RNA SYNTHESIS AND TURNOVER DURING DENSITY-INHIBITED GROWTH AND ENCYSTMENT OF *ACANTHAMOEBA CASTELLANII*

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ABSTRACT

Alterations in transcription that precede and accompany encystment (E) of suspension grown *A. castellanii* have been investigated. Comparative studies were performed on cells undergoing spontaneous E in high density stationary phase cultures or after experimental induction of E at low cell densities by deprivation of nutrients in exponential growth. Onset of growth deceleration at high cell densities was accompanied by an increase in the cellular RNA. The maximum RNA content occurred in cells at stationary phase and subsequently declined with the appearance of cysts in the cultures. On the contrary, the RNA content in cells whose growth was immediately terminated by experimental E induction remained at a constant exponential level through 5 h postinduction and then began to decline shortly before the appearance of cysts. The mature cyst formed in stationary phase cultures and after experimental E induction contained an equivalent amount of RNA (~50% of the exponential value). Comparison of the kinetics of [^{3}H]uridine incorporation demonstrated that there was an abrupt reduction in the rate of uridine incorporation into RNA with onset of growth deceleration or after growth termination in experimental E induction. The reduced incorporation of uridine into RNA could not be attributed to to a reduced uptake of the isotope by the cells or an altered capacity of the cells to phosphorylate uridine. Uridine continued to be incorporated into RNA at a reduced rate in cells throughout growth deceleration, in stationary phase, and up to 12 h postexperimental induction. Considered together, these results indicate that a buildup in RNA is not necessary for induction of encystment in acanthamoeba. The accumulated RNA in stationary phase cells appears to be due to the greater reduction in the growth rate than in transcription and the absence of RNA turnover in cells during growth deceleration. Initiation of RNA turnover appears to accompany growth termination and induction of E. The results further demonstrate that the regulation of the rate of transcription is closely coordinated with the control of growth and encystment in acanthamoeba.

INTRODUCTION

A variety of experimental systems have been explored to elucidate key metabolic events that may be involved in the regulation of cell growth and differentiation. One such system, which is receiving increased attention, is the transformation of a vegetatively growing ameba into a dormant cyst encased in a double wall. Although an example of protozoan differentiation, the encystment of the

small ameba, *Acanthamoeba castellanii*, shares features in common with the development of more highly evolved systems. For example, encystment is accompanied by morphological changes (Neff et al., 1964; Bowers and Korn, 1969), termination of cell growth (Neff et al., 1964), and production of luxury molecules (Neff and Neff, 1969; Potter and Weisman, 1971; Weisman et al., 1970). Inhibition of DNA synthesis has been reported to induce encystment (E) (Neff and Neff, 1969), whereas interruption of RNA synthesis prevents the process (Neff and Neff, 1969; Rudick, 1971). Finally, a potential role of cyclic AMP in encystment induction is suggested by results obtained on a related strain (Raizada and Murti, 1972).

Alterations in metabolic activity during acanthamoeba encystment have been investigated in cells undergoing spontaneous differentiation in stationary cultures (Byers et al., 1969; Rudick, 1971) or after experimental induction by deprivation of nutrients in exponential growth (Neff and Neff, 1969). However, results from studies utilizing the two approaches have not always yielded comparable conclusions concerning synthetic events critical to triggering encystment in acanthamoeba. This is particularly evident in definition of the alterations in transcriptive activity associated with encystment. For example, acanthamoeba underoing spontaneous differentiation appear to need a buildup of RNA (Rudick, 1971), whereas a similar accumulation of RNA cannot be demonstrated in cells experimentally induced to encyst (Neff and Neff, 1969). In an attempt to reconcile these differences, we have undertaken studies to compare RNA metabolism in exponentially growing, density-inhibited and encysting acanthamoeba. The results of these studies are the subject of this report and indicate that the rates of RNA synthesis and turnover are closely integrated phenomena in the differentiation of acanthamoeba.

MATERIALS AND METHODS

Cell Culture

A. castellanii (Neff strain) were maintained in axenic (aerated) suspension cultures in optimal growth medium (OGM) as defined by Neff et al. (1964). Stock cultures were inoculated routinely at initial cell densities of 1–2 $\times$ 10^3 cells/ml. Under these conditions, the strain exhibited a constant generation time of 7–8 h and grew exponentially to a density of 2.5–3 $\times$ 10^6 cells/ml. Thereafter, the growth rate of the cells was sharply reduced (growth deceleration period) until arrival into stationary phase at roughly 5 $\times$ 10^6 cells/ml (see Fig. 1). Unless specified, the term "density-inhibited growth" is used as an inclusive term for describing cells in post-exponential growth, even though the importance of population density in growth of acanthamoeba is yet to be clearly defined. To permit correlation of determinations made in separate experiments, culture hours were normalized to an initial cell concentration of 2 $\times$ 10^3 cells/ml. All cell counts were performed microscopically, using a Neubauer hemocytometer.

Encystment Conditions

SPONTANEOUS ENCYSTMENT: Cultures were allowed to grow to stationary density and the degree and synchrony of encystment were determined microscopically by differential cell counts (Neff et al., 1964) until 100% encystment was obtained.

EXPERIMENTAL INDUCTION: Induction of encystment was performed according to the constant pH method of Neff et al. (1964), with cells inoculated into the encystment medium (EM) at a final concentration of 2 $\times$ 10^5 cells/ml. Zero induction of encystment was taken as the time when the cells were placed with the first EM rinse (Neff et al., 1964).

