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CELL SIZE AND MACROMOLECULE
COMPOSITION DURING AGING IN CULTURES
OF ACANTHAMOEBA CASTELLANII.

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CELL SIZE AND MACROMOLECULE COMPOSITION DURING AGING 
IN CULTURES OF ACANTHAMOEBA CASTELLANII

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * * * * *

The Ohio State University
1969

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INTRODUCTION

Differentiation is generally regarded as the central process in an organism's development, for it involves the changes in structure and chemical composition that make that particular organism unique. Perhaps the least complicated, and for this reason the most useful, type of differentiation to study is characteristic of tissue culture cells and unicellular organisms which undergo physiological modifications as they age in culture. When a bacterial population passes through its growth phases, changes occur in cell size and biochemical content as the accumulation of some metabolites and the exhaustion of others subject the cells to a continually varying environment to which they must adjust (Dean and Hinshelwood, 1959; Dean, 1961; 1962). Studies with Aerobacter aerogenes (Dean and Hinshelwood, 1966; Maalire and Kjeldgaard, 1966) suggest that the bacterial cell is, thus, constantly differentiating, by regulation of macromolecule levels, in an attempt to attain an overall steady state between the cell and the current culture conditions.

Populations of eukaryotes react in a manner similar to bacteria. Both mouse (Swaffield and Foley, 1960) and HeLa cells (Saltzman, 1959), in suspension or in monolayers, pass through lag, logarithmic and stationary phases of growth, each of which is characterized by cell differences in ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and protein. Work with some protozoans such as Euglena gracilis
(Buetow and Levedahl, 1962; Pogo et al., 1966; Cook and Heinrich, 1968), Astasia longa (Buetow and Padilla, 1963) and Tetrahymena pyriformis (Prescott, 1957) indicate that as these cells age their macromolecule composition, dry weight, and respiration rate also change with alteration of the environment. Most of the studies cited, however, have simply verified that cellular modifications occur with aging. It is probably more useful to look at examples of directed change, or differentiation, that lead toward easily defined morphological states.

Sporulation and encystment are phenomena which are characteristic of many unicellular organisms. Awareness that these processes represent model systems for studying mechanisms of differentiation at a basic level is indicated by the recent appearance of many reviews in these areas (Halvorson, 1965; Halvorson et al., 1966; Vinter, 1967; Murrel, 1967; Kornberg et al., 1968).

The process of sporulation is probably the most comprehensively studied example of directed differentiation. The physiological and morphological events associated with the formation of spores have been recorded in a number of different systems, such as bacteria (Young and Fitz-James, 1959a, b; Fitz-James, 1960; Halvorson and Srinivasan, 1964; Aronson, 1965; Ryter and Szulmajster, 1965; Kogoma and Yanagita, 1967) and fungi (Gregg and Bronsweig, 1956; Sussman, 1966; Tubaki, 1966). However, studies on the induction and regulation of bacterial sporulation are made somewhat difficult because the mother cell is apparently still capable of further macromolecule synthesis.
during early stages of spore formation (Young and Fitz-James, 1959a). Thus, since the spore develops from and remains within the mother cell, there are two distinct but interacting biosynthetic systems within one bacterium.

Of the fungi, the cellular slime molds (Acrasiales) have been the most extensively studied. Gregg (1966) and Bonner (1967) have reviewed the literature concerning differentiation in the slime molds. These plants are unique in that while single cells grow indefinitely in the presence of sufficient food, under starvation conditions, the cells aggregate into a mass, part of which develops into a stalk that bears a spore containing encapsulated cells (Sussman, 1955). Cellular slime mold development, then, just as bacterial sporulation, represents a process complicated by cell-cell interactions.

Protista offer some of the best opportunities for examining simple types of differentiation. Cyst formation is common in many protozoans (Hanson, 1967) and the environmental factors controlling encystment are the same as those which terminate logarithmic growth in a population of aging cells (Hanson, 1967; Dean and Hinshelwood, 1966). For this reason, the study of organisms actively growing and then encysting in culture is an ideal means of examining both cellular aging and the events which culminate in a specific conversion from one morphological state to another.

_Acanthamoeba castellanii_ possesses a relatively uncomplicated life cycle, consisting of a vegetatively multiplying amoeboid form alternating, under unfavorable environmental conditions, with a cyst form and, thus, fits well the requirements for studying simple
cellular differentiation. This organism is especially suited to this type of investigation because it can be grown axenically and can be induced to encyst upon transfer to nutrient-free medium.

