MITOCHONDRIAL INCORPORATION OF TRITIATED THYMIDINE IN *TETRAHYMENA PYRIFORMIS*

JOHN A. PARSONS. From the Department of Biology, San Diego State College, San Diego, California

Cytoplasmic DNA is known in a number of cell types. Hoff-Jørgenson and Zeuthen (7), using a microbial bioassay method, reported the presence of cytoplasmic DNA in frog eggs. Later, Hoff-Jørgenson (8) and Elson *et al.* (4) indicated that sea urchin eggs contained a DNA reserve in the cytoplasm. Using autoradiographs of whole cells, Scherbaum (21) described cytoplasmic labeling in H^3-thymidine–fed *Tetrahymena*. In some cell types the DNA has been associated with a definite cytoplasmic structure. Examples are the kinetoplasts of *Trypanosoma* (22), chloroplasts of *Chlamydomonas* (20), and 0.3 to 0.5 μ particles in *Amoeba* (18).

Chèvremont *et al.* (2, 3) have found H^3-thymidine incorporation in chick embryo mitochondria after treatment of living cells with DNase or reduced temperatures of 16°C. The authors conclude that under these conditions DNA is synthesized in the cytoplasm and transported to the nucleus by mitochondria. Ris (19), Nass and Nass (14) and Swift *et al.* (24) have reported fibers in electron micrographs of mitochondria which resemble DNA. Meek and Moses (12), using H^3-thymidine–labeled HeLa cells, observed grains over mitochondria in electron microscope autoradiographs. However, when labeled HeLa cells were washed with an excess of cold thymidine prior to fixation, the cytoplasmic label was considerably reduced or eliminated. In H^3-thymidine-labeled *Tetrahymena*, Stone and Miller (23) have shown by electron microscope autoradiography that practically all the cytoplasmic radioactivity is associated with mitochondria. Their electron micrographs indicate that the tritiated label is located inside mitochondria. Bell and Mühlethaler (1) have recently published electron microscope autoradiographs showing H^3-thymidine labeling of mature fern egg mitochondria and proplastids. These authors interpret their results to indicate that nuclear DNA is sloughed into the mitochondria and proplastids. Finally, Guttes and Guttes (6) have reported H^3-thymidine incorporation by cytoplasmic particles in *Physarum*. The authors suggest that the particles are mitochondria based on their size and frequency.

The present report describes mitochondrial incorporation of H^3-thymidine in *Tetrahymena*. Evidence will be presented that under conditions of normal growth H^3-thymidine is continuously incorporated into a mitochondrial DNA or DNA-like polymer which is not synthesized in the nucleus.

MATERIALS AND METHODS

Tetrahymena pyriformis strains HSM and variety 6, mating type III, were grown axenically at 24°C in a defined medium modified from Kidder and Dewey (9). Culture tubes containing one ml of the medium were autoclaved for 20 minutes at 121°C, 18 pounds of pressure prior to inoculation. H^3-thymidine (Schwarz Bioresearch Inc., Mt. Vernon, New York, 3.0 and 6.0 c/mM) was used at concentrations from 50 to 100 μc/ml medium. During experimental sampling, 40 μg/ml streptomycin sulfate and 25 μg/ml sodium penicillin G were used to insure against bacterial contamination. Cells were squashed on chromic acid–cleaned slides under a No. 1 coverglass. The coverglass was removed after freezing on dry ice. Alternatively, isolated mitochondria, prepared by the method of Mager and Lipmann (11), were fixed in 10 per cent formalin. One drop of this mitochondrial suspension was placed on a slide and spread

