MACROMOLECULAR EVENTS LEADING TO CELL DIVISION IN *TETRAHYMENA PYRIFORMIS* AFTER REMOVAL AND REPLACEMENT OF REQUIRED PYRIMIDINES

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ABSTRACT

Tetrahymena pyriformis were brought to a non-growing state by removal of pyrimidines from their growth medium. During pyrimidine deprivation cell number increased 3- to 4-fold, and this increase was accompanied by one or more complete cycles of macronuclear DNA replication. Autoradiographic studies show that endogenous protein and RNA were turning over throughout starvation and that RNA breakdown products were used to support the DNA synthesis that occurred during the early period of starvation. However, after 72 hours of starvation all DNA synthesis and cell division had ceased. Feulgen microspectrophotometry shows the macronuclei of these cells to have been stopped at a point prior to DNA replication (G1 stage). After pyrimidine replacement the incorporation of H^3-uridine, H^3-adenosine, and H^3-leucine was measured by the autoradiographic grain counting method. The results indicate that RNA synthesis began to increase almost immediately, but that there was a lag of almost an hour before an increase in protein synthesis. In agreement with the autoradiographic data, chemical data also show that cellular content of RNA began to increase shortly after pyrimidine replacement but that cellular protein content did not increase until about one hour later. Pulse labeling of the cells with H^3-thymidine at intervals after pyrimidine replacement shows that labeled macronuclei first began to appear at 150 minutes; that 98 per cent of the macronuclei were in DNA synthesis at 240 to 270 minutes; and that the percentage then began to decrease from 300 to 390 minutes, at which time only 25 per cent of the macronuclei were labeled. Cellular content of DNA did not increase for at least 135 minutes after pyrimidine replacement; however, just before the first cells divided (360 minutes) the DNA content had doubled. After pyrimidine replacement the cells first began to divide at 360 minutes, and 50 per cent had divided at 420 minutes; however, all cells had not divided until 573 minutes. This technique of chemical synchronization of cells in mass cultures makes feasible detailed biochemical analysis of events leading to nuclear DNA replication and cell division.

INTRODUCTION

A cell prepares for division by completing several chains of metabolic and structural events. It appears that these chains of events can operate simultaneously and more or less independently of one another. The replication of nuclear DNA can be cited as an integral segment of one such chain of preparative events. It should be noted that although replication of DNA is generally considered

an essential prerequisite for cell reproduction, it is not necessarily an essential process for cell survival or cell function.

Auxotrophic organisms or cells which can be grown on a complex but defined medium permit the manipulation of the cells' known biochemical pathways which may be rather specifically involved in cell reproduction. This manipulation is accomplished by withdrawal of a known nutrient precursor from the growth medium. Elliott and others have worked out the minimal complete synthetic medium that will support growth and cell division in the ciliated protozoan *Tetrahymena pyriformis* (5). This organism has an absolute requirement for pyrimidines in its growth medium (6). Because pyrimidines are an integral part of the biochemical pathways which lead to RNA and DNA synthesis, they offer sites for metabolic manipulation. Studies by Lederberg and Mazia (7) have already demonstrated that pyrimidine starvation of *T. pyriformis* leads to a rapid decrease of RNA per cell, while cellular protein remains constant during a 3-fold increase in cell number before it too decreases and the cells no longer divide. Though no longer dividing, the cells are able to maintain their mobility and viability for several weeks (personal observation). Lederberg and Mazia's work also suggested that pyrimidine replacement leads to the accumulation of cellular RNA content at a faster rate than protein content, and that further analysis of this period should prove of special interest.

Since pyrimidines are essential for DNA replication in *T. pyriformis* and since this replication process is a normal prerequisite to cell division, one might expect that a period of pyrimidine starvation would bring all cells in a population to the same stage in the cell cycle, perhaps to a point somewhere before DNA replication. The replacement of pyrimidines might therefore initiate one or several rather specific biochemical pathways which would lead to a synchronous chain of events, including DNA replication, and would eventually culminate in cell division. This study deals with changes in the metabolism of RNA, DNA, and protein during pyrimidine starvation of *T. pyriformis*, but is especially concerned with the temporal pattern of biochemical events that lead to cell growth and cell division after replacement of the required pyrimidines.

The pattern of events which is found by this method of cell synchronization may differ from that disclosed by other methods, but perhaps it is only through analysis of these different approaches that we may come to know how a cell controls its growth and reproduction.