Although encystment has been studied before in *Acanthamoeba* (Band, 1961, 1963; Neff *et al.*, 1964; Griffiths *et al.*, 1966; Griffiths and Hughes, 1969) and in other protozoans (Schuster, 1963; Groh and Buhse, 1968; Brown and Finley, 1968), it has usually been induced by placing the cells in a minimal defined medium, as in bacterial sporulation studies (Slepecky, 1969), rather than following the process in the typical nutrient medium of the organism. Cysts do occur naturally in aging cultures of *Acanthamoeba* (Volkonsky, 1931) and, therefore, biochemical changes leading to encystment must occur in populations of actively growing cells as they age. Since encystment and sporulation appear to be part of the normal life cycle of many organisms, it seems more logical to study them as natural components of growth rather than as artificially induced phenomena. For these reasons, this dissertation has attempted to describe the physiological and morphological changes during aging in *Acanthamoeba castellanii* and preliminary efforts have been made to associate these changes with encystment.
MATERIALS AND METHODS

The Organism and Its Culture

The organism used in this study was originally obtained from Dr. R.J. Neff, Vanderbilt University, and was designated by him as *Acanthamoeba* sp., clone I-12. However, Page (1967) has since re-defined the genus *Acanthamoeba*, including Neff's strain, and has indicated that the latter should be called *Acanthamoeba castellanii* (Douglas, 1930). The clone I-12 is characterized by its ability to encyst readily under unfavorable conditions. The original cell line was begun in 1964 from a single cell and has not been recloned for this study.

The amoebae were grown axenically in 50 ml of medium in 250 ml Erlenmeyer flasks sealed by aluminum foil or stainless steel culture caps. Cultures were grown at 30°C in a dark incubator without aeration or agitation. Neff's (1964) optimal growth medium (OGM) was used routinely for culturing cells. It contains per liter: 7.5 g each of proteose peptone (Difco) and yeast extract (Difco), salts (246 mg MgSO₄·7H₂O, 7.4 mg CaCl₂·2H₂O, 272 mg KH₂PO₄ and 30 mg ferric citrate), vitamins (0.5 µg B₁ hydrochloride, 0.1 µg biotin, 5 x 10⁻⁴ µg B₁₂), and 1.0 N KOH to bring the pH to 7.0. Thirty percent glucose (w/v) was autoclaved separately and added aseptically to the medium at the time of inoculation to give a final concentration of 1.5%.
Autoclaving of OGM immediately after its preparation results in the formation of precipitates which interfere with macromolecule analyses and electronic counting of amoebae during the subsequent culture period. To eliminate these precipitates, the medium was heated to 85-90 C and allowed to stand at that temperature for 5-10 minutes. It was then cooled to 25-30 C with an immersion cooler. The pH was checked at this time and, if necessary, adjusted to pH 7.0 with additional 1.0 N KOH. The cooled medium was then passed under pressure through a 0.8 μm pore membrane filter preceded by a fiberglass prefiltro (Type AP 25, Millipore Corp.). After being autoclaved at 18 psi for 20 minutes, medium prepared in this manner remained precipitate-free and usable for several weeks.

Inoculations were performed aseptically under ultraviolet lamps in a hood. Stock cultures of amoebae were subcultured approximately every 2-3 days and most experiments were begun using cells in logarithmic growth.

For one series of experiments the amoebae were induced to encyst by transferring them from OGM to Neff's (1964) nutrient-free encystment medium (EM). A liter of this medium contains 7.45 g KCl, 2.42 g amine buffer (tris(2-amino-2-hydroxymethyl 1,3-propanediol), 1.83 g MgSO₄, 58.8 mg CaCl₂, 84 mg NaHCO₃ and 1.0 N KOH to make the solution pH 9.0. The EM was filtered before being autoclaved.

In the encystment studies, cells were recovered from OGM, washed twice in EM and resuspended into 5 ml of EM in 25 x 150 mm Pyrex test tubes at 5 x 10⁴ amoebae/ml. Because acanthamoebae require vigorous
aeration to encyst (Neff et al., 1964), cells cultured in EM were placed in a 30 C water bath and aerated at 4 ft$^3$/hr/l for 24 hours.

Cell Counting and Sizing

Amoebae grown in OGM were counted in an electronic particle counter (Celloscope 101-TN, Particle Data, Inc.) fitted with a 100 μ orifice. Since Neff (1964) had experienced clumping of cells and extremely high background rates when an electronic counter was used for counting acanthamoebae, various precautions were taken to try to eliminate these problems.