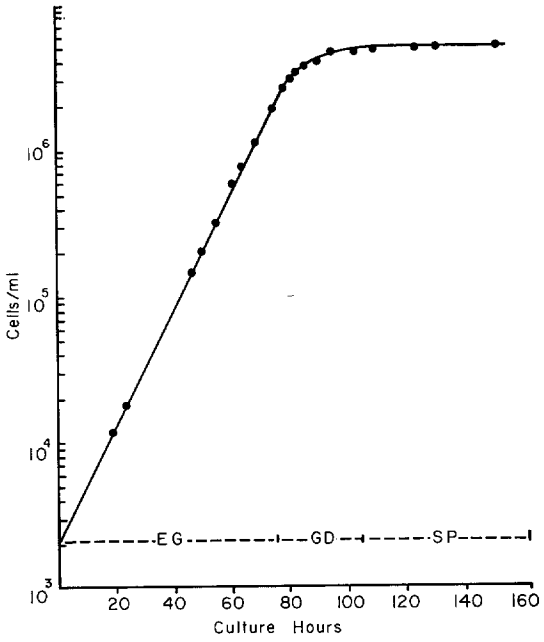

FIGURE 1 Growth kinetics of *A. castellanii* (Neff strain) grown in optimal growth medium (OGM) in axenic, aerating cultures as defined by Neff et al. (1964). Periods of exponential growth (EG), growth deceleration (GD), and stationary phase (SP) are indicated.

It should be noted that the cell concentration at which the cells were removed from optimal growth (OG) conditions and induced to encyst (i.e., 2×10^6 cells/ml) represented cells still in exponential growth (see Fig. 1). Essentially analogous results were obtained in encystment synchrony when cells were removed from OG cultures in earlier periods of exponential growth. Differential cell counts to determine degree and synchrony of encystment were accomplished according to Neff et al. (1964).

Determination of RNA

Samples of known volume and cell concentration were obtained at different times from either optimal growth or encysting cultures and the cells were concentrated by centrifugation. RNA was differentially extracted by a modification of the Fleck and Monroe (1962) technique. Samples, other than those containing young and mature cysts, were precipitated for 1 h after addition of bovine serum albumin and homogenization in 0.4 *N* $HClO_4$. After a wash, the pellet was dissolved in 0.5 *N* NaOH and hydrolyzed for 2 h at 37°C (>99% RNA hydrolyzed). The samples were then neutralized, acidified to 0.4 *N* $HClO_4$, and precipitated for at least 3 h. The supernatant, containing hydrolyzed RNA, was collected and combined with two washes of the pellet. All operations, with the exception of the alkaline hydrolysis, were performed at 0–4°C. Samples containing cysts were disrupted in a French pressure cell (American Instrument Co., Inc., Silver Springs, Md.) at a pressure of 14,000 lb/in^2 before the initial precipitation. The RNA concentration in the samples was determined by comparison to standard curves of hydrolyzed yeast RNA (Sigma Chemical Co., St. Louis, Mo.) handled in an identical manner.

Rate Studies

OPTIMAL GROWTH CULTURES: [5-^{3}H]uridine (New England Nuclear, Boston, Mass.; 28 Ci/mM) was added at a final concentration of 1 μCi/ml at specified times in growth. Samples were removed at regular intervals, and the RNA content was determined as above after an initial 10 ml saline rinse of the cell pellets. Portions of the acid-soluble material or hydrolyzed RNA were counted in 10 ml of a Triton X-100: toluene cocktail (1 part Triton: 2 parts toluene, 2,5-diphenyloxazole [PPO], 1,4-bis-[2-(5-phenyloxazole)]benzene [POPOP]) at an efficiency of 33% in a Beckman LS-150 Liquid Scintillation Counter.

ENCYSTMENT CULTURES: Cells were either: (*a*) exposed to [5-^{3}H]uridine (1 μCi/ml) from 0 induction of encystment; or (*b*) prelabeled with the isotope (1 μCi/ml) in OGM and subsequently induced to encyst in the presence of the isotope when reaching 2×10^6 cells/ml. Samples were obtained at regular intervals, rinsed with 10 ml of EM (Neff et al., 1964), and the specific activity of the RNA was determined as above.

Sedimentation Studies

RNA was prepared by phenol extraction from a known quantity of cells after exposure to a pulse label of [5-^{3}H]uridine (5 μCi/ml). Methods of extraction and analysis were as described previously (Stevens and Pachler, 1972). Radioactivity in the fractions was determined by liquid scintillation counting after precipitation of the samples with 10% ice-cold TCA and collection on filters (H. Reeve Angel & Co., Inc., Clifton, N. J.).

Chromatography

Samples were obtained from labeled cultures, rinsed with 10 ml of EM, and subsequently homogenized in 10% ice-cold TCA. After precipitation for 2 h at 0–4°C, the acid-soluble material was collected and combined with a 1-ml TCA rinse of the precipitate. TCA was extracted according to Bronstad et al. (1971), and the samples were evaporated to dryness under a stream of air. The dried samples were redissolved in 100 μl of 80% ethanol and applied onto sheets of Whatman DEAE 81 paper. The paper was developed by descending chromatography at room temperature according to Ives et al. (1963). All experimental samples were mixed with 15 μl of a solution of uridine, UMP, UDP, UTP (10 mg/ml each) before analysis, and a mixture of uridine and the nucleotides was cochromatographed. After development, the paper was dried, examined under UV light, and cut into 1-cm segments at right angles to the direction of migration. After elution with 0.5 *N* HCl for 3 h, radioactivity was determined in the eluates using the Triton: toluene cocktail. The amount of radioactivity associated with uridine and the nucleotides was calculated as the per cent of total radioactivity represented in the UV-light absorption spots of the appropriate standards.