Briefly, the results reported here show that pyrimidine deprivation stalls all the cells at a point prior to macronuclear DNA replication (G1 stage). Replacement of the required pyrimidine causes synchrony in the biochemical response of the cells. RNA synthesis increases almost immediately and is followed in about an hour by an increased protein synthesis, which then leads to the initiation of macronuclear DNA synthesis in about 2.5 hours and the eventual cell division after about 6 hours. The early biochemical events are very well synchronized, but the synchrony is quickly dissipated as the cells reach and finish their first cell division.

MATERIALS AND METHODS

Tetrahymena pyriformis HSM, a micronucleated strain, was maintained at 29°C throughout the study. The cells were cultured axenically in synthetic medium (5) enriched with 0.04 per cent (w/v) proteose peptone and with 0.05 per cent (v/v) Tween 80 and adjusted to pH 7.2–7.4 with NaOH. Although it would be desirable to maintain the cells in medium without the 0.04 per cent proteose peptone, this supplement assures a reproducible, faster, and less variable multiplication rate.

Logarithmically growing cells were deprived of pyrimidines by washing them in medium deprived of pyrimidine and proteose peptone. The cells were concentrated to 0.05 ml of medium by gentle centrifugation at 600 RPM for 2 minutes. Centrifugation was carried out in 10-ml graduated reduced capillary tip (Hopkins Vaccine Centrifuge) tubes. Excess medium and cells were aspirated off and the cells were resuspended in pyrimidineless medium by gentle mixing with a culture pipette (12, 15). The cells were washed three times in this manner, giving a dilution of at least 10,000-fold. The 0.05 ml of concentrated cells was then divided equally into two 150-ml Erlenmeyer flasks, each with 10 ml of pyrimidineless medium. At 40 hours the cultures were diluted with one volume of fresh pyrimidineless medium.

Throughout the experiment, cell multiplication was determined by growing samples of the cells in capillary culture pipettes according to Prescott (12, also see 15). At 72 hours of pyrimidine starvation an amount of sterile pyrimidines and proteose peptone equal to the original concentration in the medium was added to the starved cells.

Autoradiographic Studies

To determine RNA and protein synthesis, cells were incubated for 15 minutes in medium containing 15 μc/ml H^3-leucine (specific activity 5.0 c/mmole), with 10 μc/ml H^3-uridine (specific activity 3.73 c/mmole), or with 20 μc/ml H^3-adenosine (specific activity 1.22 c/mmole) (all from New England Nuclear Corp.). To detect DNA synthesis, 15 μc/ml H^3-thymidine (specific activity 6.70 c/mmole (New England Nuclear Corp.) was used. All cells were air dried on slides (15). Cells were fixed for 30 minutes in 3:1 ethanol:acetic acid, given three 30-minute washings in 70 per cent alcohol, and air dried.

DNA digestions were carried out with a 0.1 per cent DNase solution (Worthington) made up in a 0.003 M $NaHPO_4$, 0.007 M KH_2PO_4, and 0.005 M $MgSO_4$ (pH 6.8) at 37°C for 2 hours. RNA digestions were carried out with a 0.05 per cent RNase solution (Sigma) 5 times recrystallized at pH 6.8 at 37°C for 1 hour. Autoradiography was done with NTB3 liquid emulsion (Kodak) as outlined by Prescott (13). Slides were stained through the emulsion with toluidine blue after autoradiographic development.

The H^3-thymidine autoradiographs were exposed for an extended time, as determined by test slides, so that subjective scoring of labeled or unlabeled nuclei was at a minimum.

The quantitation of H^3-leucine, H^3-uridine, and H^3-adenosine incorporation was carried out by counting the number of autoradiographic grains over a unit area of nucleus or cytoplasm measured with the aid of a Whipple ocular disk. The background grain count based on the same area was subtracted from the actual grain count. The number of grains per unit area is proportional to the rate of incorporation per unit mass when pulse labeling is used.

The total Feulgen-positive material was determined microspectrophotometrically with a Canalco microspectrophotometer using the two-wavelength method of Patau (11).

Biochemical Analysis

To make the biochemical runs, 2.5-liter low form culture flasks were inoculated with 500 ml of pyrimidine-deficient medium plus 5×10^6 cells which had just been washed three times by centrifugation in pyrimidine-lacking growth medium. At 40 hours 500 ml of fresh pyrimidine-deficient medium was added to each flask. At 72 hours pyrimidines were added to the deficient cultures, as described above. Throughout these runs cell counts were taken by an electronic counter (Coulter Co.).