First, only amoebae grown in filtered culture medium were used for counting. Cells were fixed by the addition of 0.25 ml formalin per 50 ml culture. Since the cells adhere to the glass surface even after fixation, they were first loosened from the flask wall with a rubber policeman before being collected by centrifugation at 600 x g for 3 minutes. The cell pellet was then resuspended with a vortex mixer and counted in 0.5% (v/v) formalin-0.6% (w/v) NaCl which had been filtered in the same manner as had the growth medium. In addition, all glassware involved with cell counting was rinsed thoroughly with filtered distilled water. To ensure that the cells were randomly dispersed in the medium while being counted, they were gently agitated by hydropulsion provided by the vacuum pump of the particle counter. With this method cell clumping rarely occurred and background in cell-free counting medium or OGM was less than 20 counts per ml.
The averages of the cell concentrations obtained from three flasks at each sampling time were used to plot the growth curves and compute population doubling times. A least squares analysis of growth was employed to calculate the slope of the regression line best fitting the points in the logarithmic portion of the growth curve. The slope was then used to determine the value of the population doubling time and the normalization time. The normalization time was the value that had to be added to each sampling time in order to make zero time of the growth curve correspond to $1 \times 10^3$ amoebae/ml.

Cells grown in EM were fixed by the addition of 2 drops of formalin per 5 ml culture, centrifuged at $600 \times g$ for 5 minutes and resuspended in 0.1 M acetate buffer, pH 5.15. The amoebae were then stained in the acetate buffer using an iodine-Eosin Y staining technique developed by Mattar (1969). This stain allows trophozoites, dormant, activated and empty cysts to be differentiated from each other, thus making microscopic detection of encysted forms more reliable. The stained amoebae were counted on a haemocytometer.

Amoeba volumes were determined simultaneously with cell concentrations by the use of a 128-channel pulse-height analyzer (ND 110, Nuclear Data, Inc.) connected to the output of the particle counter. Amplified voltage pulses received from the celloscope as cells pass through the counting orifice are proportional in amplitude to the volumes of the amoebae. The analyzer determines the amplitude of each incoming pulse and categorizes it into 128 linearly related size classes (channels). The data were obtained in the form of a
cell number versus pulse-height histogram on a chart recorder (Dohrmann S-Y 850 Recorder). Glass beads and pollen of ragweed and mulberry obtained from Particle Information Service, Palo Alto, California, were used as standards to relate channel numbers to volumes.

In older cultures as cell volumes increased and exceeded the upper limit of the pulse-height analyzer, it was necessary to replace the 100 μ orifice with one of 150 μ diameter. This procedure permitted a continuous volume distribution to be obtained for cells of all ages.

Formalin fixation causes amoebae to swell. Dolphin's (1968) data indicate that volumes measured by the same method as used in this study were about 15% greater than actual cell volumes. However, the volume data presented in this work have not been corrected for this swelling.

Macromolecule Assays and Dry Mass

A. Total Protein

Samples of 5-10 x 10^4 cells were collected by centrifugation at 600 x g for 3 minutes and washed twice with 0.15 M KCl. From the final wash suspension, a 0.25 ml aliquot was removed for a cell count. The washed pellet was resuspended in 5% trichloroacetic acid (TCA) and allowed to stand for 20 minutes at room temperature. The acid-insoluble material was collected by centrifugation at 12,000 x g for 15 minutes and washed twice in 5% TCA.
Total amoeba protein was determined using the Lowry method as modified by Oyama and Eagle (1956). Five ml of C reagent (50 parts reagent A, containing 20 g Na$_2$CO$_3$, 4 g NaOH, 0.2 g NaKtartrate in 1.0 l water, to one part reagent B, containing 5 g CuSO$_4$·5H$_2$O in 1.0 l water), made up fresh daily, was added to the washed acid-insoluble pellet. This was followed by the addition of 0.5 ml standardized Folin-Ciocalteau reagent squirted rapidly into the C reagent with a syringe. The optical density was determined at a wave length of 660 nm after 30 minutes incubation at room temperature. Samples of crystalline bovine albumen (10-70 μg) were treated similarly and served as standards.

B. Nucleic Acids

All operations for nucleic acid extraction were carried out at 0-4 C. Samples of $5 \times 10^7$ amoebae were collected and washed twice in 0.15 M KCl. From the final washed cell suspension, a 0.01 ml aliquot was removed for a cell count.