RESULTS

Encystment Synchrony

Although data were available for the synchrony of encystment in experimentally induced acanthamoeba (Neff et al., 1964), the kinetics of encystment in density-inhibited, suspension-grown acanthamoeba had not been well defined. Thus initial studies were performed to verify the encystment synchrony in our experimentally induced cultures and to precisely determine the rate of

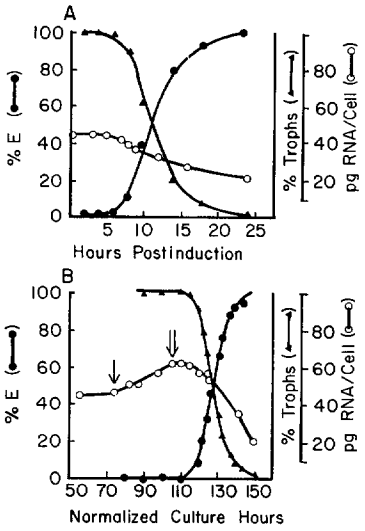

FIGURE 2 Encystment of *A. castellanii*: A, Rate of encystment (percent young and mature cysts) after experimental induction of encystment in exponentially growing cells. Cells were removed from optimal growth (OG) cultures at a cell density of 2×10^6 cells/ml and induced to encyst in encystment medium (EM), pH 8.9, at a final concentration of 2×10^5 cells/ml, according to the method of Neff et al. (1964). B, Rate of encystment (percent young and mature cysts) in stationary phase OG cultures. Single arrow denotes end of exponential growth and beginning of growth deceleration; double arrow marks end of growth deceleration and beginning of stationary phase. Encystment synchrony and RNA content represent an average of at least three separate determinations per point. To permit correlations of determinations made in separate experiments, culture hours were normalized to an initial cell concentration of 2×10^3 cells/ml.

encystment in the stationary cultures. Results of these studies are presented in Fig. 2.

In agreement with Neff (Neff et al., 1964), we found that transfer of cells from OGM to EM resulted in cessation of growth. Similarly, the induction phase, in which the cells (preencysters) maintained their typical trophozoite appearance, lasted approximately 5 h. Subsequently, the cells began to assume a rounded morphology (cyst initiators), signaling the beginning of wall synthesis. Cysts began to appear in the cultures by 8 h and reached >95% by 19 h postinduction (Fig. 2 A).

Throughout the period of induction and cyst initiation the cell number remained constant. However, at approximately the time that young cysts began to appear in the culture, the cell number began to decline. By 100% encystment, the total cell number was reduced by 40–50%. This high percentage of cell death was not noted by Neff et al. (1964) using the pH 8.9 encystment synchrony method. At this time, we can offer no explanation for the discrepant results. Nevertheless, encystment induction in exponentially growing cells would not appear to be related to the cell death since the loss of cells was not observed until after the period of induction. Moreover, we have obtained encystment synchrony, comparable to that obtained after induction at pH 8.9, by induction of exponentially growing cells in encystment medium at pH 7.0. Encystment of cells induced at pH 7.0 is accompanied by only minimal cell death in the cultures.

Analyses of a variety of cultures during growth deceleration and stationary phase (see Fig. 1) indicated that considerable time elapsed between termination of exponential growth and appearance of cysts in the optimal growth cultures. Although some variability was encountered between cultures in the total period of growth deceleration, invariably cysts began to appear in the cultures after approximately 7–10 h into stationary growth (i.e., 115–118 normalized culture hours (Fig. 2 B). Subsequent to their appearance, the cysts increased at nearly a linear rate to >90% over the next 24 h (Fig. 2 B).

It should be noted that the percentage of preencysters or cyst initiators could not be determined during the time preceding the appearance of cysts in the optimal growth cultures. In general, trophozoites at the high cell densities characteristic of stationary growth assume a rounded appearance, making it extremely difficult to distinguish preencysters or cyst initiators. Therefore, the appearance of cysts was used as the morphological marker of differentiation for comparing transcriptive activities in the induced or spontaneously differentiating cultures.

RNA Content

Preliminary studies to compare alterations in transcription in density-inhibited or encysting acanthamoeba were restricted to analysis of the RNA content. Although not shown, determinations of the RNA content in acanthamoeba from densities of 8×10^4 cells/ml through 2×10^6 cells/ml demonstrated that the average content (45 pg/cell) remained constant throughout exponential growth. Subsequent to the onset of growth deceleration, however, the RNA content began to

increase and reached a maximum of 35% greater than the exponential RNA amount at approximately the time when the maximal stationary density was reached (i.e., 5×10^6 cells/ml). The latter density represented only a twofold increase in the cell number during the growth deceleration period (see Fig. 1). During the period of RNA accumulation, the cell volume was noted to increase; a similar increase in cell volume has been found previously in aging cultures (Kjellstrand, 1968; Byers et al., 1969).

The increased level of RNA was maintained for ~9 h in stationary phase and then began to decline at the time of cyst appearance in the cultures. When 100% encystment was obtained, the RNA content had decreased to ~50% of that found in the exponential cell (Fig. 2 B).

The increase in cellular RNA during growth deceleration suggested that such an increased accumulation might be necessary for the induction of encystment in acanthamoeba. This suggestion has been made by Rudick (1971), who examined RNA synthesis in aging cultures of acanthamoeba grown in monolayer. To check this possibility, the RNA content was examined in cells induced to encyst under more strictly defined conditions.

The data in Fig. 2 A demonstrated that, contrary to the increase found in postexponential cells, the RNA content in the induced cell remained at a constant exponential level for the first 5 h after encystment induction. Subsequently, the RNA content began to decline until 100% encystment was obtained. The RNA content in the mature cyst formed after experimental induction was roughly equivalent to the amount determined in the spontaneously formed cyst (i.e., ~50% of the exponential value; Figs. 2 A and 2 B). Neff and Neff (1969) have shown a similar loss of RNA during encystment of induced cells; however, their data indicated that the decline in RNA began soon after encystment induction.

The results on the RNA content in experimentally induced, encysting cells indicated to us that a net increase in cellular RNA was not necessary for the induction of encystment. The increase in RNA in postexponential cells then could represent an increased production of RNA for unrelated encystment events during growth deceleration. A more plausible explanation would be that RNA accumulated in cells during growth deceleration as a result of a greater reduction in the growth rate than in the rate of transcription. Subsequent experiments were performed to investigate the rates of RNA synthesis in cells during exponential growth, growth deceleration, and encystment.