The total protein content of the cells was measured by the spectrophotometric method of Lowry *et al.* (8), using known concentrations of bovine serum albumin as the standard. Determinations were made on duplicate samples which contained about 3.4 million saline-washed cells per sample.

Estimation of the RNA and DNA content of the cells was carried out as described by Blum and Padilla (1). The procedure is outlined as follows. Duplicate 40-ml samples of cells at a concentration of about 87,000 per ml were washed with saline by centrifugation. The cells were concentrated and extracted at room temperature with 4 ml of 95 per cent ethanol, 5 ml of 50:50 (v/v) ether–absolute ethanol, and 4 ml of absolute ethanol, in this order. The concentrated cells were extracted twice for 15 minutes with 2 ml of 0.2 N $HClO_4$ at 0°C and then hydrolyzed for 16 hours at 30°C with 4 ml of 1 N NaOH in Parafilm-covered centrifuge tubes. The hydrolyzed samples were then neutralized with 4 ml of 1 N HCl, chilled at 0–3°C, and 4 ml of cold 1 N $HClO_4$ added to each tube. The tubes were then centrifuged at 800 *g* in the cold for 5 minutes. The supernatant was saved and the pellet washed with 2 ml of cold 1 N $HClO_4$ with the aid of a Vortex Jr. stirrer, the mixture was again centrifuged, and the supernatants were combined. The combined supernatants were measured at 260 mμ and at 315 mμ in a Zeiss PMQ-II spectrophotometer. The difference in absorbance at the two wavelengths was used to compute the RNA content, using a conversion factor derived from De Deken-Grenson and De Deken (4). The remaining pellet was extracted with 2.0 and 4.0 ml of 1 N $HClO_4$ at 90°C for 10 and 5 minutes, respectively. The supernatants were combined and clarified by centrifugation at 8000 *g* for 10 minutes. The absorbance at 267 mμ and 315 mμ was then measured. The content of DNA was computed from the difference in absorbance at these two wavelengths. A conversion factor derived from De Deken-Grenson and De Deken (4) was used to determine the amount of DNA. All values were converted to μg RNA or DNA per 10^6 cells.

RESULTS

Cell Division in Pyrimidine-Starved and Refed Cells

Two groups of 15 pairs of dividing cells were selected with a braking pipette from a logarithmically growing culture and washed four times in either complete or pyrimidineless medium (5000- to 10,000-fold dilution) using depression slides. The cells were then injected into capillary culture pipettes and periodically counted. The cells in the complete medium divided for the first time between 170 and 275 minutes with a mean genera-

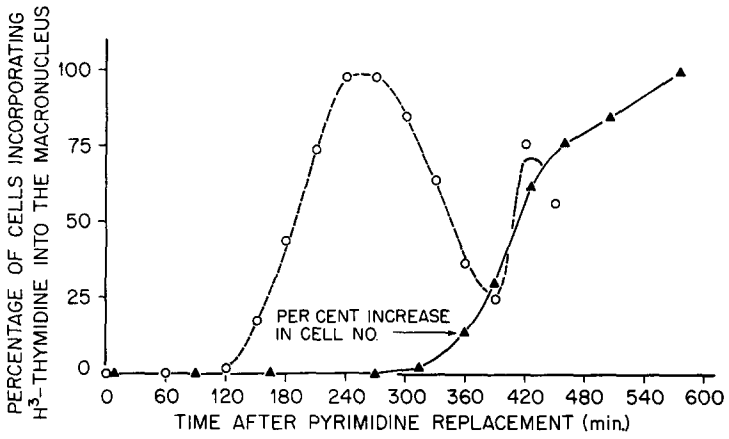


FIGURE 1 Percentage of cells incorporating H^3-thymidine (open circles) when given a 15-minute pulse of the radioisotope, plotted against time after replacement of pyrimidines to cells deprived of pyrimidines for 72 hours. At least 100 cells were analyzed for each point. Solid triangles show the percentage increase in cell number after the "shift-up" to complete medium.

tion time of 225 minutes; the second division was complete by 570 minutes. Half of the cells in the pyrimidineless medium had divided by 300 minutes, but the second division was not complete until 46 hours, and no further cell division took place. After 72 hours of starvation, the tips of the culture pipettes were broken off, and the pyrimidine-starved cells were put into complete medium. In the complete medium these cells divided again between 360 and 495 minutes, with a mean of 410 minutes.

