H^3-THYMIDINE DERIVATIVE POOLS IN RELATION TO MACRONUCLEAR DNA SYNTHESIS IN *TETRAHYMENA PYRIFORMIS*

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ABSTRACT

The formation of a soluble H^3-thymidine derivative pool has been examined in *Tetrahymena pyriformis* as a function of macronuclear DNA synthesis during the cell life cycle. An autoradiographic technique which allows the detection of water-soluble materials within a cell has shown that these cells do not take up and retain exogenous H^3-thymidine during G1 or G2. Uptake of H^3-thymidine is restricted to the S period of the cell cycle. Additional autoradiographic experiments show, however, that a soluble pool of H^3-thymidine derivatives persists from the end of one DNA synthesis period to the beginning of the next synthesis period in the subsequent cell cycle. Since this persisting pool cannot be labeled with H^3-thymidine, the pool does not turn over during non-S periods.

INTRODUCTION

Previous experiments with single *Tetrahymena* have shown that macronuclear DNA synthesis can be initiated in the absence of essential amino acids but that the DNA increase is limited to 20 per cent of the G1 value (10). For this 20 per cent increase, H^3-thymidine of the medium is not utilized. If amino acid deprivation is imposed after DNA synthesis has begun, synthesis goes to completion (100 per cent increase) with no apparent impairment of incorporation of H^3-thymidine of the medium into DNA. The experiments have been interpreted in the following way. Thymidine kinase and thymidylate synthetase (and possibly other enzymes concerned directly with production of deoxyribonucleoside triphosphate pools) are normally synthesized at the beginning of DNA synthesis and disappear when DNA synthesis is completed. In the absence of essential amino acids, thymidine kinase and thymidylate synthetase cannot be produced, and DNA synthesis is limited by the size of the preexisting deoxyribonucleotide pools carried over from the previous round of DNA synthesis in the preceding cell cycle.

Although these results and interpretations are in agreement with several reports (1, 5, 11) describing the cyclic appearance of thymidine kinase in relation to DNA synthesis in the cell cycle, the interpretation would be considerably enhanced by direct demonstration of a continuing pool of thymidine derivatives and the lack of both pool formation and turnover during the G1 and G2 periods. Consequently, we have investigated the soluble intracellular pool of H^3-thymidine and its derivatives over the cell life cycle in *Tetrahymena* utilizing two different methods: (*a*) an autoradiographic procedure for detecting water-soluble

cellular materials, and (*b*) autoradiography of the dilution of labeled DNA during subsequent cell cycles.

METHODS

Sterile stock cultures of *Tetrahymena pyriformis* HSM were maintained in a synthetic medium (3) plus 0.04 per cent proteose peptone. Groups of cells (25 to 100 dividing pairs) were synchronized with respect to division by selecting dividing cells with a braking pipette from an early logarithmic culture, and were incubated in capillary culture tubes at 29°C (9). Under these conditions the average generation time is 225 minutes and macronuclear DNA synthesis occupies a period extending from between 60 and 75 minutes to between 135 and 150 minutes after division. For more detail of the cell cycle under these conditions, see reference 10.

Soluble Material Autoradiography

To test whether H^3-thymidine is taken up into a soluble intracellular pool which is retained within the living cell, the following experiments were performed. Synchronized groups of cells were incubated at room temperature for 15 to 60 minutes in 10 μc/ml H^3-thymidine (New England Nuclear Corp., sp. act. 6.6 c/mmole) in the growth medium at various times in the cell cycle. After incubation with H^3-thymidine, cells were transferred with a braking pipette through non-radioactive medium to remove exogenous H^3-thymidine. The final dilution factor was always at least 10,000-fold. As a further control on the efficiency of the washing procedure, fresh cells (including cells in S) were cultured in the last wash medium. Autoradiographs showed that the control cells did not incorporate any radioactivity. The experimental cells were then air dried on slides by placing a single cell in a small drop of medium and drawing off the excess medium as rapidly as possible. The actual drying of the cell took approximately 1 second. The washing and drying procedures together required from 10 to 15 minutes after the cells were removed from the labeled medium.