Rate of RNA Synthesis

EXPONENTIAL GROWTH, GROWTH DECELERATION, AND STATIONARY PHASE: Continuous exposure of suspension-grown acanthamoeba to [^{3}H]uridine showed that the isotope was incorporated into RNA at a constant rate throughout logarithmic growth (Fig. 3). The rapidity with which the constant rate of isotope incorporation into RNA was attained suggests that the specific activity of the uridine precursor pool utilized for RNA synthesis is equilibrated very quickly after addition of labeled precursor to the medium. The continued increase in acid-soluble (AS) radioactivity (up to 1.5 h; Fig. 3) is not completely understood but could reflect delayed saturation of a second uridine pool that is not used in transcription of the bulk cellular RNA. This statement is supported by recent work of Plagemann (1972) who has shown the existence of compartmentalized

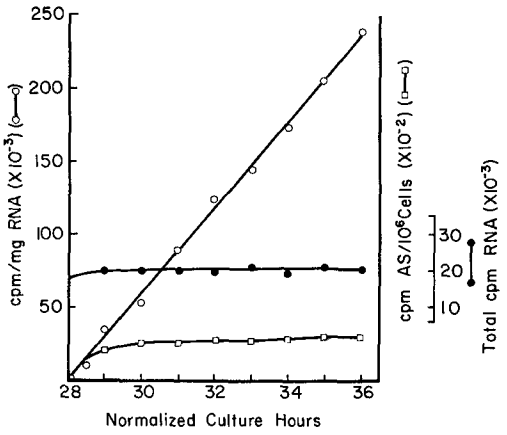

FIGURE 3 RNA synthesis during exponential growth. [^{3}H]uridine was added to suspension grown acanthamoeba and small portions of cells were removed from the flasks at indicated times. Incorporation of radioactivity into the acid-soluble pools and RNA was determined as described in Methods and Materials. To determine stability of radioactivity in macromolecular RNA during logarithmic growth, cells were grown for one generation with [^{3}H]uridine (1 μCi/ml), washed free of the labeled medium, and inoculated into fresh OGM containing unlabeled uridine (3.8×10^{-5} M). (Surviving RNA, ●——●; acid-soluble pools, □——□). Points are representative of data accumulated from at least two separate experiments and an average of duplicate samples in single experiments.

uridine pools, only one of which provides ribonucleotides for RNA synthesis.

Examination of the radioactivity in macromolecular RNA during an 8 h chase of labeled, exponentially growing cells in the presence of excess, unlabeled uridine demonstrated that the major portion of the synthesized RNA was conserved (Fig. 3). Similar stability of RNA has been found in other rapidly dividing cells (Feinendegen et al., 1961; Emerson, 1971).

Experiments to analyze RNA synthesis during the transition from exponential growth to growth deceleration indicated that the rate of [^{3}H]uridine incorporation into RNA dropped to 55% of the exponential rate at a time concomitant with the termination of logarithmic growth (Fig. 4). The abrupt decline in incorporation did not appear to be due to an exhaustion of available labeled precursor; the level of radioactivity within the acid-soluble pool material remained at saturation during and after the period of the rate drop (Fig. 4).

To determine whether the subexponential rate of uridine incorporation into RNA was maintained until the maximum increase in RNA had occurred, cells in late growth deceleration (i.e., 9 or 22 h postexponential) were exposed to [^{3}H]uridine. In agreement with results obtained on higher cell types (Plagemann et al., 1969; Weber and Rubin, 1971), we found that density-inhibited acanthamoeba had a reduced capacity for uptake of the precursor. After equivalent periods of exposure, the level of acid-soluble radioactivity in cells labeled in late growth deceleration was 55% of that obtained in exponential cells (Fig. 5). However, the reduced uptake of the isotope by the cells could not totally account for the decreased uptake of the uridine into RNA. The rate of uridine incorporation into RNA in cells labeled 9–21 h after termination of logarithmic growth was only 35% of the exponential rate; the rate of incorporation in cells pulsed between 22–25 h postexponential growth dropped to 20% of the exponential value (Figs. 5 A and 5 B).

Assuming that the size of the pools did not change in exponential and density-inhibited growth and that the reduced uptake reflects, therefore, a decreased specific activity of the uridine pool, correction of the reduced uridine incorporation into RNA for the lowered acid-soluble pool radioactivity should be possible. Such calculations indicated that the relative rate of [^{3}H]uridine incorporation into RNA, assumed at the end of logarithmic growth (i.e., 55%), was

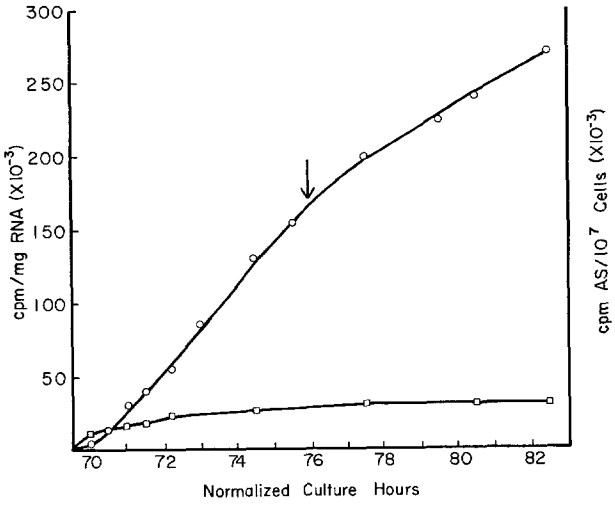

FIGURE 4 RNA synthesis in cells entering growth deceleration. [^{3}H]uridine (1 μCi/ml) was added to OG cultures at a cell concentration of 1.8 $\times$ 10^6 cells/ml. Rate of [^{3}H]uridine incorporation was followed up and through the transition (denoted by arrow) from exponential growth into growth deceleration (cell concentration, 3.6 $\times$ 10^6 cells/ml). Radioactivity in RNA (○——○) and acid-soluble pools (□——□) was determined as described in Methods and Materials. Points are representative of data accumulated from at least two separate experiments and are an average of duplicate samples in a single experiment.