When mass cultures of cells were pyrimidine-starved for 72 hours and then returned to a complete medium, it was 360 minutes before the first cells began to divide. At 420 minutes, 50 per cent had divided; however, all cells had not divided until 573 minutes (Fig. 1). There was a 15 to 18 per cent division index at the peak of division activity. Cell mobility was not noticeably affected by starvation or refeeding.

DNA Synthesis in Pyrimidine-Starved and Refed Cells

Fig. 1 shows that there is a 150-minute lag between the "shift-up" (defined as a change to an environment capable of supporting a faster growth rate) to complete medium and the first appearance of H^3-thymidine–labeled macronuclei. The percentage of cells with labeled macronuclei then rises rapidly so that 98 per cent of the cells show label in their macronuclei when pulsed at 240 or 270 minutes. The percentage then decreases rapidly between 300 and 390 minutes, at which time a low of 25 per cent of the macronuclei is labeled. Analysis of this curve from peak to peak or from trough to trough suggests a new cell cycle time of 3½ to 4 hours; this is comparable to the normal cell cycle in complete medium. The experiment shown in Fig. 1 has been repeated with the same results.

In this "shift-up" period the micronucleus never appears labeled before the macronucleus. A peak of labeled micronuclei (33 per cent) was recorded at 390 minutes; however, this percentage is considered a low estimate because some of the labeled micronuclei may not be in close enough proximity to the photographic emulsion to register tritium disintegrations.

The effect of pyrimidine deprivation on DNA synthesis was also studied by microspectrophotometric measurement of Feulgen-positive material in macronuclei of cells starved of pyrimidines for 72 hours and in newly divided cells (assuring measurement of a pre-DNA synthetic period or G1 condition) that were growing on complete medium. The data are shown in Fig. 2 with the 95 per cent confidence limits of the mean of the relative amounts of Feulgen-positive material in the macronucleus. The two groups of cells have the same mean and are not significantly different from each other in DNA values. Doubling of the values of the control daughter cells, as well as the 95 per cent confidence limits, as shown in Fig. 2, illustrates what might be expected after comple-

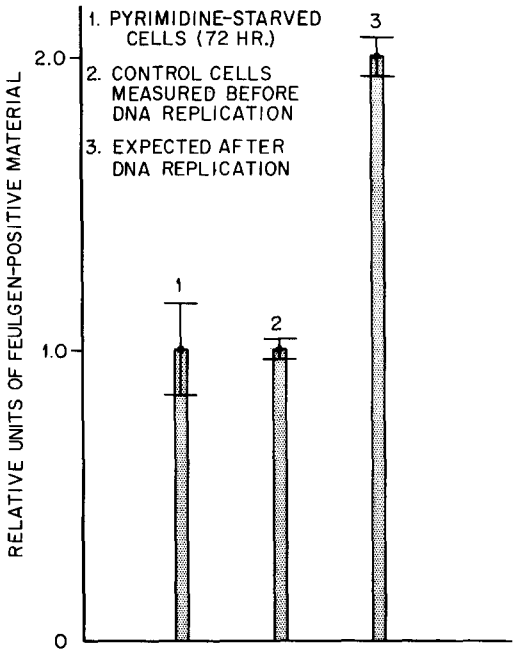


FIGURE 2 Relative units of Feulgen-positive material of cells measured immediately after division (control cells) and cells that were deprived of pyrimidines for 72 hours. The values for the control daughter cells were doubled, as were the 95 per cent confidence limits, in order to illustrate the point that the controls and pyrimidine-starved cells would be different from cells that have completed DNA replication.

tion of DNA replication. Cells deprived of pyrimidines and the newly divided daughter cells that were grown in the complete medium show that DNA synthesis had been arrested in the pyrimidine-starved cells at some point near the beginning of macronuclear replication. The biochemical data shown in Fig. 3 confirm the finding that there is an interval of at least 135 minutes before cellular DNA content increases. The chemical data also show that cellular DNA content is doubled just before the first cell division. The autoradiographic, spectrophotometric, and chemical data show quite clearly that pyrimidine starvation stalls cells at a point prior to macronuclear DNA replication and that after replacement of pyrimidine there is a period of more than 2 hours before all cells rather synchronously enter DNA synthesis to replicate the cellular content of DNA before their first cell division. The drop in cellular content of DNA at 7 hours (Fig. 3) is due to the division of cells between 6 and 7 hours. This phenomenon is also apparent for cellular RNA and protein content (see Fig. 3).