under a coverglass which was removed after freezing. Slides were air dried and passed through 70 per cent, 95 per cent, 100 per cent ethanol, xylene-ethanol, and xylene, each containing approximately 200 μg unlabeled thymidine/ml. Nitro-BT (Dajac Laboratories, Philadelphia) staining as described by Nachlas *et al.* (13) was used to identify mitochondria. Enzymatic digestion of slides was carried out at 37°C in a solution of 0.1 M Tris buffer (pH 7.5), 0.0025 M $MgSO_4$ containing one mg/ml DNase (Calbiochem Co., Los Angeles, EC 3.1.4.5). A control digestion was done with the Tris-$MgSO_4$ buffer without DNase. Other extractions were done with a solution of one mg/ml RNase (Calbiochem, EC 2.7.7.16) at 37°C, 5 per cent trichloroacetic acid (TCA) at 90°C, and 10 per cent perchloric acid at 4°C and 37°C. Slides were dipped in liquid nuclear track emulsion, type NTB (Kodak) and stored in light-tight desiccators at 4°C for periods of 1 to 3 weeks. Development and fixation were carried out at 18°C in Kodak D-19 (2 minutes) and Acid Fixer (8 minutes). Autoradiographs were analyzed with oil immersion phase-contrast microscopy.

RESULTS AND DISCUSSION

Initially, mitochondria were identified from Nitro-BT stained slides of whole and squashed *Tetrahymena*. The use of succinate as a substrate provided specific staining of areas with succinic dehydrogenase activity. All cytoplasmic structures in the 1 to 5 μ size range which remained after alcohol and xylene dehydration stained light blue. The particles identified by Nitro-BT staining and mitochondria prepared by the method of Mager and Lipmann (11) corresponded in size and appearance to the dark bodies identified with phase-contrast in squash preparations (Fig. 1). Further identification of mitochondria in autoradiographs was done with phase-contrast alone.

Unsquashed *Tetrahymena* cover an area of approximately 1200 μ^2 in autoradiographs. The squash technique employed spreads the cell's contents over at least 20,000 μ^2 of slide area. Good squashes showed mitochondria retaining shapes

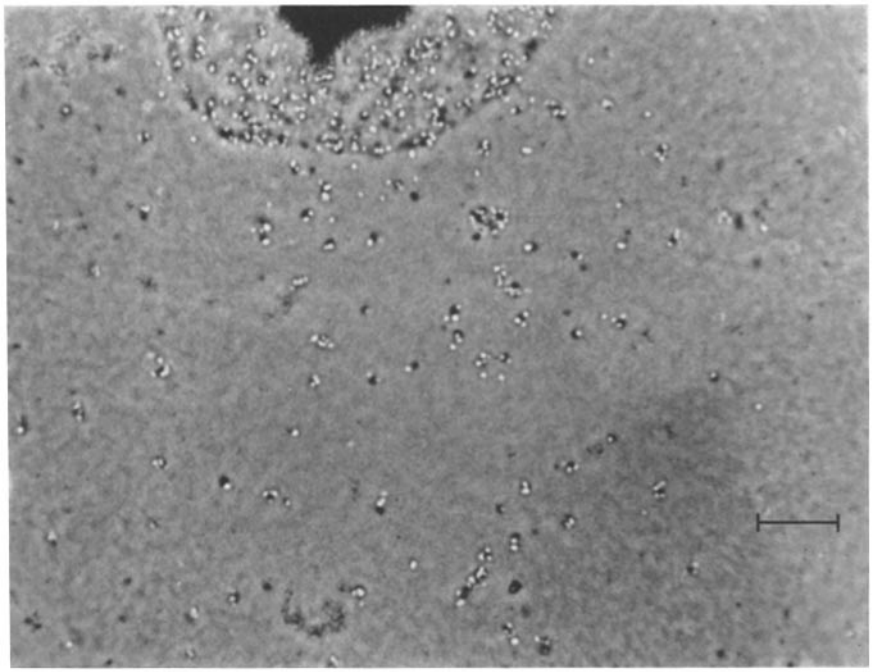


FIGURE 1 Phase-contrast photomicrograph of a squash autoradiograph. The *Tetrahymena* cell was grown in H^3-thymidine for one population doubling time. The cell was then squashed on a glass slide so that the cytoplasm spread over a wide area. The main cell body is visible at the upper edge of the figure with the nucleus covered by a black mass of autoradiograph grains. Mitochondria show below the cell as small black spots, single autoradiograph grains as smaller white spots. The scale is 10 μ in all figures.