Although details of the autoradiographic technique for detection and location of intracellular water-soluble materials have been described elsewhere (8), a brief description is given here. A thin film of emulsion was obtained by dipping a nickel-chrome wire loop (3.5 to 4 cm in diameter) into NTB3 liquid emulsion at 30°C. The film was allowed to air dry for an hour and then placed over the cells on the slide. Next, warm, moist air (breath) was blown at the slide, causing the film to collapse against and to adhere to the slide and specimen. In a test of the procedure, labeled nucleosides which were dried down on a slide in a circumscribed area and covered with a dry film showed no detectable diffusion or displacement.

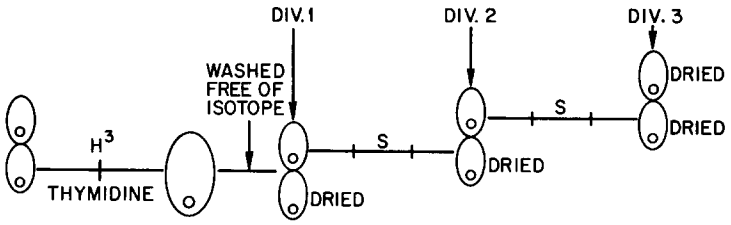


FIGURE 1 Illustration of experimental design for the dilution of isotope through two cell cycles after labeling with H^3-thymidine. In some cases both daughter cells of the second division were killed; in others, one was killed and the other allowed to go to the third division.

To demonstrate soluble radioactive materials within a cell, a film of emulsion diluted 1:1 with H_2O was prepared as described and allowed to gel but not dry. This film contained enough moisture to cause limited diffusion of water-soluble components out of the cell and into the surrounding emulsion. Exposure times were from 1 to 3 weeks.

Dilution of Incorporated H^3-Thymidine

To determine whether a soluble thymidine derivative pool which can be used in DNA synthesis persists within this cell from one S period to the next, the following experiment was performed (Fig. 1). Individual cells were equally labeled by incubating in H^3-thymidine medium (29°C) from 60 minutes after division (the beginning of S) until the appearance of a cleavage furrow (the end of G2). The dividing cell was then removed from the medium containing isotope and throughly washed through several changes of fresh medium using a braking pipette. Upon separation of the two daughter cells, one was dried on a slide and its sister allowed to go through another cell cycle, which included another DNA synthesis period. At the second division, both daughter cells were dried on a slide, or one daughter cell

was dried and its sister allowed to go through yet another cell cycle and the resulting daughter cells were dried on a slide. All cells of one line were dried on the same slide, extracted for 1 to 2 hours with 3:1 alcohol–acetic acid, then covered with liquid emulsion for autoradiography.

RESULTS

Soluble Material Autoradiography

Cells which were incubated with H^3-thymidine before (between 5 minutes and 60 minutes after division) or after (between 150 minutes after division and the next division) macronuclear DNA synthesis (S), washed thoroughly, dried rapidly on a slide, and then covered with dry emulsion film exhibited no radioactivity above background over the nucleus or cytoplasm (Figs. 2 and 3). These results indicate that during G1 (before DNA synthesis) and G2 (after DNA synthesis) either the cells are not able to take up H^3-thymidine of if they do take it up it can be washed out of the cell with non-radioactive medium. Cells incubated during macronuclear DNA synthesis (75 to 90 minutes after division) and then covered with a dry film showed heavy labeling over the macronucleus (Fig. 4). Cells which were similarly incubated with H^3-thymidine and then thoroughly extracted with a large volume of acetic acid–aclohol before applying dry emulsion are shown in Fig. 5. Both extracted and non-extracted cells show radioactivity only in the macronucleus. To answer the question whether all the radioactivity in the macronucleus in unextracted cells was incorporated in a water- or acid-insoluble state, cells were incubated with H^3-thymidine during S, washed thoroughly, dried rapidly, and covered with the wet emulsion film, which allows limited diffusion of soluble radioactivity out of the cell. An example of the resulting autoradiographs is shown in Fig. 6. It can be seen that, although there still is a heavy concentration of grains over the macronucleus, there is also a gradient of silver grains extending from the macronucleus over the cytoplasm and around the cell.