Nucleic acid extraction was performed using a modified method of Munro and Fleck (1966) and Fleck and Begg (1965). The washed pellet was resuspended in 3 ml of cold 0.15 M KCl to which was added cold 0.6 N perchloric acid (PCA) to a final concentration of 0.2 N PCA. After 10 minutes, the mixture was centrifuged and the nucleic acid-containing pellet washed twice with cold 0.2 N PCA. Two ml of 0.3 N KOH were added to the pellet and the mixture was incubated for 1.0 hour at 37 C. The hydrolysate was cooled to 0 C and then protein
and DNA were precipitated by the addition of 1.25 ml of 1.2 N PCA. After standing 10 minutes, the precipitate was pelleted by centrifugation and the ribonucleotide-containing supernatant was carefully removed and saved. The DNA-containing pellet was washed twice with 1.0 ml 0.2 N PCA, each time adding the washes to the original ribonucleotide solution. This fraction was diluted to 0.1 N PCA and the optical density at 260 nm determined. Samples of yeast RNA were treated similarly and used as standards.

DNA was extracted from the final pellet by heating it at 70°C for 15 minutes in 1.0 N PCA. The extraction procedure was repeated twice, each time saving the supernatants. The combined supernatants were then assayed for deoxyribose using the Burton reaction as modified by Giles and Meyers (1965). Aliquots of salmon sperm DNA (10-100 μg) served as standards. All samples were incubated at 30°C overnight and the optical density determined at 595-700 nm against a 1.0 N PCA blank. Occasionally, this modification results in the formation of a black precipitate (Burton, 1969) which was removed by centrifugation prior to spectrophotometer reading.

C. Glycogen

Two methods were employed to assay for amoeba glycogen. The first was adapted from McCready et al. (1950) and Cook (1967). Samples of 2-4 x 10^5 cells were washed three times in 0.15 M KCl and then extracted twice with boiling 55% ethanol to remove free sugars. Following centrifugation, the cell residue was hydrolyzed at 25°C for
10 minutes in 2 ml 1.0 N NaOH. Two ml 50% PCA were added to the hydrolysate and the samples chilled to 0-5 C. Six ml of cold anthrone reagent (2 mg/ml cold concentrated H₂SO₄) were then squirted into the tubes. The samples were placed in a boiling water bath for 10 minutes, cooled to room temperature and the optical densities read at 630 nm.

In the second assay, the glycogen was first isolated and then hydrolyzed to glucose. These procedures were adapted from the work of Bowers and Korn (1968). Samples of 5 x 10⁵ washed cells were subjected to hydrolysis by 30% KOH for 3 hours at 90 C. The solubilized glycogen was precipitated by the addition of ethanol to a final concentration of 65%. A volume of saturated Na₂SO₄ equivalent to 1% of the volume of the ethanol used was added as a coprecipitant (Van Handel, 1965). The glycogen was collected by centrifugation, dissolved in distilled water and reprecipitated in 65% ethanol until the washes were neutral in pH. The washed glycogen was hydrolyzed to glucose in 1.0 N H₂SO₄ for 1.0 hour at 100 C and the final solution neutralized with 1.0 N NaOH.

The assay for glucose was adapted from Dubois et al. (1951) and Dubois (1956). An aliquot of 0.05 ml 80% phenol was added to the glucose solution, followed by 5 ml concentrated H₂SO₄. After incubating 30 minutes at 25-30 C, the optical densities at 490 nm of the samples were determined.
D. Dry Mass

Samples of $10^8$-$10^9$ cells were washed three times in 0.15 M KCl and transferred onto tared aluminum planchets. These were dried overnight at 110°C and were then dessicated over Drierite and concentrated H$_2$SO$_4$ until constant weights were achieved. Control planchets were used to correct for the weight of the salts in the suspending medium.

Electron Microscopy

Pellets of washed amoebae containing $10^7$ cells were prepared for electron microscopy using the methods of Bowers and Korn (1968). Cells were collected at two different times during growth, logarithmic and population growth deceleration phases. Amoebae were fixed in suspension with 3% glutaraldehyde in 0.1 M NaH$_2$PO$_4$ buffer, pH 6.8, containing 1.0 mM CaCl$_2$ for 1 hour at room temperature. After centrifugation, the cells were rinsed in four changes of cold phosphate buffer over a 2 hour period and then were postfixed with cold 1% osmium tetraoxide in the same phosphate buffer for 2 hours in an ice bath. The fixed amoebae were then dehydrated through a graded series of cold ethanol (35-100%), taken through propylene oxide (two 15 minute washes) and embedded in Epon 812. Thin sections were stained on copper grids for 5 minutes in 1% uranyl acetate and 5 minutes in lead citrate. The sections were examined with a RCA EMU-36 electron microscope at 50 kV and photographed at magnifications of 2800-17,000.
Labeling Experiments

