RELATIONSHIPS BETWEEN NUCLEIC

ACID SYNTHETIC PATTERNS AND ENCYSTMENT

IN AGING UNAGITATED CULTURES OF

ACANTHAMOEBA CASTELLANII

V. L. RUDICK

From the Graduate Program in Zoology and the Academic Faculty of Microbial and Cellular Biology, The Ohio State University, Columbus, Ohio 43210

ABSTRACT

Changes in the levels of DNA and RNA syntheses have been studied in unagitated cultures of *Acanthamoeba castellanii* during the phases of logarithmic multiplication (LM) and population growth deceleration (PGD). Pulse-labeling experiments show that the rate of DNA synthesis decreases at the same time that DNA per cell is known to drop by 50%. The drop in DNA content has been explained by demonstrating with hydroxyurea that the majority of LM amebas can replicate once when DNA synthesis is inhibited and, therefore, must be in G_2, whereas the PGD amebas cannot multiply in the presence of inhibitor and, therefore, must be in G_1. The inhibition of DNA synthesis in LM or PGD cells has been shown to induce encystment. The rate of RNA synthesis, as illustrated by pulse-labeling experiments, increases 25% in late LM–early PGD while RNA per cell increases 75%. The rate of synthesis then decreases 65%. The majority of accumulated RNA has been demonstrated to be ribosomal by disc electrophoresis. By using actinomycin D at different stages during the RNA build-up, the ability of the amebas to encyst has been shown to depend on the presence of this RNA. The observations on DNA and RNA are discussed with respect to the occurrence of cysts in the cultures during PGD.

INTRODUCTION

Previous studies performed by Byers et al. (1969) on unagitated cultures of *Acanthamoeba castellanii* have revealed that there is a 75% increase in RNA per ameba, without a significant rise in cellular protein, and a 50% decrease in the DNA per cell during the transition from logarithmic multiplication (LM)[1] to population growth deceleration (PGD). These changes are followed by an increase in the relative number of cysts.

These authors demonstrated that variations in the amounts of macromolecules per ameba are initiated at the same time that the dissolved oxygen content of the culture medium decreases during mid- to late LM. Furthermore, they proposed that changes in nucleic acid levels may be associated with the ameba's preparation for encystment. Work by other authors has also given evidence that changes in nucleic acid metabolism are important in encystment (Neff and Neff, 1969; Bauer, 1967; Bowers and Korn, 1969).

---

[1] *Abbreviations used in this paper:* EM, encystment medium; HU, hydroxyurea; LM, logarithmic multiplication; OGM, optimal growth medium; PGD, population growth deceleration; PPO, 2,5-diphenyloxazole; TCA, trichloroacetic acid.

In an attempt to gain some insight into the events which occur during what appears to be a simple type of cellular differentiation, our laboratory has begun an intensive study of the encystment process in *A. castellanii*. This report examines the relationship of the RNA build-up and the drop in DNA per cell with aging and preparation for encystment in the trophozoite. A subsequent paper by Mattar and Byers (1971) will deal with the cyst form and the process of excystment.

MATERIALS AND METHODS

Cell Culture and Labeling Technique

Acanthamoeba castellanii (Neff's *Acanthamoeba* sp., clone I-12) was routinely cultured axenically in 50 ml of optimal growth medium (OGM) in 250-ml Erlenmeyer flasks as described previously (Byers et al., 1969). Except where noted, amebas were inoculated
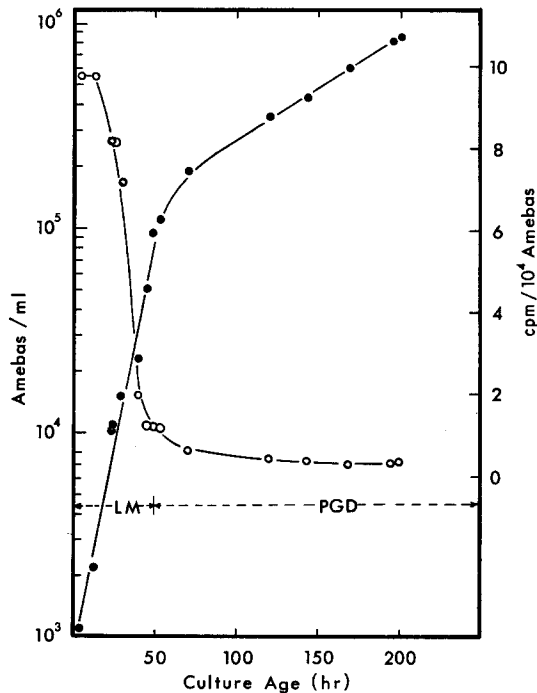