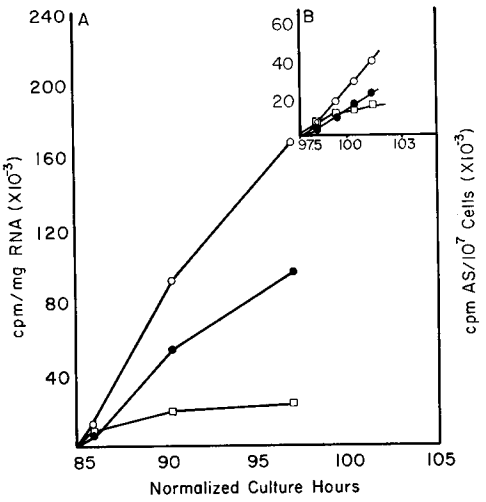

FIGURE 5 RNA synthesis in cells in growth deceleration. Cultures were continuously exposed to [^{3}H]uridine (1 μCi/ml) from 9 to 20.5 h postexponential growth (85–96.5 normalized culture hours), (A), or were pulsed with [^{3}H]uridine (1 μCi/ml) from 22 to 25.5 h postexponential growth (98–101.5 normalized culture hours), (B). Incorporation of radioactivity into the acid-soluble (AS) pools and RNA was determined as described in Methods and Materials. AS (□——□); RNA (uncorrected) (●——●); RNA (corrected for reduced [^{3}H]uridine uptake into AS pools) (○——○). Points are an average of duplicate samples in a single experiment and representative of data acquired from three separate experiments.

maintained through the first 15 h of the growth deceleration period (Fig. 5 A). During the latter half of growth deceleration, the rate dropped to 36% of the exponential rate. The assumption that the pool size did not vary was supported by the constancy of the acid-soluble pool 260 nm absorption in exponentially growing and density-inhibited acanthamoeba and the data of Weber and Rubin (1971) that showed no significant changes in pool sizes in logarithmically growing and contact-inhibited chick embryo fibroblasts and 3T3 mouse cells.

The above results support our contention that the increase in RNA in cells during growth deceleration resulted from a greater reduction in the growth rate of the cells in comparison to the decline in the rate of transcription. We can roughly estimate that cells undergoing a single doubling and synthesizing RNA at a relative rate of 55% for 15 h and 36% for 14 h during growth deceleration would have accumulated approximately 33% of the exponential RNA amount. This figure is in close agreement with the determined 35% increase, but represents a conservative estimate since uridine incorporation into RNA could not be accurately measured during the last 5 h of growth deceleration. However, limited incorporation of uridine into RNA was obtained during stationary phase when the RNA content remained constant. It would appear then that during growth deceleration there was little or no turnover of RNA but, with the onset of stationary phase, RNA turnover was initiated at a rate equivalent to its synthesis.

ENCYSTMENT: Results of the studies to investigate the rate of transcription during encystment of experimentally induced, exponential cells are given in Figs. 6 and 7. Addition of the labeled precursor to the cells at 0-induction demonstrated that, after an initial lag, [^{3}H]uridine was incorporated into RNA at only ~50% of the exponential rate (Fig. 6). The reduced incorporation rate appeared to be maintained throughout the first 10 h of encystment, at which time no further increase in the specific activity of the RNA could be detected. The reduction in the rate of uridine incorporation into RNA could not be attributed to a decreased capacity for uridine uptake by the encysting cell; the level of radioactivity in the acid-soluble pool material in the encysting cell was not significantly different from the level attained by the exponential cell (see Figs. 3 and 6).

Although the decrease in uridine incorporation into RNA in the above experiment could be tentatively interpreted to mean that the rate of RNA synthesis was reduced with the induction of encystment, the results were complicated by the turnover of RNA that was occurring during encystment. RNA could be synthesized at the exponential rate, but, due to turnover of newly synthesized RNA species, the rate of increase in the specific activity of the RNA would be reduced. Therefore, additional experiments were carried out to follow the rate of [^{3}H]uridine incorporation into RNA during encystment of prelabeled exponential cells (see Methods and Materials for experimental details).

The results from this type of experiment, which are given in Fig. 7, verified that the decrease in uridine incorporation into RNA was a normal event accompanying the transfer of exponential cells to the encystment environment. Although the

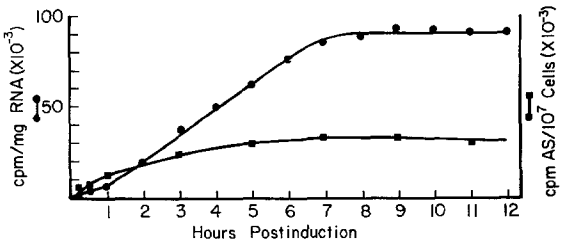

FIGURE 6 RNA synthesis in experimentally induced encystment. Cells in exponential growth were experimentally induced to encyst (see legend of Fig. 2 A for details) in EM containing [^{3}H]uridine (1 μCi/ml). Incorporation of radioactivity into acid-soluble pools (■——■) and RNA (●——●) was determined as described in Methods and Materials. Points are representative of data accumulated from two separate experiments.

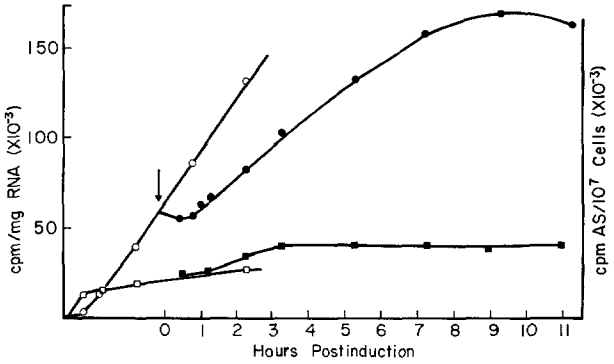

FIGURE 7 RNA synthesis during encystment of prelabeled cells. Acanthamoeba were labeled with [^{3}H]-uridine (1 μCi/ml) in exponential growth (1.8×10^6 cells/ml) When the cell density reached 2×10^6 cells/ml, a portion of the culture was experimentally induced to encyst (see legend of Fig. 2 A for details) in EM containing [^{3}H]uridine (1 μCi/ml). Incorporation of radioactivity into acid-soluble pools (■——■) and RNA (●——●) was determined as described in Methods and Materials. The rate of uridine incorporation into RNA was followed for several hours in the cells remaining in the OG culture (○——○); acid-soluble OG (□——□). Points represent an average of duplicate samples in a single experiment.

level of isotope was maintained in the EM, the rate of uridine incorporation into RNA underwent an abrupt drop to 50% of the exponential rate by the first hour of encystment (Fig. 7). Contrary to the data obtained from the first experiment, this rate was maintained only through the first 5 h; between 5 and 10 h, the rate of incorporation underwent a further decline to ~25% of the exponential value.