For a typical labeling experiment, cells were grown in OGM supplemented with cold precursor at a final concentration of $10^{-5}$ M. Cells from either 25-30 hour cultures with cell concentration of 1-2 x $10^4$ cells/ml or 70-80 hour cultures at 2-3 x $10^5$ cells/ml were removed from the flask walls, centrifuged and reinoculated into the original medium at a concentration of 2 x $10^4$ amoebae/ml. For one experiment only, the cells were reinoculated at a concentration of 2 x $10^6$ amoebae/ml. The radioisotope was added as the diluted cells were mixed with a magnetic stirrer. Two ml aliquots of the radioactive cell suspension were rapidly distributed to 2 dram, 17 x 60 mm vials, sealed with aluminum foil caps and incubated at 30 C. The medium in the vials was 1.0 cm deep and, thus, there were the same number of amoebae per cm$^2$ of surface area in the vials as were found in the 1.0 cm deep 50 ml logarithmically growing cultures.

Duplicate unlabeled cultures were started at the same time and were used for determining changes in cell number during the experiment. Controls contained medium and isotope but no amoebae.

Cells were continuously labeled for 7-9 hours. At various intervals during the experiment, uptake was monitored by fixing 3 culture vials and 3 control vials with 2 ml of cold 10% TCA. Amoebae were TCA-extracted in an ice bath for several hours to overnight. The extracted cells from each vial were then transferred to conical centrifuge tubes, centrifuged, the supernatant discarded and the cells lysed by the addition of 0.5 ml sodium dodecyl sulfate (SDS)
solution (1% w/v SDS, 1% v/v mercaptoethanol, 0.01 M tris buffer, pH 8.5, 0.1 M NaCl and 0.002 M ethylenediaminetetraacetate). Lysis was completed by placing the suspension in a boiling water bath until total liquification occurred (Bratt and Robinson, 1967). After cooling, the volume in the tubes was brought up to 3 ml with water containing 0.02% carrier RNA. The macromolecular components of this solution were precipitated with TCA (final concentration 5%) in the cold and collected as precipitates on 0.45 μ pore millipore filters. Each filter was washed with 20 ml 5% TCA in 5 ml aliquots. The dried filters were placed in scintillation vials containing 10 ml of toluene scintillation fluid (4 g PPO and 150 mg dimethyl POPOP in 1.0 l toluene) and the radioactivity counted in a TriCarb Liquid Scintillation Spectrometer (Packard Instrument Co.). Controls were treated similarly. In addition, each time labeled cells were sampled, three vials of unlabeled amoebae were fixed with 1.0 drop formalin/vial and counted in the particle counter.

During some labeling experiments, hydroxyurea, an inhibitor of DNA synthesis (Adams and Lindsay, 1967), was added to the medium at a final concentration of 30 mM simultaneously with the isotope. Radioactive cultures containing the inhibitor were treated in the same manner as were labeled uninhibited cultures and unlabeled amoebae which had been exposed to hydroxyurea were counted periodically throughout the experiment as were uninhibited cells.
RESULTS

Growth Curves

An average growth curve for 13 experiments is represented in Figure 1. The values for this curve were obtained by first fitting curves of individual populations to the data from each experiment by a least squares analysis for the logarithmic portions and by eye for the remainder. The individual curves were then normalized to a common initial concentration of $1 \times 10^3$ amoebae/ml and the average curve obtained by calculating the mean cell concentrations at 25 hour intervals.

The average doubling time for the 13 experiments was 7.48 with a standard deviation of ±0.9 hours. Since cultures were always begun from logarithmically growing stocks, the average age being 36.75 hours when cultures were started for nucleic acid analyses and 24 ± 1 hour for all other experiments, it was thought that there would be no significant lag phase and, indeed, with the standard sampling procedure, none was observed. For this reason, and since growth was terminated prior to maximum stationary phase, the growth curves for these cultures may be divided into two phases, logarithmic multiplication (LM), lasting about 50 hours, and population growth deceleration (PGD), following 50 hours on the normalized time scale.
Figure 1. An average population growth curve for cultures of A. castellaniii. The data are from 13 experiments. Each point represents the mean cell concentration for a 25 hour interval.
Populations were usually sampled at the time of inoculation and not again until 8-10 hours had elapsed. As indicated, data obtained in this manner suggested that LM begins at zero time. However, when populations were sampled at 0.5 hour intervals from the time of inoculation, deviations from logarithmic growth became evident. Figures 2a and 2b show the results from an experiment in which amoebae from LM and PGD were reinoculated at LM cell concentrations into cell-free medium recovered from their respective original 50 ml cultures. A 1-2 hour lag phase and evidence for some synchrony in cell division were observed using either age inoculum. The details are missed in the standard sampling procedure because the lag and the following accelerated rates of division combine to produce a doubling in cell number in 7-8 hours which, of course, is the rate established in LM.