To test whether a soluble pool continues after the S period, cells were incubated with H^3-thymidine for 60 minutes toward the end of the cell cycle (from about 120 minutes after division to 180 minutes). They were then washed thoroughly and allowed to continue to division. The dividing cells were air dried on slides and covered with gelled, wet emulsion. Fig. 7 shows that these cells still contained detectable soluble radioactivity at a time when the cells were not synthesizing macronuclear DNA.

Because the cells used in the autoradiography of the soluble material were not fixed, the radioactive material diffusing into the emulsion might represent degradation products resulting from endogenous DNase. To test this possibility, a logarithmically growing culture of cells was incubated with H^3-thymidine for 60 minutes, then washed thoroughly by centrifugation, and samples were withdrawn at 2, 4, 3, 6, and 8 hours from the end of labeling time. During this sampling time, cells which were in S during the incubation period divided (generation time 225 minutes, S about 75 to 90 minutes) and went through another S period in non-radioactive medium. After application of the wet, gelled emulsion, the number of labeled cells showing pools decreased with increasing sample time. None of the labeled cells in the last sampling times exhibited pools. If the halos are produced by DNase activity, all labeled cells should have exhibited "pools" regardless of the time of sampling. The absence of radioactive halos in the last samples indicates that the radioactive pools demonstrated by this procedure consist of soluble components present in the cell at the time of drying and are not the result of DNase degradation of insoluble DNA at the time of film application.

Dilution of Incorporated H^3-Thymidine

The DNA dilution experiment (Fig. 1) was designed to determine whether a thymidine derivative pool which is used in DNA synthesis persists between succeeding S periods. If no continuing soluble pool of thymidine derivatives exists from one synthetic period to another, grain counts after acid-alcohols extraction should be reduced by 50 per cent at each subsequent division. If a continuing, soluble pool does exist, the daughter cells of the second division would be expected to show less than a 50 per cent decrease in grain count. In the latter case, the products of a third division would be expected to halve the grains of the second division if the thymidine derivative pool completely turns over within a single S period, but should show less than a 50 per cent decrease if the pool takes more than one S period to turn over.

The results of the labeled DNA dilution experiment (outlined in Fig. 1) are given in Tables I and II, which summarize two different experiments. Table I gives the number of silver grains over the macronucleus of the first and second division daughter cells for 14 clones. Student's t test was

applied to determine whether the total number of grains over the two second division daughter cells was significantly different from the total number of grains over the first division daughter cell. This assumes that the daughter cells of the first division distributed the radioactivity equally between the two and does not allow for any variation between the two sister cells. However, the cells were randomly selected to cancel any prejudice for any given cell. The results of the test indicate that the total number of grains in the two second division daughter cells is significantly higher than in the first division daughter cell. In Table II the same analysis is carried out on 9 cell lines that were allowed to reach the third division after labeling. In this test, the grain value for a single daughter cell which was dried at the second division was doubled and this value compared with that for the first division daughter cell. Again, Student's t test indicates significantly more total grains in the second division products than are present in the first division cell. However, when the same analysis is carried out on the third division products as compared with the second division daughter cell, the total number of grains is not significantly different.

DISCUSSION

The role of DNA precursors in controlling DNA synthesis has recently been reviewed by Lark (6). One general conclusion drawn is that synthesis of precursors for DNA is temporally associated with DNA synthesis. In our study on *Tetrahymena*, the observation that H^3-thymidine enters a soluble pool only near the time of initiation of or during DNA synthesis is consistent with this conclusion and with evidence that thymidine kinase is synthesized at the onset of DNA synthesis in other cell systems (1, 5, 11). This is also in agreement with another study on *Tetrahymena* (7) in which cells were exposed to H^3-thymidine in G1, washed thoroughly, and allowed to continue to division. Autoradiographs of such cells showed no incorporation of the isotope into DNA during macronuclear S, indicating that during G1 the thymidine did not enter a cellular pool which could be reretained and utilized by the cell in DNA synthesis.