The lag in uridine incorporation into RNA (Figs. 6 and 7) after transfer of exponential cells to the EM deserves mention at this point. The lag reflects the cell's reaction to methods used for encystment induction, i.e., the necessity of washing and centrifuging the cells before subculture into the EM (Neff et al., 1964). An analogous lag was encountered when exponentially growing cells were handled identically but subcultured back into OGM; however, in the latter situation, [^{3}H]uridine incorporation into RNA resumed at the exponential rate after the initial lag of 1 h.

Considering the results of the kinetic studies with respect to the RNA determinations made in the experimentally induced, encysting cell, the following conclusions seem justified. Although RNA synthesis was occurring, the net amount of RNA remained constant in early encystment (up to 5 h) (Fig. 2 A). Thus, turnover of RNA was initiated at or soon after the onset of induction and occurred at a rate equivalent to the rate of synthesis for the first 5 h. The continued increase in the specific activity of the RNA after transfer of prelabeled exponential cells to the EM further implied that there was a preferential turnover of preencystment (old) RNA species during the first 5 h. RNA synthesis occurring at the exponential

rate in the presence of this degradation would have resulted in the specific activity of the RNA increasing at a rate faster than that found in exponential growth. Since this did not occur, we may assume that the decreased rate of uridine incorporation into RNA (Fig. 7) reflected a true reduction in the rate of transcription with encystment induction.

Between 5 and 10 h postinduction, the RNA content was declining (Fig. 2 A), which indicated that the rate of RNA degradation was in excess of the rate of synthesis. A determination of the rate of synthesis by analyses of [^{3}H]uridine incorporation during continuous exposure of cells from 0-induction would indicate the maximal rate that could be taking place since turnover may still be restricted primarily to old (preencystment) RNA species. This theory was supported by the further decrease in uridine incorporation into RNA to one fourth of the exponential rate between 5 and 10 h encystment in the prelabeling experiment (Fig. 7). Although the continued increase in the specific activity of the RNA after 5 h could be obtained in an absence of synthesis, [^{3}H]uridine incorporation into RNA was obtained in pulse exposures in cells up to 12 h postinduction (see Sedimentation Studies). Considering that RNA synthesis was detected at a time when the specific activity of the RNA remained constant (Figs. 6 and 7; i.e., 10–12 h encystment), we may further conclude that after 10 h, preferential turnover of newly synthesized RNA species (i.e., those made during encystment) was occurring.

Chromatographic Analyses

Throughout the above studies, we have assumed that the reduced incorporation of uridine into RNA during onset of density-inhibited growth or encystment reflected a true change in the rate of transcription. The reduced incorporation could not be explained by a precursor limitation due to the reduced capacity of the cell for uptake of the isotope. However, the possibility remained that the cell had an altered capacity to phosphorylate uridine. This, in turn, might result in a change in the rate of [^{3}H]uridine incorporation into RNA which would not represent a true change in the rate of RNA synthesis. To investigate this possibility, the level of radioactivity in uridine and uridine nucleotides was analyzed during transition from exponential growth to growth deceleration or after experimental encystment induction. Similar studies in later periods of growth deceleration were hampered by the lower level of radioactivity incorporated into the acid-soluble pools.

Chromatographic analyses, shown in Table I, demonstrated that the percentage of activity represented in uridine and the combined nucleotides did not vary significantly during the periods when abrupt changes in the rate of uridine incorporation into RNA were found. The observation that the highest proportion of radioactivity was found consistently in uridine is in contrast to the situation found in mammalian cells, in which the highest percentage of activity is found in the nucleotides after exposure to [^{3}H]uridine. Undoubtedly, in acanthamoeba, precursors derived from phosphorylation of uridine via a salvage pathway are competing at all times with the bulk of uridine precursors synthesized by the normal de novo pathway which is not suppressed by the exogenously supplied [^{3}H]uridine. Nevertheless, the lack of an apparent change in the percentage of radioactivity in the combined nucleotides does

TABLE I

Distribution of Radioactivity during Exponential Growth, Growth Deceleration, and Encystment

Culture hour	Cells/ml	% of total radioactivity			
		Uridine	UMP	UDP	UTP
73	1.9×10^6	75.00	9.50	10.20	5.00
74	2.1×10^6	71.30	11.90	12.20	4.03
75	2.3×10^6	74.80	11.98	9.36	3.80
76	2.5×10^6	73.50	11.70	10.50	4.10
Hours of Post encystment induction					
0.5		79.20	8.87	8.10	3.77
1.0		75.30	10.20	9.78	4.60
2.0		75.10	9.30	11.40	4.20
3.0		81.30	6.20	8.20	4.09

Distribution of radioactivity in uridine and uridine nucleotides after chromatographic analysis of acid-soluble pool material. Cells in exponential growth (1.7×10^6 cells/ml) were labeled with [^{3}H] uridine (1 μCi/ml). When the cell density reached 2×10^6 cells/ml, a portion of the culture was induced to encyst (see legend of Fig. 2 A for details) in encystment medium containing [^{3}H] uridine (1 μCi/ml). Chromatography of the acid-soluble pool radioactivity was performed as described in Methods and Materials.

not support the argument that the altered rate of uridine incorporation into RNA is due to an altered capacity of the cell to phosphorylate uridine.