FIGURE 1 Thymidine-^{3}H incorporation by cells during culture aging. Closed circles, population growth curve. Amebas grown in 50-ml unagitated cultures under standard conditions. Phases of logarithmic multiplication (*LM*) and population growth deceleration (*PGD*) are indicated. Open circles, thymidine-^{3}H incorporation. Cells were pulse labeled for 100 min with 5 μCi/ml thymidine-^{3}H and then were fixed in 5% TCA. Each point represents average data from three flasks.

at 10^3 cells per milliliter. Under these conditions logarithmic multiplication (LM) lasts about 50 hr and then the cultures enter population growth deceleration (PGD) (see Fig. 1). For labeling experiments, however, cells were grown in 2-dr, 17 × 60-mm vials or 10-ml Erlenmeyer flasks sealed with aluminum foil caps and incubated at 30°C. The vials (flasks) contained 2 ml OGM which was 1.0 cm deep and, thus, there should have been about the same number of amebas per square centimeter of bottom surface area in the vials as were found in the 1.0 cm-deep 50-ml cultures.

Pulse labeling of either RNA or DNA was accomplished by adding 5 μCi/ml uracil-^{3}H or thymidine-^{3}H (Schwartz Bio Research Inc., Orangeburg, N.Y.), supplemented with the appropriate cold precursor at a final concentration of 10^{-5} M, to the cell cultures. After 100 min, the amebas were fixed by the addition of 2 ml cold 10% trichloroacetic acid (TCA). Cells were TCA-extracted at 0°–5°C for at least 24 hr and were then solubilized by the method of Bratt and Robinson (1967). The macromolecular components were collected as TCA precipitates on 0.45 μ membrane filters. Each filter was washed with 20-ml 5% TCA in 5-ml amounts. The dried filters were placed in scintillation vials containing 10 ml of toluene scintillation fluid (4 g 2,5-diphenyloxazole [PPO] and 150 mg 1,4 bis-2-[4 methyl-5-phenyloxazolyl] benzene in 1.0 liter toluene) and the radioactivity was counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Background controls consisted of medium plus isotope minus amebas. Each time that cells were pulse labeled, three vials of unlabeled amebas were fixed with 1.0 drop formalin per vial and the cell cell concentration was determined in an electronic particle counter (Celloscope 101, Particle Data Inc, Elmhurst, Ill.).

Studies of Cyst Formation after Inhibition of DNA Synthesis

Amebas used for inhibition studies were grown in the same manner as cells used for labeling experiments. Hydroxyurea (Sigma Chemical Co., St. Louis, Mo.), an inhibitor of DNA synthesis (Adams and Lindsay, 1967), was added to the medium at a final concentration of 30 mM. This concentration of hydroxyurea (HU) completely inhibits DNA synthesis in both LM and PGD cells without affecting either RNA or protein synthesis (Rudick, 1969, unpublished observations). For one series of experiments, in which cyst induction in OGM was followed, the cultures were aerated by agitation at 100 cycles/min in a reciprocating shaking water-bath (Eberbach Corp., Ann Arbor, Mich.). In this experiment, fixed trophozoites and cysts were counted on a hemocytometer.

RNA Isolation and Disc Gel Electrophoresis

ISOLATION: All operations were performed at 0°–5°C. A minimum of 10[7] amebas were centrifuged at 1.5 g and washed once in 0.15 M KCl. Cells were resuspended in 8 volumes 0.05 M sodium acetate (pH 5.1), containing 5 mM $MgCl_2$, and 1.0 volume 5% sodium dodecyl sulfate. To this was added an equal volume of 90% phenol, containing 0.1% 8-hydroxyquinoline as a preservative, and 100 µg/ml purified bentonite (Fraenkel-Conrat et al., 1961). Cells were homogenized in a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, N.J.), shaken for 15 min, and then centrifuged at 15,000 g for 10 min. The aqueous phase and the interphase were removed and shaken for 10 min with an amount of phenol equivalent to three-fourths of the original volume, and then an additional 100 µg/ml bentonite was added to the suspension. After centrifugation, the RNA was precipitated from the upper four-fifths of the aqueous phase by the addition of 2 M NaCl (final concentration 0.1 M) followed by 2 volumes 95% ethanol. The mixture was allowed to stand overnight at −20°C and then centrifuged at 12,000 g for 10 min. The RNA pellet was rinsed two to three times with 95% ethanol and resuspended in the E buffer of Bishop et al. (1967) (40 mM Tris, 20 mM sodium acetate, 1 mM NaEDTA, pH adjusted to 7.2 with glacial acetic acid). DNA contamination of the RNA preparation was negligible, so that DNase treatment was not required. The concentration of RNA in solution was determined by the optical density of the solution at 260 nm.