One question posed by these observations is whether the formation of a precursor pool plays a role in initiating DNA synthesis or is a consequence of DNA synthesis. Earlier work (10) on *Tetrahymena* cells deprived of essential amino acids in G1 showed that there is an increase in DNA (microspectrophotometry) although the cells do not utilize exogenously supplied H^3-thymidine. This has been interpreted to mean that DNA synthesis is initiated at the expense of a preexisting pool and that amino acid deprivation interferes with the synthesis of new thymidine kinase and thymidylate synthetase and therefore prevents continuation of DNA synthesis. The results contained in the present report show more directly that a soluble pool of H^3-thymidine derivatives is present from the

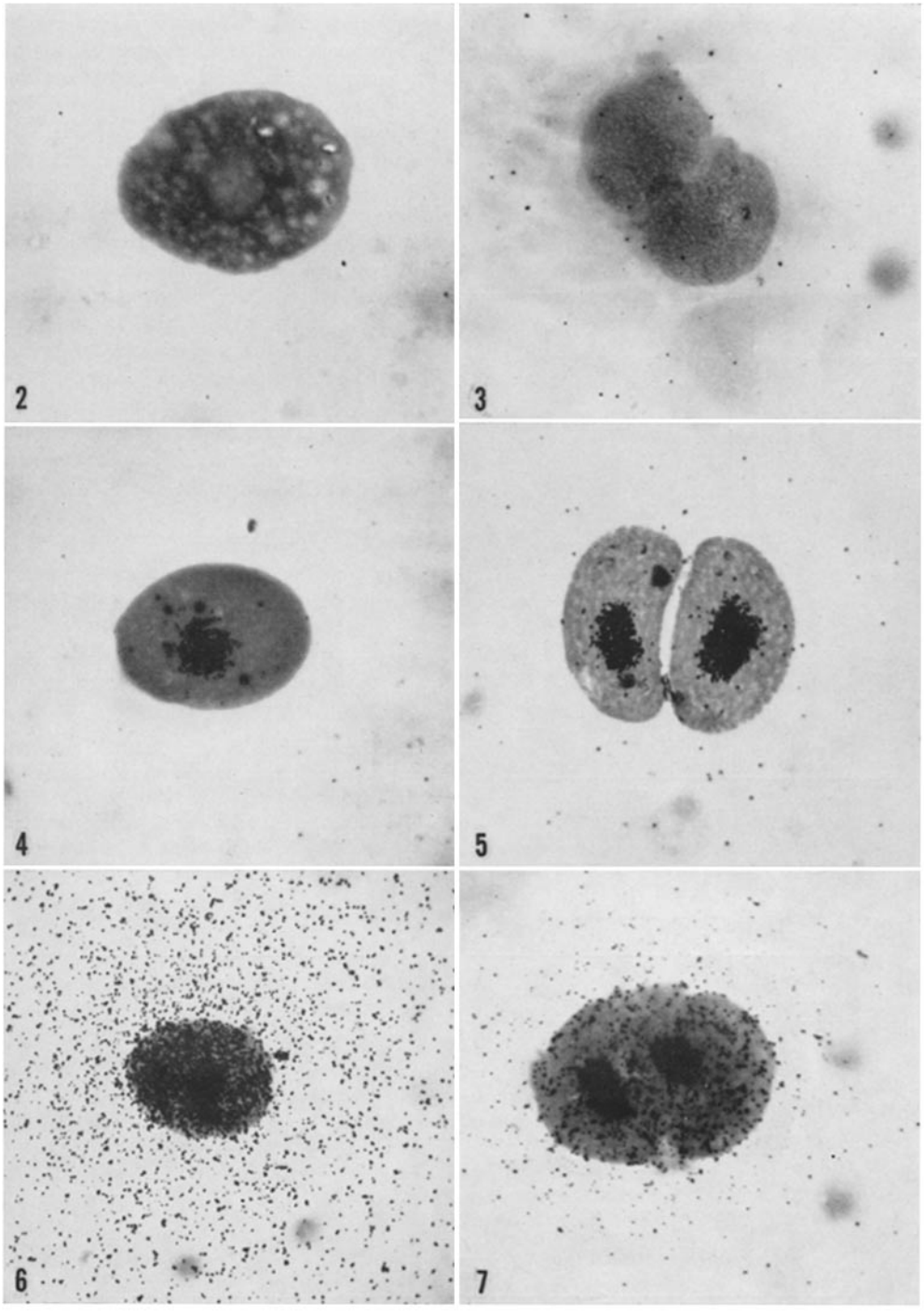
FIGURES 2 AND 3 Cells exposed to H^3-thymidine for 15 minutes in G1 (Fig. 2) and G2 (Fig. 3), washed thoroughly in cold medium, dried, and covered with dry emulsion. The G2 cell had started cytokinesis by the time of drying. The absence of label in both instances indicates that thymidine does not enter a soluble thymidine derivative pool during these stages of the cell cycle. Fig. 2, × 900; Fig. 3, × 800.