When fresh instead of recovered medium was used, and the cells were washed in 0.15 M KCl before reinoculation, the lag phase of PGD amoebae was extended to 2.5 hours whereas LM cells continued to show a 1-2 hour lag. Nevertheless, both populations doubled within 8 hours.

Amoebae inoculated into either fresh or recovered medium were disturbed by the scraping required to detach the cells from the flask walls, by the washing procedure before reinoculation into the fresh medium, and by the stirring used to suspend them in the medium during the 0.5 hour it took to distribute cells to replicate sample vials. A semi-quantitative estimate was made by eye of the time it took for the amoebae to resettle to the flask bottom after inoculation. Approximately 1.5-2.0 hours elapsed before the cells had attached to the
Figure 2. Growth of *A. castellanii* during the first generation following inoculation. Each point of these curves is an average of 3 samples. A—LM Cells inoculated into cell-free recovered medium. B—PGD cells transferred to cell-free recovered medium.
glass and this may explain the observed lag phases. It is probable that a lag phase occurred in 50 ml cultures as well as in 2 ml ones, but this was not checked.

Cellular Changes During Growth

a. Volume Distributions

Volume distributions were followed as a function of population age. Figure 3 depicts volume distributions at three culture ages. The curves were prepared from a recorded histogram which indicated the number of amoebae in each of 128 size classes each having a volume range of 970 μ³. Modal volume, i.e. the most frequent volume, which is marked by a dotted line in Figure 3, increased after the completion of exponential growth. All of the distributions were skewed to the right, and as the populations aged, the distributions became wider, indicating an increase in the variance of the populations.

In order to compare the skewed volume distributions at different culture ages, the volume measurements were transformed to another parameter in an attempt to obtain a normal distribution. Using methods suggested by Bliss (1967) and first used for A. castellanii by Dolphin (1968), a normal distribution was obtained, as indicated by a straight line on probability paper, by plotting the diameters of spheres equivalent in volume to the cells versus cell frequency. Figure 4 illustrates three probability plots computed at different times during growth. Volume intervals were chosen which correspond to diameter intervals of 0.5 μ and the relative cumulative frequency
Figure 3. Volume distributions of A. castellanii growing in optimal growth medium. The total number of cells measured is indicated for each distribution. A- Twenty-four hour culture. B- Forty-seven hour culture. C- One hundred and twenty hour culture.
Figure 4. Probit plot of diameters of spheres equivalent in volume to cells. A represents cells from a 24 hour culture; B represents cells from a 47 hour culture; and C represents cells from a 120 hour culture.
was determined at these intervals. The relative cumulative frequency of the total, transformed distribution was then plotted on probability paper versus the diameter. The slope of these curves is a measure of the variation of the cell size in the distribution. As the population aged, the variation increased, as shown by the decreased slope. The average cell diameters were read from such plots as the value corresponding to 50% probability.

Changes in modal and average cell volumes as a function of population age are illustrated in Figure 5, curves A and B. Modal volumes were taken from volume distribution histograms such as those in Figure 3. Calculation of modal volume from diameters obtained with probability plots provided a means of checking on the accuracy of modal volumes read from the histograms.

The minimal modal volume obtained was 2830 ± 249 μ^3 for 25 cultures aged 25-50 hours on the normalized scale. During PGD, the modal volume of the individual cells gradually increased to a maximum value of 3800-4000 μ^3 which was achieved prior to the beginning of stationary phase. LM cells had diameters averaging 18.02 μ while those of PGD amoebae averaged 31.30 μ.

Since the volume histograms were not normally distributed, average cell volumes were obtained by dividing the distributions into 5-channel intervals (0-128 channels) and measuring the percentage of cells falling into each interval with a planimeter. From these data, the average 5-channel interval was calculated at each sampling time. By knowing the μ^3 of volume per channel obtained from standardization
Figure 5. Changes in modal and average volumes as a function of population age in *A. castellanii*. Curve A represents modal cell volumes. Each point represents the average of modal volumes collected over a 25 hour interval. Standard deviations are shown for groups having 3 to 25 separate measurements. Curve B shows average cell volumes. Each point represents the mean of average volumes for groups of 3-6 samples of approximately the same normalized age.
of the multichannel analyzer with the pollens and glass beads, it was possible to compute average cell volumes as a function of age.