ELECTROPHORESIS: Acrylamide and N,N'-methylenebis-acrylamide (Eastman Organic Chemicals, Rochester, N.Y.) were purified according to Loening (1967). Preparation of 8-cm × 5-mm 6% polyacrylamide gels and 2.4% polyacrylamide gels containing 0.5% agarose (Bausch and Lomb, Inc., Rochester, N.Y.) was done according to the methods of R. A. Tassava (1969, unpublished observations). After 30 min of polymerization, gels were prerun in E buffer (Bishop et al., 1967) at 0°–5°C, 5 ma per tube and 50 v for 0.5–1.0 hr, using a regulated high-voltage power supply. Samples of RNA (25–50 µg) were loaded on the gels in 50 µl of E buffer containing 10% RNase-free sucrose (Mann Research Labs Inc., New York). Electrophoresis was performed at 0°–5°C, 5 ma per tube for 90 min.

After electrophoresis, gels were removed from the tubes, fixed in 1.0 M acetic acid for 15 min, and scanned at 260 nm in a Gilford gel scanner attached to a recorder. The areas under the peaks were determined from the chart recordings with a planimeter and were calculated as percentages of total RNA on each gel. By using the values for RNA per cell obtained by Byers et al. (1969), it was then possible to calculate the amounts of each class of RNA at various culture ages.

RNA Inhibition and Cyst Formation

Amebas were induced to encyst by transferring them from OGM to Neff's (Neff et al., 1964) nutrient-free encystment medium (EM) that undergoes a progressive increase in pH. Cells were recovered from OGM, washed twice in EM, and resuspended in 5 ml of EM in 25 × 150-mm Pyrex tubes at 5 × 10^{4} amebas per milliliter. One-half of the cultures contained 15 µg/ml actinomycin D (Calbiochem, Los Angeles, Calif.). This concentration of antibiotic has been shown to be greater than 90% effective in preventing RNA synthesis in A. castellanii (Mattar, 1970). All cultures were placed in a 30°C water bath and aerated at 4 ft[3]/hr per liter for 24 hr. Amebas were fixed and stained using an iodine–eosine Y staining technique developed by Mattar. Cyst numbers were determined in a hemocytometer.

RESULTS

DNA Studies

PULSE LABELING: Fig. 1 illustrates the pattern obtained when cells are pulse labeled with thymidine-[3]H. The amount of radioisotope incorporated into DNA is constant during the
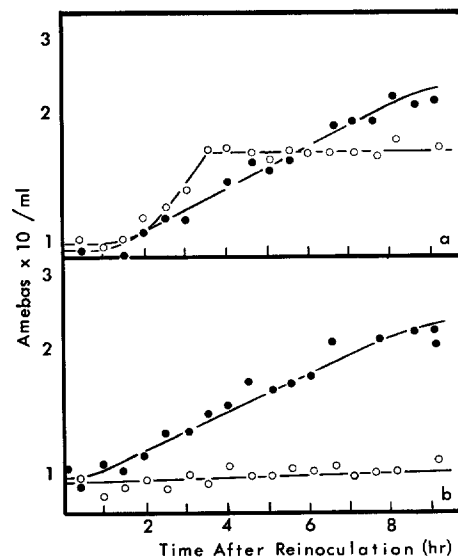

FIGURE 2 Influence of hydroxyurea on multiplication of LM and PGD amebas. Cells from unagitated 50-ml cultures in LM or PGD were aseptically harvested and then reinoculated at 10[4] amebas per milliliter into the same medium in the presence (open circles) or absence (closed circles) of 30 mM hydroxyurea. (a) Inoculum from culture in LM. (b) Inoculum from culture in PGD. Each point represents data from three replicate cultures.
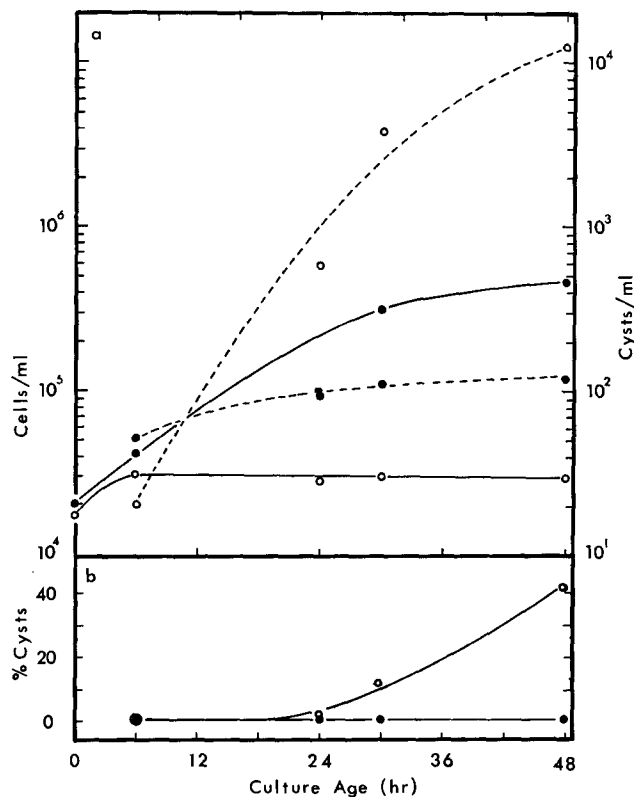
FIGURE 3 Influence of hydroxyurea on cyst formation. Amebas from 50-ml unagitated cultures in LM were harvested and used to inoculate 50-ml cultures of fresh OGM at LM concentrations. The cultures were then agitated at 100 cycles/min in a water bath shaker. Each point is an average for three flasks. Closed circles, plain OGM; open circles, OGM plus 30 mM hydroxyurea. (a) Growth curves for total cell numbers (solid line) and cysts (broken line). (b) Cysts as per cents of total cell populations in the experimental and control cultures.