Sedimentation Studies

Recent reports have indicated that an integral relation exists between the production of ribosomal RNA (rRNA) and cell growth. For example, the rate of rRNA production is abruptly reduced during contact-inhibited growth of mammalian cells (Emerson, 1971). Even though rRNA synthesis is required during differentiation, the greatly reduced growth rate of the differentiating cell suggests that a similar alteration may occur in the rate of rRNA transcription. To check this possibility, we attempted to compare the rate of rRNA synthesis during density inhibition or induction of encystment in acanthamoeba.

As shown in Fig. 8, the rate of rRNA formation in exponentially growing acanthamoeba is extremely rapid. After only a 25-min exposure to [^{3}H]uridine, the mature rRNA species (i.e., 26S and 18S; Stevens and Pachler, 1972) contain the major amount of radioactivity. The small peak of activity seen at approximately "37S" (arrow: Fig. 8) may be a precursor rRNA molecule, but this could not be definitely shown. RNA of sufficiently high specific activity could not be obtained in acanthamoeba in pulses short enough to characterize this molecule.

Nevertheless, it was apparent from a comparison of the relative specific activities in the radioactive profiles shown in Fig. 8 that the production of rRNA species becomes drastically decreased at or soon after termination of exponential growth by density inhibition or induction of encystment in acanthamoeba. The slight increase in the radioactivity in the "37S" region of the encysting cell profile is not understood; conceivably, in addition to a reduced rate of rRNA precursor synthesis, a delay in processing of the molecule also occurs. Although not shown, cells in stationary phase were pulsed. However, no definitive analysis could be made of the profile from the limited incorporation that was obtained in the three major RNA species. The latter was due in part to the greatly reduced uridine uptake by the cells in stationary phase.

It is also of interest, in the profiles shown in Fig. 8, to compare the relative specific activity of the RNA sedimenting at 4S (>80% transfer RNA [tRNA]; Stevens and Pachler, 1972). The synthesis of the 4S RNA appeared to be depressed in the density-inhibited cells, whereas production of this

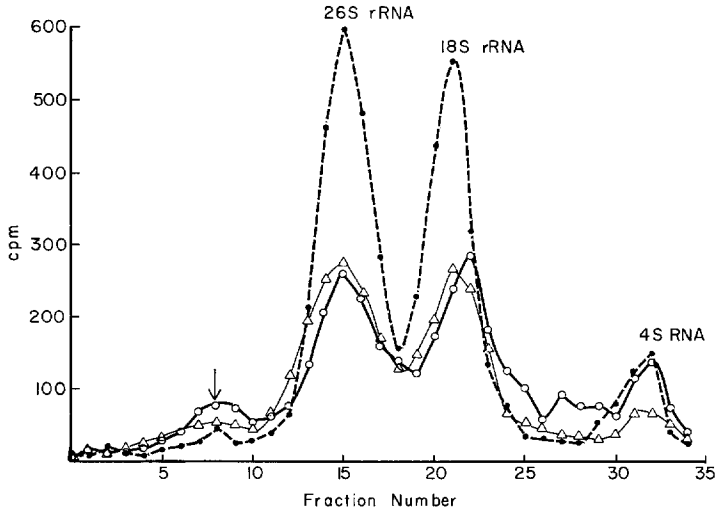

FIGURE 8 Pulse-labeled whole cell *Acanthamoeba* RNA. Cells in exponential growth (8×10^5 cells/ml) (●——●), growth deceleration (2.9×10^6 cells/ml) (△——△), and experimentally induced to encyst (see legend of Fig. 2 A for details) (○——○) were exposed to [^{3}H]uridine for 25 min. Whole cell RNA was extracted as described in Methods and Materials. The profiles have been normalized to a constant cell number. The radioactivity in RNA obtained from cells pulsed in growth deceleration has been corrected for a 20% reduction in uridine uptake into the cells.

RNA during early encystment seemed comparable to the exponential level. The normal or possibly enhanced rate of 4S formation would compare to the increase in this component in late growth deceleration (before cyst formation) in acanthamoeba grown in unagitated cultures (Rudick, 1971).

The encystment profile presented in Fig. 8 was characteristic of cells pulsed through the first 5 h of encystment. During this time the amount of bulk rRNA and 4S RNA remained constant. By 7 h postinduction, rRNA synthesis appeared to be terminated since only negligible activity was associated with the mature rRNA species. Although not illustrated, radioactive profiles obtained from cells pulsed at intervals between 7 and 12 h into encystment showed that [^{3}H]uridine was incorporated mainly into the 4S RNA and RNA sedimenting between 9 and 12S in the sucrose gradient. During this interval, bulk rRNA and 4S RNA underwent a decline.

It should be emphasized that the radioactive profiles shown in Fig. 8 were normalized to a constant cell number and were not altered by introduction of steps in the extraction technique to obtain rapidly sedimenting, heterodisperse RNA (Penman, 1966). In addition, correction was made in the radioactive profile of the density-inhibited cell for a 20% reduction in uridine uptake during the short pulse. A similar correction was not necessary for the encysting profile, since the level of activity attained in the acid-soluble pool during the pulse was found to be equivalent in the encysting and exponential cells.

DISCUSSION

As growth of acanthamoeba becomes limited at high cell densities, the rate of uridine incorporation into RNA abruptly decreases. This situation is analogous to that found in exponentially growing cells removed from OGM and inoculated at low cell densities (i.e., 2×10^5 cells/ml) into a nonnutrient encystment medium. The apparent reduction in the rate of uridine incorporation into RNA is concluded to reflect a true alteration in the rate of transcription. The reduced labeling of the RNA could not be attributed to a precursor limitation, either from a reduced uptake of isotope into the cells or from an altered capacity of the cells to phosphorylate the precursor. Although the density-inhibited acanthamoeba exhibited a reduced capacity for [^{3}H]uridine uptake, the shift in transcription rate during transition from logarithmic growth to growth deceleration was determined by prelabeling cells in exponential growth. As noted in Fig. 4, the acid-soluble pool activity remained constant before, during, and after the decline in RNA synthesis.