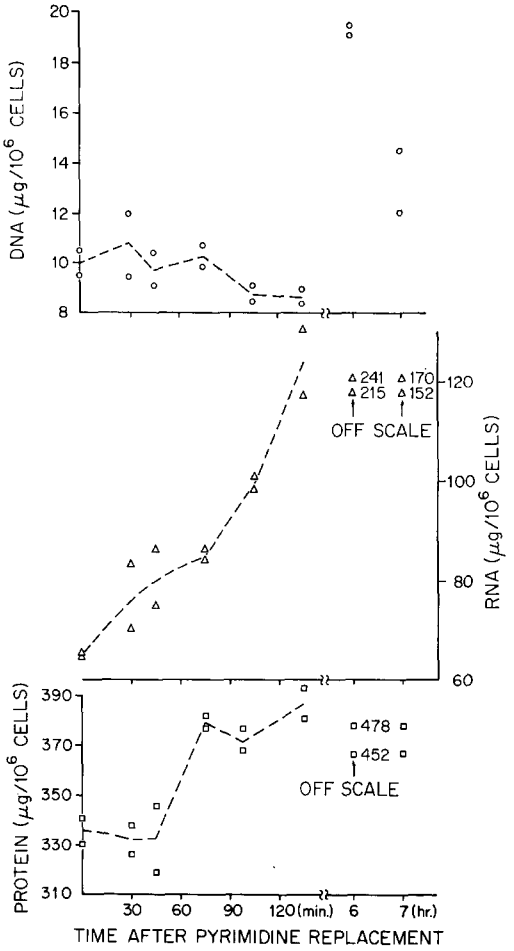


FIGURE 3 Changes in the cellular content of DNA (top), RNA (middle), and protein (bottom). Contents are expressed as $\mu g/10^6$ cells.

RNA Synthesis in Pyrimidine-Starved and Refed Cells

There was an obvious decrease in cytoplasmic basophilia (using toluidine blue stain) during the period of pyrimidine starvation. This observation is in agreement with biochemical studies showing a rapid loss of cellular RNA in pyrimidine-starved cells (7).

To get at the effect of deprivation on general RNA synthesis, starved cells were shifted to the complete medium and samples of cells were given a 15-minute pulse exposure to H^3-uridine or to H^3-adenosine at intervals. Using the incorporation of H^3-uridine or H^3-adenosine as a measure of RNA synthetic rate, the autoradiographic analysis of

these cells indicates that RNA synthesis begins to increase shortly after the shift to complete medium, with no apparent lag period (Table I; Figs. 4 and 5). The magnitude of differences from one experiment to another is due in large part to different durations of autoradiographic exposure. The incorporation of H^3-uridine (as measured by the autoradiographic grain count) over a constant unit area of the macronucleus or cytoplasm shows that differences are significant, as shown by the lack of overlap in the 95 per cent confidence interval of the mean, as early as 30 to 45 minutes after the "shift-up." Cytoplasmic and nuclear incorporation almost parallel each other over the 2 hours of measurement, and both show more than a 100 per cent increase in grain counts during the first 105 minutes after the "shift-up." As no DNA synthesis is occurring during this period, it can be assumed that the H^3-uridine is specifically measuring RNA synthesis.

The incorporation of radioisotope as an indication of synthetic rate may be subject to criticisms (such as problems of precursor pool sizes or permeability problems); therefore, it was believed that measurement of cellular RNA content was necessary to confirm the autoradiographic information. Fig. 3 shows that the cellular content of RNA begins to increase very shortly after pyrimidine replacement and that the increase continues at least up to the time of cell division. The relatively small confidence limits of the autoradiographic data show that there is considerable synchrony in the RNA synthetic response in the early period after pyrimidine replacement.

It is impossible to check the RNA turnover (defined as breakdown of macromolecular compounds to smaller molecular weight compounds and resynthesis of these smaller molecules back to macromolecular compounds) of pyrimidine-starved cells using H^3-uridine, because this com-