nitial 20 hr of growth. At mid-LM, the rate of DNA synthesis begins to decrease. If the decrease is plotted logarithmically, it is found that the synthetic rate drops to half in 9.0 hr. During early PGD, the rate continues to drop, but more slowly, reaching a plateau at 150 hr. The rate of DNA synthesis has been repressed 96% by the latter time.

INHIBITION EXPERIMENTS: The previously reported (Byers et al., 1969) 50% decrease in DNA content per cell in the period from mid-LM (25 hr) to the beginning of PGD (50 hr) can be explained if it is assumed that most LM amebas contain the G_2, postsynthetic phase, amount of DNA and that the DNA synthetic cycle is inhibited during PGD in G_1 or at the transition between G_1 and S, the presynthetic and synthetic phases, respectively. If PGD cells are blocked at

this transition point and, thus, require DNA synthesis in order to multiply, then inhibition of DNA replication should prevent cell multiplication in PGD cultures but allow LM amebas, with the G_2 amount of DNA, to complete at least one cell doubling. In accordance with this hypothesis, LM and PGD amebas were exposed to hydroxyurea (HU) and cell growth was followed for at least one generation time.

Fig. 2 presents the data from the cell growth studies $\pm$ HU. LM or PGD cells grown in the absence of HU doubled within approximately 7.5 hr after a 1–2 hr lag phase. When LM amebas were exposed to the inhibitor, there was a 60–65% increase in cell number. In contrast, the level of PGD cells remained relatively constant in the presence of HU during the entire experiment. The same results were obtained whether the cells were

TABLE I

The Influence of 30 mM Hydroxyurea on Cell Growth and Per Cent Encystment in OGM

Cell concentration of inoculum stock (cells/ml)	Initial culture concentration after inoculation (cells/ml)	% cysts 48 hr after inoculation		Relative increase in cell concentration*	
		+HU	−HU	+HU	−HU
1 × 10⁴ (LM)	1–2 × 10⁴	41.7	0.03	1.8	23
1 × 10⁴ (LM)	2–3 × 10⁵	15.0	0.31	1.8	4.5
3 × 10⁵ (PGD)	1–2 × 10⁴	31	0.85	0	35
3 × 10⁵ (PGD)	2–3 × 10⁵	10	3.0	0	3.1

* Relative increase, cell concentration after 48 hr/initial culture concentration.

inoculated into fresh medium or into the medium from which they had been harvested.

In order to determine whether amebas in which DNA synthesis is inhibited would encyst, LM and PGD cells were collected from the medium in which they were growing and reinoculated into the same medium ± HU at two different cell concentrations. Cell growth and cyst formation were then studied. The greatest degree of encystment was seen in cultures containing LM amebas reinoculated at LM cell concentrations (Fig. 3). After 48 hr in the presence of HU, 41.7% of these cells encyst and, as expected from the studies illustrated in Fig. 2 *a*, the total cell number increases 1.8-fold and then remains constant. In the absence of HU, only 0.03% of the cells encyst and total cell growth is normal.

Table I presents the results obtained when encystment is followed using different cell concentrations and LM and PGD-aged amebas as inocula. Both LM and PGD cells encyst in the presence of HU after inoculation at either LM or PGD initial culture concentrations. However, encystment is better at LM culture concentrations for both LM and PGD cells. Furthermore, LM-aged amebas appear to form a greater percentage of cysts than do PGD cells after inoculation at either initial culture concentration: 1.3-fold and 1.5-fold greater at LM and PGD culture concentrations, respectively. In general, the different-aged inocula gave the same growth curves at both culture concentrations in the absence of HU. In the presence of the drug, the PGD inocula showed no growth and the LM inocula approximately doubled.