Although definitive analyses could not be made, the apparent reduction in rRNA synthesis during onset of growth deceleration and during early encystment of acanthamoeba seems noteworthy. Equivalent reduction of rRNA synthesis has been demonstrated in contact-inhibited, nondifferentiating cell populations (Emerson, 1971; Weber, 1972) and during sporulation of bacteria (Hussey and Losick, 1971). In the latter situation, termination of rRNA synthesis occurred in advance of other RNA species. A similar early shutdown of rRNA production was shown for encysting acanthamoeba in the present studies (see Sedimentation Studies). It should be emphasized, however, that the reduced capacity of the encysting cell for synthesis of rRNA is not taken to mean that rRNA synthesis is unnecessary for encystment. Results to be reported in a separate communication strongly suggest that wholesale production of ribosomes may be essential for encystment of acanthamoeba. The requirement for rRNA synthesis during encystment has been suggested from previous studies on acanthamoeba (Biggs, 1970; Rudick, 1971) and differentiation of the cellular slime molds (Cocucci and Sussman, 1970).

Considering the decline in the rate of transcription with respect to the induction of encystment, it would appear that this metabolic alteration is secondary to some other event triggering induction of differentiation in acanthamoeba. This statement is based on the extended length of time between onset of growth deceleration, during which the rate of RNA synthesis is reduced, and encystment in stationary-phase OG cultures (see Figs. 2 B, 4, and 5). The close association between encystment and growth termination in either stationary phase or experimentally-induced cultures indicates that the trigger for induction is closely tied in to molecular events regulating cell growth.

From the above discussion, it may be speculated that the true induction period of encystment in density-inhibited cultures is that period in which the cell density remained constant before the appearance of cysts (see Figs. 1 and 2 B). The studies on exponential cells induced to encyst indicate

that initiation of RNA turnover closely accompanies induction of encystment. A similar phenomenon has been shown during differentiation of cellular slime molds (Sussman, 1967). However, turnover of RNA in the OG cultures did not appear to begin until termination of growth in stationary phase. During this time the RNA content was constant (see Fig. 2 B), although incorporation of [^{3}H]uridine into RNA could be detected.

The studies on RNA turnover during encystment are of further interest since they indicate that the amount of RNA degraded, which was primarily preencystment-synthesized RNA species, is closely regulated by the RNA content of the exponential cell. The density-inhibited cell accumulates RNA during growth deceleration, which can be explained by the greater decrease in the growth rate than in the rate of transcription. However, no net increase in RNA can be detected in the experimentally-induced exponential cell. Nevertheless, the amount of RNA in the mature cyst formed in stationary cultures is approximately equivalent to that found in cysts derived from experimental induction (i.e., ~50% of the exponential value).

The results of the present studies and the conclusions derived from them can be compared to those obtained by Byers et al. (1969). Analyses of RNA content in acanthamoeba (Neff strain) grown in unagitated (nonaerated, monolayer) cultures indicated that the RNA content remain constant through logarithmic growth and increased to 75% greater than the exponential amount during growth deceleration. The maximum increase occurred at a time preceding the appearance of cysts in the cultures.

The greater increase in RNA during aging of cells in unagitated cultures can be explained by the longer period of growth deceleration. This period was approximately 150–200 h (see Fig. 8, Byers et al., 1969) vs. 30- to 36-h growth deceleration period found in the present study of suspension grown acanthamoeba. The results of Byers et al. (1969) would thus appear compatible with our conclusion that the buildup in RNA in postexponential cells is not a necessary requirement for encystment, but merely reflects a greater reduction in the growth rate of the cells in comparison to the decrease in the transcription rate. That only limited encystment (i.e., 4%) was obtained, even after 200 h postexponential growth, in the unagitated, aging cultures (Byers et al., 1969), would support this statement.

However, a later publication on the relation of RNA synthesis to encystment of acanthamoeba grown in unagitated cultures provided data and conclusions contrary to ours (Rudick, 1971). This study indicated that cellular RNA increased to 75% greater than the *early* exponential value from mid-logarithmic growth to the end of growth deceleration. The buildup in the level of RNA was further demonstrated to be the result of an increased rate of RNA synthesis from mid-exponential growth to a maximum in early growth deceleration. Although the characteristics of Rudick's cultures appeared to be the same as in the cultures of Byers et al. (1969) (i.e., generation time, period of growth deceleration, and time and degree of encystment), Rudick (1971) concludes that a buildup in RNA is necessary for the mechanism of induction of encystment in acanthamoeba.

The apparent dissimilarities in Rudick's (1971) results and ours cannot be fully explained at this time. The greater increase in RNA by the end of growth deceleration in Rudick's cells can be accounted for by using the same reasoning as applied to the study of Byers et al. (1969) (see above). However, the changing level of RNA in logarithmic growth in acanthamoeba grown in unagitated cultures has not been found in suspension grown acanthamoeba. Conceivably, the discrepant results could be due to different methods of analysis, but until additional information is available, we must leave open the possibility that RNA metabolism during growth and encystment of acanthamoeba may not be identical in cells grown under agitated (suspension) and unagitated (monolayer) conditions. Nevertheless, we feel that our results on analysis of RNA synthesis is acanthamoeba undergoing encystment either in stationary growth or after experimental induction strongly imply that a buildup of RNA is not necessary for induction of encystment. The results further demonstrate that the rates of RNA synthesis and turnover in acanthamoeba are closely regulated by the events that control cell growth and differentiation.

The authors gratefully acknowledge the excellent technical assistance of Mrs. Barbara O'Dell.

This study was supported by designated research funds from the Veterans Administration, Project No. 5148-01, and by Grant AI 09608 from the National Institutes of Health awarded to A. R. Stevens (Larkin), Principal Investigator.

Received for publication 13 November 1972, and in revised form 12 January 1973.

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