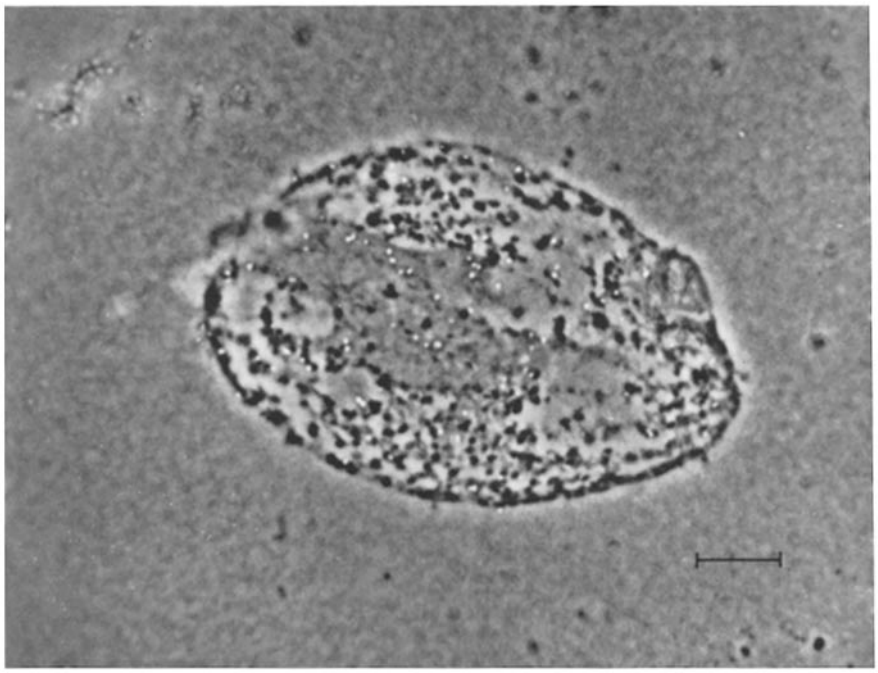
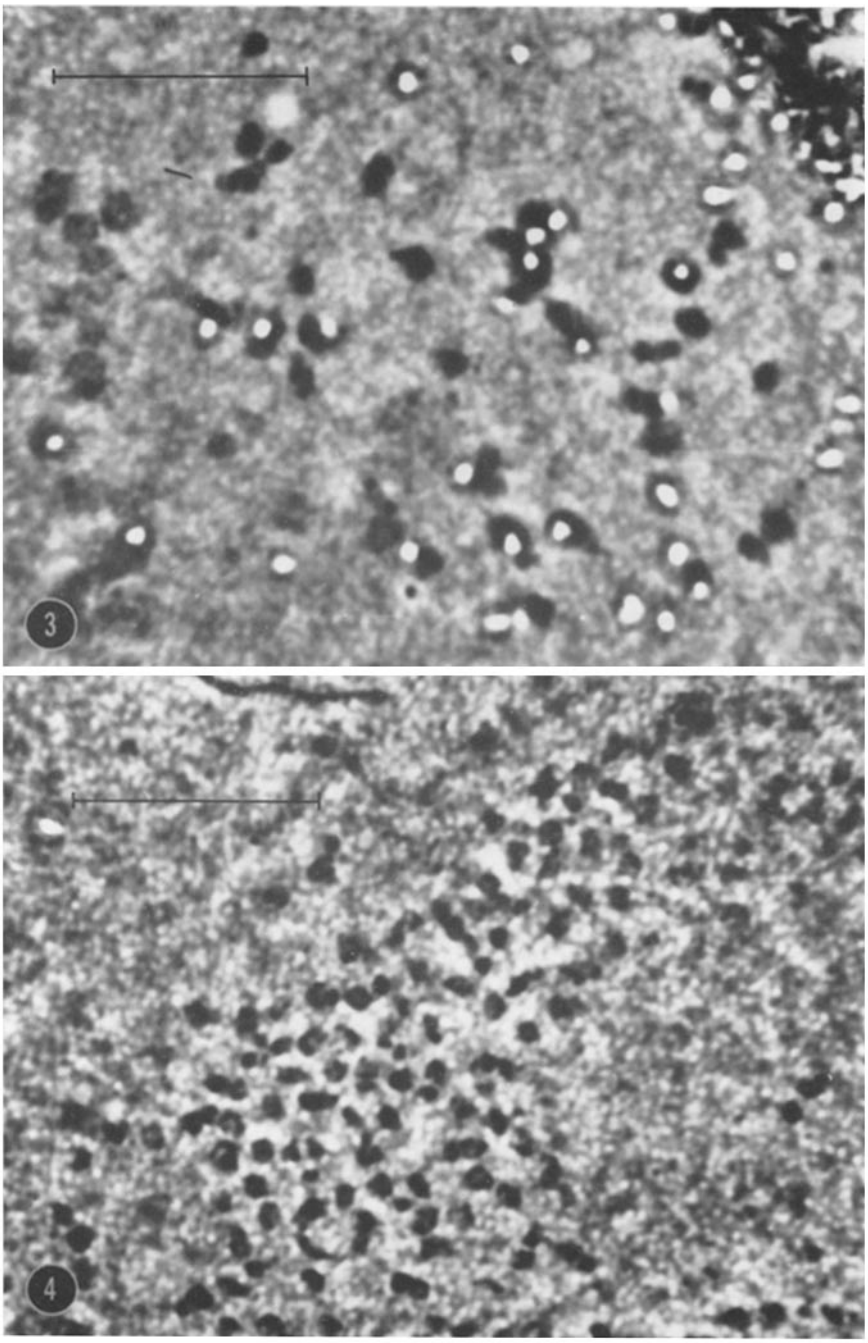