RNA Studies

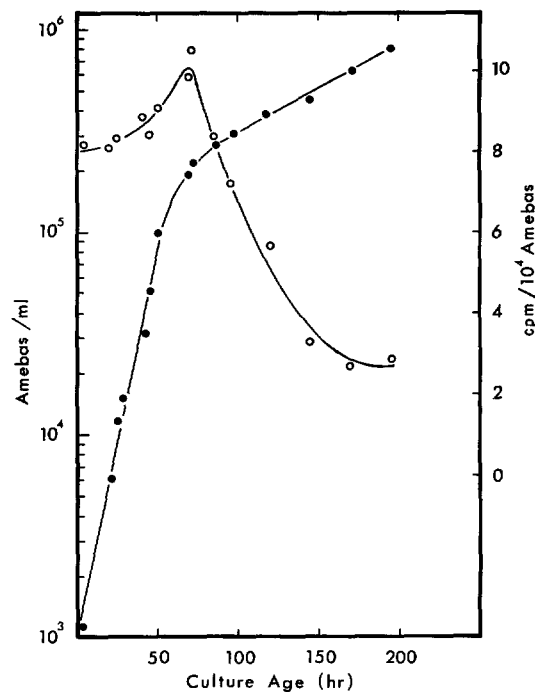PULSE LABELING: Fig. 4 illustrates the pattern obtained when cells are pulse labeled with

FIGURE 4 Uracil-³H incorporation by cells during culture aging. Closed circles, population growth curve. Cells cultured as in Fig. 1. Open circles, uracil-³H incorporation. Cells were pulse labeled for 100 min with 5 µCi/ml uracil-³H and then were fixed in 5% TCA. Each point is an average for three flasks.

uracil-³H. The rate of RNA synthesis begins to increase in mid-LM (25 hr) and reaches a maximum in early PGD (∼ 70 hr), a 1.23-fold increase over the initial value. It then begins to decrease until about 150 hr when it plateaus at a level 65% below the mid-LM value.

ELECTROPHORESIS: A typical electrophoretic pattern obtained for total RNA on a 2.4%

polyacrylamide gel is depicted in Fig. 5. From our preliminary comparisons of RNA from *A. castellanii* with bovine liver 28S and 18S RNA and with yeast 4S RNA, it appears that the three major peaks represent RNAs with sedimentation constants of approximately 30S, 20S, and 4S. The 4S peak can be resolved on a 6% polyacrylamide gel into two fractions believed to represent 5S and 4S RNA. Small peaks between the ribosomal
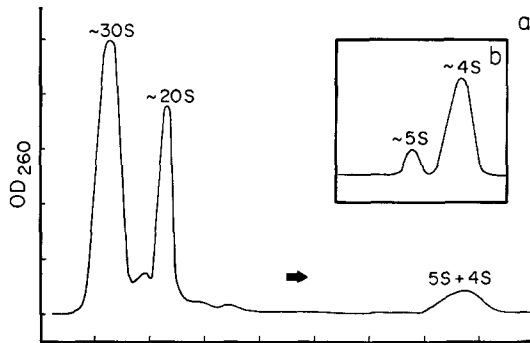

FIGURE 5 Polyacrylamide gel electrophoretic fractionation of *Acanthamoeba* RNA. The main figure and *inset* represent spectrophotometric scans of individual gels. (a) Total RNA separated on a 2.4% gel. (b) Fractionation of the 5S and 4S peaks on a 6% gel. Arrow indicates direction of migration.

components and after the 20S fraction were present in all RNA preparations. These have not yet been identified and are referred to here as "other" RNA to distinguish them from the major RNA peaks (30S, 20S, 5S, and 4S). The latter four RNA fractions increased approximately two-fold during the first 50–60 hr of growth (Fig. 6). The level of ribosomal components (30S, 20S, and 5S) reached a plateau at 75 hr and then remained constant. In contrast, the level of 4S RNA rose eightfold late in PGD. The other RNA decreased 50% by 75 hr and then was maintained at this depressed level through PGD. The table in Fig. 6 shows that the total RNA in 150 hr-old PGD amebas is enriched for ribosomal and 4S RNA at the expense of the other RNA which is abundant in 24 hr-old LM cells.