TABLE I

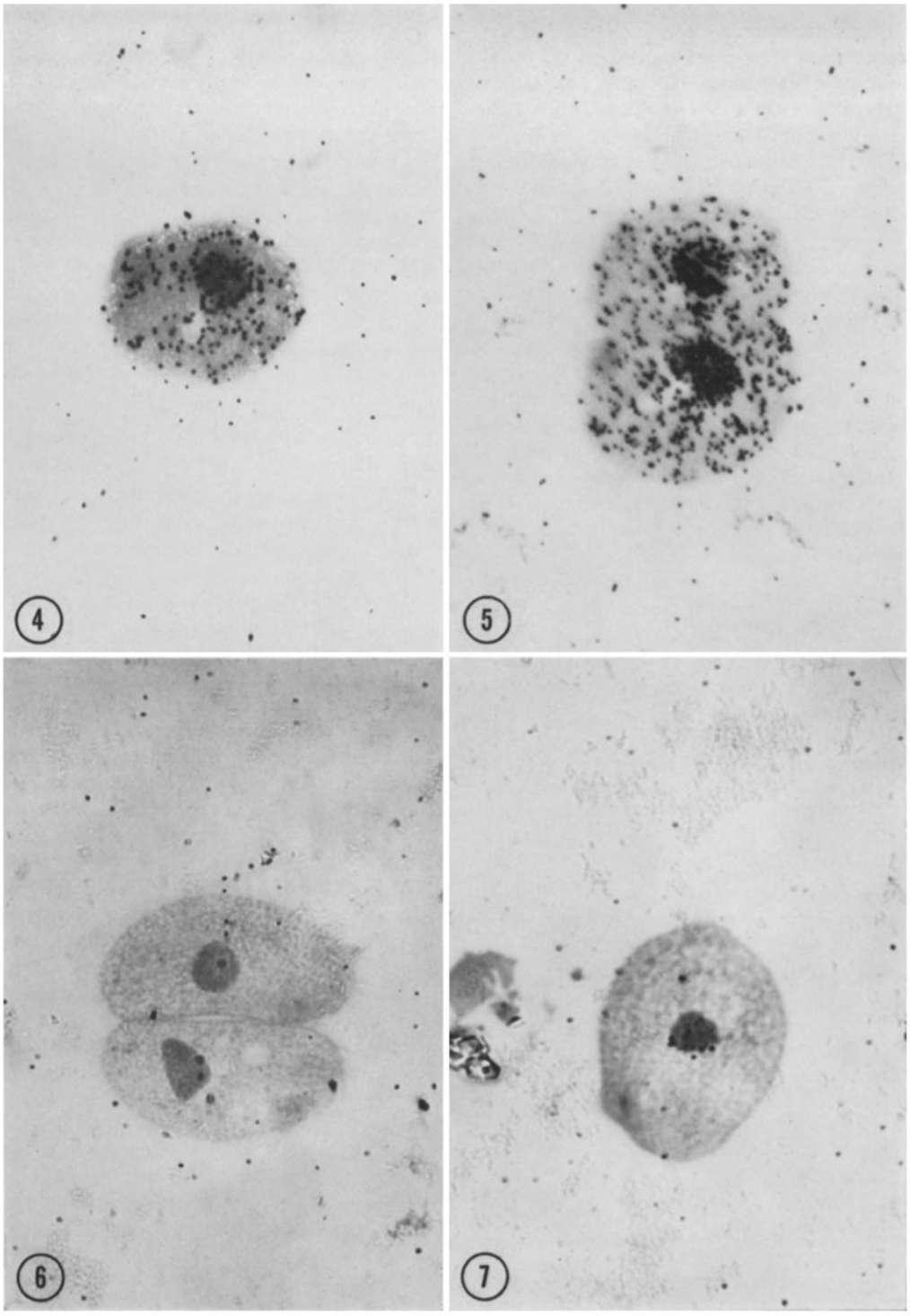
Results of Autoradiographic Grain Counts to Measure RNA Synthesis

Cells exposed to	Time after pyrimidine replacement	Average no. grains above background*	95 % confidence interval of $\bar{x}$
	min.	$\bar{x}$	
	Over cell's cytoplasm		
H^3-uridine (experiment 1)	0–15	2.7	±0.30
	30–45	6.3	±0.50
	60–75	9.2	±0.63
	90–105	13.7	±0.50
	120–135	8.7	±0.60
	150–165	11.9	±2.82
	Over cell's nucleus		
H^3-uridine (experiment 2)	0–15	21.0	±0.70
	30–45	34.4	±0.30
	60–75	49.8	±1.55
	90–105	46.3	±1.22
	Over cell's cytoplasm		
	0–15	8.6	±0.27
	30–45	10.0	±0.42
	45–60	25.6	±0.69
	90–105	22.2	±1.52
	Over cell's nucleus		
H^3-adenosine	minus 15–0	28.5	±1.99
	0–15	40.5	±4.32
	30–45	56.8	±4.39
	Over cell's cytoplasm		
	minus 15–0	11.3	±4.42
	0–15	18.4	±4.03
	30–45	20.8	±3.90

* Area over 10 cells was analyzed for each average. Data are expressed per unit area over nucleus or cytoplasm, using a Whipple ocular disk. Same unit area was used for nucleus and cytoplasm.