During LM, the average volume computed for 10 cultures was $3062 \pm 384 \, \mu^3$ (Figure 5, Curve B). Cellular volume began to increase in PGD, reaching a plateau level at approximately 125 hours. The maximum average volume for 6 cultures older than 100 hours was $4930 \pm 408 \, \mu^3$.

b. Dry Mass

As the population aged, cellular dry mass increased (Figure 6), but changed less than average amoeba volume. During the first 100 hours, dry mass was relatively constant at 623 pg per cell. In late PGD, it increased 15-20% to reach a maximum value of 738 pg per amoeba at 200 hours.

c. Electron Micrographs

Average cell volume increased approximately 63% in the transition from log phase to PGD as compared with a 15-20% addition to dry mass. To determine whether part of the enlarged PGD cell volume was due to vacuolization, electron micrographs were taken of LM and PGD amoebae. All of the whole cells were photographed at a total magnification of 9800 so that it would be possible to compare sizes of cellular structures from cell to cell. Plates I-IV illustrate that as the amoebae aged there was an increase in the number of membrane-bound vacuoles seen in the cytoplasm. Many of these, especially in
Figure 6. Changes in average dry mass of A. castellanii as a function of culture age. Each value on the curve represents a single sample from one experiment.
PLATE I

Electron micrograph of logarithmically growing A. castellanii. Examples of membrane-bound vacuoles are indicated by solid arrows. Nucleus (1); nucleolus (2); mitochondrion (3). 9800 X.
PLATE II

Electron micrograph of logarithmically growing

A. castellani. Examples of membrane-bound vacuoles
are indicated by solid arrows. 9800 X.
PLATE II
PLATE III

Electron micrograph of *A. castellanii* in PGD phase of growth. Examples of empty membrane-bound vacuoles are indicated by solid arrows. Vacuoles containing cellular debris (1); nucleus (2); nucleolus (3); mitochondrion (4). 9800 X.
PLATE IV

Electron micrograph of *A. castellanii* in PGD phase of growth. Examples of vacuoles are indicated by solid arrows. 9800 X.
old PGD cells Plate III), contained what appears to be cellular debris (Plate V), whereas vacuoles from LM amoebae were usually empty.

d. Protein Content

The average protein contents of amoebae are presented in Figure 7. A straight line calculated by the least squares method was fitted to the data but not illustrated, and suggested a slight increase in the protein content from approximately 420 pg per cell at 20 hours to 475 pg per cell at 220 hours. However, the slope of the least squares line was not significantly different from zero when tested by a t statistic at the 0.05 level of significance.

If cell protein content is considered to be constant during LM and PGD, then there was an average of 445 ± 52 pg protein per amoeba. There was, however, much variation around the sample means of the protein values as compared with the other macromolecule data. Some of this variation might have been due to incomplete TCA extraction of amino acids and other small molecular weight compounds prior to assaying for protein, or since each protein determination was performed on cells from one 250 ml flask, it may have been a result of variation among individual cultures. Nucleic acid data were obtained by combining the contents of a large number of flasks together to obtain a single point, thus, any random variation among individual cultures was canceled out before the nucleic acids were assayed.
PLATE V

Electron micrograph of *A. castellani* in PGD phase of growth. Enlarged area of the cell showing membrane-bound vacuole containing cellular debris (1). Mitochondrion (2); lipid droplet (3). 16,800 X.
Figure 7. Changes in average protein content of *A. castellani* as a function of culture age. The data are from 3 experiments. (●) mean value for six flasks. (□) protein values grouped at 25 hour intervals.
By grouping the data, as in Figure 7, into 25 hour intervals, the pattern of protein per cell during aging is more distinct. During LM, 70–75% of the dry mass, 445 ± 38 pg, was protein. There may have been a slight increase of 15–20% in cellular protein in PGD followed by a small drop after 130 hours.

e. RNA Content

The average amounts of RNA per amoeba throughout growth are illustrated in Figure 8. The cultures were inoculated from stocks which were at $4 \times 10^4$ amoebae/ml, a cell concentration that corresponds to a normalized age of 37 hours, and, therefore, the zero time point has been made equal to the average 37 hour value.

By 25 hours of normalized growth, i.e., mid-log phase, the amount of RNA per cell dropped to a minimum of 41 pg or 6.7% of the average dry mass. During late LM and early PGD, amoeba RNA increased sharply to about 70 pg per cell and thereafter continued to rise more slowly until a level of 77 pg per amoeba was reached prior to stationary phase. At this time in growth, cellular RNA was 10.2% of the dry mass.

f. DNA Content

The values for average DNA content per amoeba (Figure 9) were obtained from five separate experiments. As in the curve of RNA versus normalized culture age, the zero time point has been made equal to the average 37 hour value. After 50 hours, a straight line
Figure 8. Changes in average RNA content of *A. castellani* as a function of culture age. The data are from 5 experiments. Each point represents a single measurement.
Figure 9. Changes in average DNA content of *A. castellanii* as a function of culture age. The data are from 5 experiments. Each point represents a single measurement.
calculated by the least squares method was fitted to the data. The slope of the least squares line was not significantly different from zero when tested by a t statistic at the 0.05 level of significance.