INHIBITION EXPERIMENTS: In order to determine whether the build-up of RNA illustrated in Fig. 6 is related to encystment, amebas at different culture ages were induced to encyst in EM ± actinomycin D. Fig. 7 illustrates that 69% of the cells induced at an age (24 hr) when RNA per cell is minimal are able to encyst in the absence of actinomycin D and that 74% can encyst when induced at 100 hr, an age when RNA per cell is maximal. In contrast, with actinomycin D only 10% of the 24-hr cells encyst. The amebas
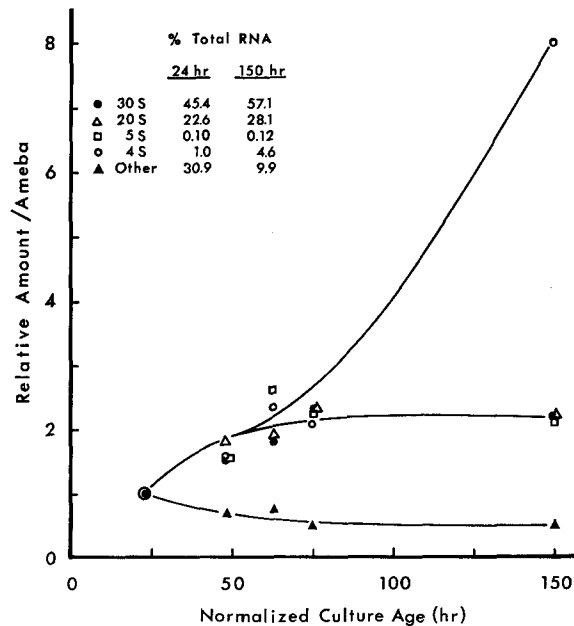

FIGURE 6 Changes in the relative amounts of five different RNA fractions during culture aging and the period of RNA accumulation. The table indicates the percentage of the total RNA that each fraction contributes at two different culture ages. The total RNA increases about 1.75 × from 24 to 150 hr.
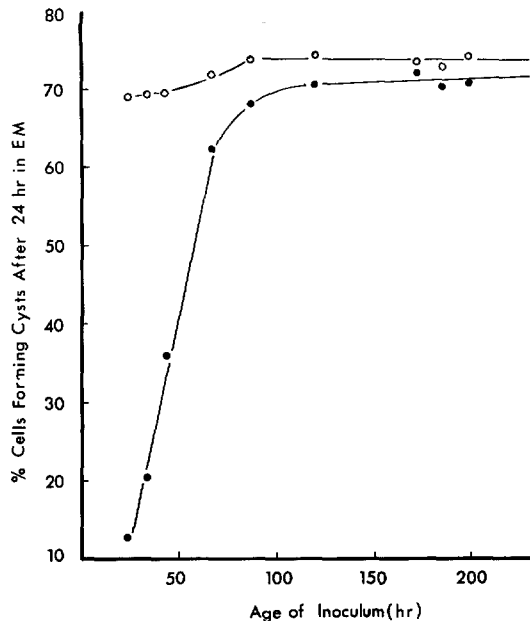
FIGURE 7 The influence of actinomycin D on the encystment of amebas from cultures of different ages. Cells were grown in 50-ml unagitated cultures in OGM. Then, at the ages indicated on the abcissa, they were transferred to EM ± actinomycin D in aspirator flasks and were aerated. After 24 hr in aerated EM, cultures were examined and cyst fractions were determined. The approximate state of the cells before being transferred to EM with regard to RNA synthesis and accumulation can be determined by examining Figs. 4 and 6 at the appropriate culture ages. Closed circles, EM + actinomycin; open circles, plain EM.

become less sensitive to the presence of the drug as they age, with maximum insensitivity reached by about 100 hr when 71 % are able to encyst.

DISCUSSION

Cysts begin to appear in OGM cultures in early PGD (Byers et al., 1969) and continue to increase through 100 hr after the beginning of this phase. Thus, the onset of PGD appears to trigger encystment, an observation which has been confirmed both by Byers et al. and by Neff and Neff (1969). However, it is also possible that the induction of encystment may occur as early as late LM and that the mechanism of induction is related to changes in macromolecule levels, particularly in levels of RNA and DNA.

The RNA per ameba has been shown to increase in actively multiplying LM *A. castellanii* (25-70 hr) and then to level off before the appear-

ance of cysts in the population during PGD (Byers et al., 1969). This build-up in RNA occurs due to an increased rate of RNA synthesis (Fig. 4), but the plateau level is maintained after a 65% decrease in the synthetic rate.

On the basis of the electrophoretic data, the majority of the accumulated RNA is seen to be ribosomal (table, Fig. 6), although 4S RNA shows the greatest relative increase.