FIGURE 2 Phase-contrast photomicrograph of an autoradiograph showing a partially squashed cell. The cell was pulse labeled for 30 minutes with H^3-thymidine at a stage when nuclear DNA synthesis was not occurring. The nucleus is visible in the center of the cell. Photographic grains over the nucleus are in contact with mitochondria remaining on the nuclear surface.

characteristic of those in the living cell. Nuclei usually remained intact within the cell cuticle. The increased resolution provided by the squash technique furnished an excellent means of determining intracellular localization with light microscope autoradiography. Mitochondria in squashes as well as those isolated from sucrose homogenates showed H^3-thymidine labeling in autoradiographs.

Kidder and Dewey (9) have shown that *Tetrahymena* requires a dietary source of pyrimidines but is unable to grow on thymine as the sole pyrimidine source. If this ciliate is unable to convert thymine into RNA precursors, the thymine should be incorporated into DNA alone. Squashes incubated in RNase solution for periods up to 24 hours showed no change in labeling. Digestion of slides for 16 hours in three changes of DNase solution completely removed the mitochondrial and nuclear labels (Figs. 3 and 4). Control digestions in water and Tris-$MgSO_4$ buffer for 24 hours did not reduce labeling. Extraction with hot TCA for 1 hour and hot perchloric acid for 24 hours, both of which remove DNA as well as RNA (16), removed nearly all cellular label. Twenty-four hours of extraction in cold perchloric acid, which removes only RNA (16), had no effect. In all cases, procedures which extracted the nuclear label also removed the mitochondrial label. The results indicate that the mitochondrial label is in DNA or a DNA-like material and not in RNA or any other cellular material.