FIGURES 4 AND 5 Fig. 4 is an autoradiograph showing the amount of H^3-adenosine incorporated into a cell starved of pyrimidines for 72 hours (15-minute exposure to the isotope). Fig. 5 shows two individual cells that were pulsed with H^3-adenosine from 30 to 45 minutes after pyrimidine replacement, then air dried and fixed on slides for autoradiography. Notice the increased incorporation of H^3-adenosine in Fig. 5. × 600.

FIGURES 6 AND 7 The cells were pulsed with H^3-uridine. The cells in Fig. 6 were from a culture that was grown in complete medium, then RNase digested and autoradiographed. The cell in Fig. 7 was treated the same, except that it was grown in pyrimidineless medium. These typical cells suggest that H^3-uridine–labeled products may be used to support DNA synthesis in pyrimidine-starved cells (see text for explanation). × 600.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pound fulfills their pyrimidine need. Therefore, a series of experiments was conducted using the purine nucleoside H^3-adenosine. The data in Table I indicate that RNA synthesis is occurring in pyrimidine-starved cells, presumably by turnover of preexisting RNA (also see Fig. 4). The H^3-adenosine data agree with the H^3-uridine data in showing a rapid rise in RNA synthesis after the shift to complete medium.

Another experiment was performed to see whether the RNA turnover that occurs during pyrimidine deprivation could supply the cell with pyrimidine bases that could then be used for the one or two cycles of nuclear DNA replication that occur during the earlier stages of pyrimidine starvation. Logarithmically growing cells were given a 15-minute pulse of H^3-uridine. The cells were then washed free of exogenous H^3-uridine. Half of the cells were placed in complete medium; the other half were washed and resuspended in pyrimidineless medium. Cell proliferation was followed after resuspension of the cells. Each group of cells was sampled after a 3-fold increase in cell number. This took about 9 hours in the complete medium and about 22 hours in the pyrimidineless medium. The fixed cells were then digested with RNase and others were digested with both RNase and DNase before autoradiographic preparation. Basophilia was removed from the cytoplasm of the RNase-treated cells, and the autoradiographs showed that the cytoplasm was almost completely free of silver grains (Figs. 6 and 7). In these cells, the silver grains appeared almost exclusively over the nucleus. In the cells that were treated with RNase and DNase, no autoradiographic silver grains appeared over any part of the cells. The nucleus of cells in the complete medium was larger than that in the pyrimidine-deprived cells. Analysis of 200 cells grown in complete medium, then fixed and RNase-digested before autoradiography, gave an average of 1.00 ± SE 0.09 silver grains per macronucleus, and only 1.5 per cent of the cells had 4 or more silver grains. An analysis of 50 pyrimidine-starved cells gave an average of 6.16 ± SE 0.42 silver grains per macronucleus, and 82 per cent had 4 or more silver grains. The enzyme digestion experiments have been reproduced to give the same results.

TABLE II

Results of Autoradiographic Grain Counts to Measure Protein Synthesis

Cells exposed to	Time after pyrimidine replacement	Average no. grains above background*	95 % confidence interval of $\bar{x}$
	min.	$\bar{x}$	
H^3-leucine (experiment 1)	minus 15–0	83.2	±10.9
	15–30	82.9	±9.0
	45–60	85.1	±10.9
	90–105	120.7	±11.2
	135–150	121.6	±9.9
H^3-leucine (experiment 2)	minus 15–0	7.2	±0.72
	0–15	6.9	±1.26
	30–45	8.4	±2.13
	60–75	10.5	±1.42
	90–105	11.0	±1.79
	120–135	13.4	±1.76

* Cytoplasmic area over 10 cells was analyzed for each average. Data are expressed per unit area of cell's cytoplasm.

Protein Synthesis in Pyrimidine-Starved and Refed Cells

Tritiated leucine incorporation continues in cells starved of pyrimidines for 72 hours, indicating turnover of proteins in the absence of net protein synthesis (7).

The rate of H^3-leucine incorporation, used as an indication of the protein synthetic rate, does not show the same immediate increase as RNA, but shows a lag of 60 to 90 minutes before its rate increases significantly (Table II). The magnitude of the differences between experiments 1 and 2 in Table II is due to differences in autoradiographic exposure times. The quantitative difference is about a 50 per cent increase; the RNA incorporation rate is increased more than 100 per cent.

