](image)

**Figure 23**

Macronucleus at 5 to 5½ hours showing nuclear fragments containing nucleoli (FR) in the cytoplasm. X 10,000.

![Figure 24](image)

**Figure 24**

A dividing macronucleus at 5½ hours, stained with phosphotungstic acid. Note the diffusely distributed ribosomes. X 6,000.
events, that is, immediately following the fifth heat shock, the chromosomes would increase again. That this does not happen can be observed by comparing Figs. 8 and 9; indeed the number is even less than during the earlier stage.

REFERENCES


FIGURE 25
A dividing macronucleus slightly later than Fig. 24, showing the daughter macronuclei. Note the reappearance of RNA bodies (RB). × 3,000.

FIGURE 26
A single daughter macronucleus immediately following cleavage. Note the finger-like processes, at lower right, directed toward the opposing daughter macronucleus. No RNA bodies are visible. × 64,000.

FIGURE 27
A daughter macronucleus (7 hours) showing RNA body (RB). × 11,000.
FIGURE 28
Macronucleus 1 hour after division (71½ hours). Note conspicuous nucleoli within blebs (B). X 13,000.

FIGURE 29
A schematic representation of the macronuclear events during the heat-shock treatment and subsequent division cycles. See text for explanation.