FIGURE 4 A cell incubated with H^3-thymidine during S, washed, dried on a slide, and covered with dry emulsion film. Essentially all the silver grains are located over the macronucleus. × 750.

FIGURE 5 Cells treated as in Fig. 4 except that they were extracted with 3:1 alcohol–acetic acid before being covered with dry emulsion. × 900.

FIGURE 6 A cell treated as in Fig. 4 except that wet emulsion film rather than dry film was applied. A comparison with Fig. 4 indicates that a soluble component was extracted from the macronucleus by the wet film. × 600.

FIGURE 7 A cell pulse-labeled during S, washed, and allowed to go to division before being dried and covered with wet emulsion film. The halo of grains indicates that a soluble component has remained in the cell through G2. × 800.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end of one DNA synthetic period to the next but does not turn over during G2 or G1. Our observations also indicate that this persisting pool is utilized in DNA synthesis during the next S period and is turned over completely during one S period or less.

Accordingly, the sequence of events in *Tetrahymena* is probably the following: (*a*) DNA synthesis is initiated and continues for a short time at the expense of a pool formed during the preceding S period, and (*b*) the turnover of the pool stimulates the synthesis of additional phosphorylated precursors, thus producing the uptake of exogenous thymidine into the intracellular pool. According to this interpretation, the immediate precursor pool would have no influence on the initiation of DNA synthesis, but rather DNA synthesis would be the stimulus for the turnover of the thymidine derivative pool.

The evidence that *Tetrahymena* utilizes a preexisting pool of thymidine derivatives for some DNA synthesis in each cell cycle raises the possibility that S may actually begin at an earlier point in the cell cycle than is indicated by the uptake of H^3-thymidine. If the appearance of thymidine kinase depends upon the initiation of DNA synthesis with consequent depletion of the preexisting pool, it is possible that DNA synthesis may be in progress for some minutes before the incorporation of H^3-thymidine (from the medium) into DNA can be detected.

By using a dry emulsion film, we have also been able to show that the soluble thymidine derivative pool exists exclusively in the nucleus. This is also in agreement with other studies which indicate that thymidine nucleotides are localized mainly in the nucleus (2, 4).