The increase in total RNA in *A. castellanii* is very similar to that found in sporulating bacteria (Young and Fitz-James, 1959). Furthermore, Bauer (1967) has observed, and Bowers and Korn (1969) have confirmed, that conditions leading to encystment in *A. castellanii* result in the intracellular elaboration of stacks of membranes, the outside of which are densely coated with ribosomes. The rise in RNA level, particularly in ribosomal RNA components, may be partly due to the formation of ribosomes which would function in protein synthesis for cyst wall construction (Neff et al., 1964; Bauer, 1967). The eightfold rise in 4S RNA is not surprising in the light of evidence from sporulating organisms such as bacteria (Slepecky, 1969) and slime molds (Sauer et al., 1969), which also suggests that this component increases greatly before spore formation.

More direct evidence that the RNA build-up is related to encystment was obtained in the actinomycin D experiments. Actinomycin D was shown to interfere with cyst formation if administered before RNA accumulated in the cells. If the amebas were exposed to it at later times, the ability to form cysts became progressively insensitive to the inhibition of RNA synthesis. Maximum insensitivity corresponded with the beginning of the final plateau level reached by RNA in mid-PGD (Byers et al., 1969). Similar results have been obtained in sporulating *Bacillus cereus* (Aronson, 1965) and the slime mold, *Physarum polycephalum* (Sauer et al., 1969). In the former study the results were interpreted as reflecting the formation of stable messenger-RNA during vegetative growth of the bacteria which would later be used for the elaboration of spore components. Long-lived RNA (Tyler, 1967) and stored ribosomes (Brown and Dawid, 1968) are not uncommon in eukaryotes, and, in view of the actinomycin D data, it would appear that a similar phenomenon may be operating in *A. castellanii*. First, there is an enrichment both for ribosomal RNA and for 4S RNA before encystment. The former accumulates well before cysts

begin to appear in the cultures, so that the ribosomes resulting from this late LM–early PGD synthesis may not be used until late PGD when the amount of 4S RNA is enhanced. Second, evidence for long-lived RNA in *A. castellanii* has recently been obtained by Mattar (1970) in our laboratory. In brief, he has shown that there is some carry-over of RNA from trophozoite to cyst, and Mattar and Byers (1971) present evidence that some of this RNA is capable of promoting protein synthesis in a cell-free system.

The drop in *A. castellanii* DNA level (Byers et al., 1969) is coincident with the decrease in the rate of DNA synthesis from mid-LM to the end of LM. Since the rate of DNA synthesis drops logarithmically with a half time equal to 9.0 hr, it decreases approximately 50% every generation until 50 hr.

If it is assumed that most LM amebas contain the G_2 amount of DNA and that the DNA synthetic cycle is inhibited in the transition between G_1 and S, as was suggested in this paper and by Byers et al. (1969), then the decrease in DNA per ameba is explained. Direct evidence for this hypothesis was obtained by exposing LM and PGD cells to hydroxyurea (Fig. 2). If 100% of the LM cells had been in G_2, then all of them should have doubled, in spite of the fact that hydroxyurea was inhibiting DNA synthesis. However, the LM inocula had a normalized age of 30 hr, an age when DNA synthesis was already partially inhibited (Fig. 1) and when DNA per cell had dropped about 20% (Byers et al., 1969). These two facts, plus the fact that some fraction of the LM cells had to be in S phase, explain why the inhibited LM cells only increased 1.7-fold rather than twofold. In contrast, PGD amebas, which were hypothesized to be blocked in the transition between G_1 and S, were completely prevented from multiplying. These data, in addition, lend support to the observations reported by Neff and Neff (1969) which suggest that S is very short in logarithmically multiplying *A. castellanii*, occurring during the first 20% of the growth-replication cycle, and that G_2 is 75–80% of the cycle.

Neff and Neff (1966; 1969) reported that inhibitors of DNA synthesis induce encystment in *A. castellanii*, and this also has been found in the present study. Both LM and PGD amebas encyst in the presence of hydroxyurea. But LM amebas appear to be more competent to encyst than do PGD cells under the same conditions. On the basis of the actinomycin D experiments in the present study, it might be expected that PGD cells would be more competent to encyst since they had accumulated RNA. However, in order to induce maximal encystment, it was necessary to aerate the cells by agitation, and M. Rudick (1970) has demonstrated that agitation causes an increase in cellular RNA content in LM cells. Thus, possibly agitated LM cells do not have to undergo an additional RNA accumulation phase in order to achieve competence for encystment. The important point, however, is that either LM or PGD amebas, which had been exposed to hydroxyurea and hence contained the G_1 amount of DNA, were able to encyst.