Short pulse labels of cell populations revealed many cells not undergoing nuclear DNA replication while the isotope was present in the medium (Fig. 2). All autoradiographs of cells pulse-labeled for 15 minutes showed grains on approximately 10 per cent of the mitochondrial population, even when no nuclear label was present. It is apparent that the mitochondrial label is being synthesized continuously and is not being synthesized in the nucleus. Background was less than one grain per 1000 μ^2 in areas near mitochondria as well as in control areas of the slide. The low background suggests the possibility of DNA synthesis at the mitochondrion itself. Prescott *et al.* (17) have proposed that the cytoplasmic H^3-thymidine



FIGURES 3 and 4 Phase-contrast photomicrographs of squash autoradiographs. The cells were labeled with H^3-thymidine for one population doubling time, and autoradiographs were incubated for 7 days. The nucleus can be seen at the upper right corner of Fig. 3. The slide in Fig. 4 was treated for 16 hours in three changes of DNase before being dipped in the photographic emulsion.

TABLE I

Average mitochondrial grain count ± standard deviation of H^3-thymidine–labeled variety 6, mating type III *Tetrahymena* cells during growth in unlabeled medium. Figures in parentheses are calculated from shorter autoradiograph exposure data.

	N*	Autoradiograph exposure time (*days*)				
		7	14	28	(56)	(112)
Zero time	11	1.135 ± 0.107				
Division 1	11		1.045 ± 0.077			
Division 2	11			1.051 ± 0.068		
Division 3	8			0.544 ± 0.092	(1.088)	
Division 4	5			0.284 ± 0.066		(1.136)

* Number of cells counted, one hundred mitochondria per cell.

incorporation in *Amoeba* might result from a cytoplasmic DNA polymerase acting with primer DNA molecules ingested with food. Association of the label with a cytoplasmic organelle and the growth of *Tetrahymena* on a defined medium devoid of DNA rules out this possibility.

The classic work of Ephrussi (5) demonstrated that the yeast "petite" mutant results from inheritable factors located in the cytoplasm. Evidence suggests that the genetic factors are associated with mitochondria themselves. Luck (10), using H^3-choline–labeled *Neurospora*, has shown that autoradiograph grains of isolated mitochondria follow a Poisson distribution over three mass doublings. Similarly, short pulse labels revealed a random distribution of label among all mitochondria. Luck's data provide evidence for the mitochondrial division theory.

Since the mitochondrial label in the present experiments is not of nuclear origin and not washed out as a low molecular weight component, slides were prepared for a statistical analysis of the label distribution over several cell cycles. Analysis of these slides showed that the average mitochondrial grain count remained constant over four generations when autoradiograph exposure times were doubled for each cell doubling. The results indicate that the mitochondrial label is stable over four generations (Table I). Furthermore, this label remained randomly distributed throughout the mitochondrial population as would be predicted by the mitochondrial duplication theory (15). It is possible that the present experiments have detected a genetic DNA in this organelle. However, it is also possible that *Tetrahymena* mitochondria represent an evolutionary transition from kinetoplasts. Further studies on the presence of DNA in higher animal and plant mitochondria will be required before we can ascertain its functional significance.

SUMMARY

1. Phase-contrast autoradiography has revealed continuous incorporation of H^3-thymidine by the mitochondria of *Tetrahymena pyriformis*.

2. Enzymatic digestion and acid extraction indicate that the H^3-thymidine is in the form of DNA or a DNA-like material.

3. Pulse labeling indicates that mitochondrial incorporation of H^3-thymidine is occurring at periods in the cell growth cycle when nuclei are not synthesizing DNA.

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Note added in proof: Since this manuscript was submitted for publication, Luck and Reich have reported the isolation of a mitochondrial DNA from *Neurospora* which had a different buoyant density from the nuclear DNA. LUCK, D. J. L., and REICH, E., *Proc. Nat. Acad. Sc.*, 1963, **52,** 931.

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