TABLE I

Comparison between the Number of Silver Grains over the Nucleus in First and Second Division Cells

Division 1: 1 cell (T_1)	Division 2: 2 sister cells	Sum div. 2 (T_2)	d ($T_2 - T_1$)
102	47, 57	104	+2
23	11, 11	22	−1
62	32, 30	62	0
36	24, 22	46	+10
28	19, 25	44	+16
28	16, 21	37	+9
48	30, 32	62	+14
40	24, 21	45	+5
18	15, 18	33	+15
54	38, 25	63	+9
70	33, 29	62	−8
23	12, 10	22	−1
17	11, 14	25	+8
21	13, 12	25	+4
			+82

$\bar{d} = 5.85$ $S\bar{d} = 1.86$
$t = 3.136$ $P = 0.01$–0.001

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TABLE II

Comparison between the Number of Silver Grains over the Nucleus in First, Second, and Third Division Cells

Division 1: 1 cell (T_1)	Division 2: 1 cell	2 × div. 2 (T_2)	d_1 ($T_2 - T_1$)	Division 3: 2 sister cells	Sum div. 3 (T_3)	d_2 ($T_3 - \frac{1}{2} T_2$)
39	31	62	+23	11, 13	24	−7
44	34	68	+24	18, 14	32	−2
58	27	54	−4	12, 23	35	+8
27	23	46	+19	12, 13	25	+2
51	31	62	+11	14, 25	39	+8
46	30	60	+14	8, 12	20	−10
30	15	30	0	4, 8	12	−3
30	27	54	+24	13, 16	29	+2
81	61	122	+41	27, 29	56	−5
			+160			−7

$\bar{d}_1 = 17.77$ $S\bar{d}_1 = 4.109$ $\bar{d}_2 = 0.777$ $S\bar{d}_2 = 2.09$
$t = 3.7136$ $P = 0.01$–0.001 $t = 0.3703$ $P = 0.80$–0.70

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REFERENCES

1. BOLLUM, F. J., and POTTER, V. R., Nucleic acid metabolism in regenerating rat liver. VI. Soluble enzymes which convert thymidine to thymidine phosphates and DNA, *Cancer Research*, 1959, **19**, 561.
2. BRANTON, D., and JACOBSON, L., Dry, high resolution autoradiography, *Stain Technol.*, 1962, **37**, 239.
3. ELLIOTT, A. M., BRONWELL, L. E., and GROSS, J. A., The use of *Tetrahymena* to evaluate the effects of gamma radiation on essential nutrilites, *J. Protozool.*, 1954, **1**, 193.
4. FEINENDEGEN, L. E., and BOND, V. P., Differential uptake of H^3-thymidine into the soluble fraction of single bone marrow cells determined by autoradiography, *Exp. Cell Research*, 1962, **27**, 474.
5. HOTTA, Y., and STERN, H., Molecular facets of mitotic regulation. I. Synthesis of thymidine kinase, *Proc. Nat. Acad. Sc.*, 1963, **49**, 648.
6. LARK, K. G., Cellular control of DNA biosynthesis, *in* Molecular Genetics, (J. H. Taylor, editor), New York, Academic Press, Inc., 1963, 153.
7. MCDONALD, B. B., Synthesis of deoxyribonucleic acid by micro- and macronuclei of *Tetrahymena pyriformis*, *J. Cell Biol.*, 1962, **13**, 193.
8. MILLER, O. L., JR., STONE, G. E., and PRESCOTT, D. M., Autoradiography of water soluble materials, *in* Methods in Cell Physiology, (D. M. Prescott, editor), New York, Academic Press, Inc., 1964, 371.
9. STONE, G. E., and CAMERON, I. L., Methods for using *Tetrahymena* in studies of the normal cell cycle, *in* Methods in Cell Physiology, (D. M. Prescott, editor), New York, Academic Press, Inc., 1964, 127.
10. STONE, G. E., and PRESCOTT, D. M., Cell division and DNA synthesis in *Tetrahymena pyriformis* deprived of essential amino acids, *J. Cell Biol.*, 1964, **21**, 275.
11. WEISSMAN, S. M., SMELLIE, R. M. S., and PAUL, J., Studies on the biosynthesis of deoxyribonucleic acid by extracts of mammalian cells. IV. The phosphorylation of thymidine, *Biochim. et Biophysica Acta*, 1960, **45**, 101.