Thus, two physiological events which occur before encystment and may be required for cyst induction are a build-up in RNA, the majority of which is ribosomal, and a decrease in cellular DNA from the G_2 to the G_1 or early S value. The factor(s) which induce changes in these macromolecule levels and terminate active growth have not yet been identified definitely. Such environmental elements as pH changes, nutrient deprivation, and waste accumulation appear not to be causative agents since both LM and PGD amebas reinoculated into the medium in which they had been growing at LM cell concentrations, as was done in some of the experiments presented here grow well. Byers et al. (1969) have implicated the decrease in oxygen tension as one factor which may induce encystment. Crowding may be another factor. Either or both of these changes in environment would probably be sufficient to induce the variations in DNA and RNA levels described and necessitate encystment in order to preserve the species.

REFERENCES

ADAMS, R. L. P., and J. G. LINDSAY. 1967. Hydroxyurea: Reversal of inhibition and use as a cell-synchronizing agent. *J. Biol. Chem.* **242**:1314.

ARONSON, A. I. 1965. Characterization of messenger RNA in sporulating *Bacillus cereus. J. Mol. Biol.* **11**:576.

BAUER, H. 1967. Ultrastruktur und Zellwand-bildung von *Acanthamoeba* sp. *Vierteljahresschr. Naturforsch. Ges. Zurich.* **112:**173.

BISHOP, D. H. L., J. R. CLAYBROOK, and S. SPIEGEL-MAN. 1967. Electrophoretic separation of viral nucleic acids on polyacrylamide gels. *J. Mol. Biol.* **26:**373.

BOWERS, B., and E. KORN. 1969. The fine structore of *Acanthamoeba castellanii* (Neff strain). II. Encystment. *J. Cell Biol.* **41:**786.

BRATT, M. A., and W. S. ROBINSON. 1967. Ribo-nucleic acid synthesis in cells infected with New-castle Disease Virus. *J. Mol. Biol.* **23:**1.

BROWN, D. D., and J. B. DAWID. 1968. Specific gene amplification in oocytes. *Science (Washington).* **160:**272.

BYERS, T. J., V. L. RUDICK, and M. J. RUDICK. 1969. Cell size, macromolecule composition, nuclear number, oxygen consumption and cyst formation during two growth phases in unagitated cultures of *Acanthamoeba castellanii. J. Protozool.* **16:**693.

FRAENKEL-CONRAT, H., B. SINGER, and A. TSUGITA. 1961. Purification of viral RNA by means of bentonite. *Virology.* **14:**54.

LOENING, U. E. 1967. The fractionation of high-molecular weight ribonucleic acid by polyacryla-mide gel electrophoresis. *Biochem. J.* **102:**251.

MATTAR, F. E. 1970. Changes in cellular morphology and macromolecule synthesis during excystment of *Acanthamoeba castellanii.* Ph.D. Dissertation. The Ohio State University, Columbus.

MATTAR, F. E., and T. J. BYERS. 1971. Morphological changes and the requirements for macromolecule synthesis during excystment of *Acanthamoeba castel-lanii. J. Cell. Biol.* **49:**507.

NEFF, R. J., and R. H. NEFF. 1966. Induction of differentiation by antibiotics, antimetabolites, and cytostatic agents. *J. Cell Biol.* **31:**80 A. (Abstr.)

NEFF, R. J., and R. H. NEFF. 1969. The biochemistry of amoebic encystment. *Symp. Soc. Exp. Biol.* **23:**51.

NEFF, R. J., S. RAY, W. BENTON, and M. WILBORN. 1964. Induction of synchronous encystment (dif-ferentiation) in *Acanthamoeba* sp. *In* Methods in Cell Physiology. I. D. Prescott, editor. Academic Press Inc., New York. 55.

RUDICK, M. J. 1970. The environmental control of cytochrome oxidase activity in *Acanthamoeba castel-lanii.* Ph.D. Dissertation. The Ohio State Univer-sity, Columbus.

SAUER, H. W., K. L. BABCOCK, and H. P. RUSCH. 1969. Changes in RNA synthesis associated with differentiation (sporulation) in *Physarum poly-cephalum. Biochim. Biophys. Acta.* **195:**410.

SLEPECKY, R. A. 1969. Synchrony and the formation and germination of bacterial spores. *In* The Cell Cycle. G. M. Padilla, G. L. Whitson, and I. L. Cameron, editors. Academic Press Inc., New York. 77.

TYLER, A. 1967. Masked mRNA and cytoplasmic DNA. *In* Control Mechanisms in Developmental Processes. M. Locke, editor. Academic Press Inc., New York. 170.

YOUNG, I. E., and P. C. FITZ-JAMES. 1959. Chemical and morphological studies of bacterial spore formu-lation. I. The formation of spores in *Bacillus cereus. J. Biophys. Biochem. Cytol.* **6:**467.