Chemical measurements of the cellular content of protein (Fig. 3) show that there is a period of at least 45 minutes before the cellular protein content increases. The cellular content has increased at 75 minutes, indicating that a net synthesis of protein must have occurred between 45 and 75 minutes after pyrimidine replacement. Therefore the chemical and the autoradiographic data seem to agree and complement each other on this lag period in protein synthetic rate.

DISCUSSION

The Pyrimidine Deprival Period

During pyrimidine starvation one may assume two complete cycles of DNA synthesis even though DNA synthesis was not underway for the first cycle. This assumption is based on the observa-

tion that dividing cells will go on to complete two more cell divisions after being placed in the pyrimidine-free medium. Presumably, during pyrimidine starvation the cells have an endogenous pyrimidine reserve that may be used to complete these two cell and nuclear cycles. Indeed, the conversion of labeled RNA via turnover (breakdown and resynthesis) to supply pyrimidine precursors for DNA synthesis is indicated by the results of the RNase and RNase plus DNase digestion procedures. Because the cells starved of pyrimidines for 72 hours and the newly divided log cells (which are known to be in a stage prior to DNA replication of the macronucleus) do *not* have a significant difference in their Feulgen-positive material, one may assume that prolonged pyrimidine starvation stops cells somewhere near the beginning of macronuclear DNA replication. The chemical data show that the DNA is indeed doubled before the first cell division.

An early decrease of RNA per cell occurs during pyrimidine starvation (7). This decrease may also be accounted for by the breakdown of ribosomal RNA and conversion of the breakdown products (presumably pyrimidine derivatives) to DNA. The eventual decrease of cellular protein during pyrimidine starvation (7) also suggests ribosomal breakdown. That H^3-adenosine is incorporated into the cells starved for 72 hours shows that RNA synthesis (without net increase in cellular RNA) is occurring even at this late period of starvation. Presumably this RNA synthesis is turnover of preexisting RNA. Work of this laboratory (unpublished) shows that the number of protein-synthesizing centers (polyribosomes) in the cell is reduced after pyrimidine starvation for 72 hours.

How does pyrimidine starvation contrast with amino acid starvation (histidine and tryptophan) as described by Stone and Prescott in this same organism (16)? They saw one cycle of DNA synthesis after amino acid deprivation if DNA synthesis had already started, but only 20 per cent increase in Feulgen-positive material with *no* H^3-thymidine incorporation if DNA synthesis had not started. Cell division ceases shortly after amino acid deprivation (16). It will be interesting to follow the sequence of events after amino acid replacement to starved cells. Perhaps DNA synthesis will start immediately instead of showing a lag as occurs after pyrimidine replacement. The role of endogenous reserves in supporting cell proliferation has been suggested from experiments utilizing heat shocks to induce division synchrony in *T. pyriformis*, in which it was shown that cells may be placed in balanced salt solution and will still continue to divide for two or three more cycles (3). Presumably, endogenous reserves are built up during the heat shocks and enable cell division to take place for some time in the absence of any exogenous organic nutrient. The definite but limited cell proliferation that occurs during the early stages of pyrimidine starvation also suggests the use of endogenous reserves which for a time support DNA replication and cell division. The data also suggest that cell division can occur in the absence of net increase in cellular RNA but that DNA replication is an essential prerequisite to cell division.

The Pyrimidine Replacement Period

The sequence of macromolecular events shown in this study is similar to that described for a "shift-up" in bacterial growth rate (*e.g.*, by means of changing from poor to rich medium); however, the present study relates the synthetic events more directly to the individual cells, and it is clear that essentially all the cells are synchronized in terms of their chemical response. Many studies have demonstrated the sensitivity of RNA metabolism to changes in the cell's environment. This has been especially well documented in studies with unbalanced growth in bacteria (9, 10, 14). Indeed, a pattern of cellular responses has emerged from the study of unbalanced growth. In particular, this temporal pattern shows RNA to respond first, either to "shift-up" or to "shift-down" in the growth conditions; protein synthesis follows, then DNA synthesis, and finally cell division. By this pattern of cellular events the cell is presumably able to adapt and survive in a changing environment.

The pyrimidine withdrawal and replacement method of synchronizing mass cultures of cells should lend itself to analysis of the chains of metabolic and structural events which must precede DNA replication and cell division.

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