The average log phase DNA content was 2.1 pg per amoeba, approximately 0.3% of the dry mass. During late LM, DNA per cell dropped to a new plateau level of 1.1 to 1.3 pg and remained relatively constant until growth was terminated.

g. Glycogen Content

Following total protein, glycogen was the most abundant macromolecule (Figure 10). During early LM, the average glycogen content was about 100 pg per cell, 18% of the dry mass. However, this value dropped until a minimum of 50 pg per amoeba was reached at the end of LM. Cellular glycogen began to rise early in PGD to a maximum level of 130 pg, approximately 20% of the dry mass. From 75-150 hours of growth, the glycogen content was relatively constant, but after 150 hours, it again dropped to about the late LM value.

Relationship of RNA Levels to Cyst Formation

A 75% increase in RNA content per amoeba occurred during the transition from LM to PGD without a significant rise in cellular protein (Figures 7 and 8). Since the change in RNA was followed by an increase in the relative number of cysts in these cultures (Byers et al., 1969), it was postulated that some of the RNA might be used by the amoebae during the encystment process. In an attempt to
Figure 10. Changes in average glycogen content of *A. castellanii* as a function of culture age. The data are from 4 experiments. (•) Mean value for six flasks. (□) Total glycogen values grouped at 20 hour intervals with 12–36 values per point.
examine this hypothesis, cells were grown in OGM and were then removed at various times during LM and PGD. At each sampling, one half of the cells were placed in EM containing 15 μg/ml of actinomycin D, an inhibitor of RNA synthesis (Reich, 1963). Mattar (1969) has shown this concentration of actinomycin D to be effective in preventing RNA synthesis in *A. castellanii*. The remaining cells were inoculated into EM minus the inhibitor and served as controls. All cells were aerated for 24 hours to induce encystment and, then the numbers of trophozoites and cysts in experimental and control cultures were determined.

The degree of encystment was shown to be related to RNA accumulation (Figure 11). If acanthamoebae were transferred to EM from logarithmically growing OGM cultures, before RNA had accumulated in the cells, about 69% of the trophozoites were induced to encyst. If the amoebae were removed from OGM cultures in late PGD, after the RNA build-up, and placed in EM, between 74-75% encysted. In contrast, if EM contained actinomycin D, only 11% of the LM cells formed cysts while about 71% were able to encyst if taken from PGD cultures.

Relation of DNA Levels to Phases of the DNA Synthetic Cycle

The experiments to determine DNA content in *A. castellanii* showed that there was a 50% decrease in DNA content per cell during population aging. This decrease can be explained if it is assumed that most LM cells contain the G₂, postsynthetic phase, amount of DNA and that the DNA synthetic cycle is inhibited in G₁, the presynthetic phase, during PGD. If this were true, then DNA replication would
Figure 11. Encystment of A. castellanii transferred from OGM to EM. The data are from 2 experiments. Curve A represents the percentage of original cells induced to encyst upon transfer to EM. Curve B illustrates the percentage of original cells to form cysts when transferred to EM containing actinomycin D.
have to occur before cell division could resume when PGD amoebae were inoculated into fresh medium. This synthesis might be detected as a burst of incorporation of radioactive DNA precursors when PGD cells are inoculated into fresh medium, as contrasted to a continuous incorporation when LM amoebae are transferred to fresh medium. In order to test this possibility, washed LM and PGD cells were inoculated separately at a concentration of $2 \times 10^4$ cells/ml into fresh medium containing $2 \mu$C/ml thymidine-H$^3$ (sp. act. 0.36 mC/mM). A preliminary experiment showed that if LM amoebae were inoculated into fresh medium at high cell concentrations ($2 \times 10^6$ cells/ml), there was no incorporation of isotope. Duplicate cultures of LM and PGD amoebae, at $2 \times 10^4$ cells/ml, lacking the radioisotope were maintained to follow the growth curves of each during the period of labeling.

The results of the experiment to monitor DNA synthesis are presented in Figures 12 and 13. The growth curves (Figures 12B and 13B) have been normalized to an initial concentration of $1 \times 10^4$ amoebae/ml for convenience in comparing data. Curves 12A and 13A show that there was little difference in the rate of uptake between LM and PGD cells. Both age cells exhibited lags in division immediately upon transfer to fresh medium; the LM cells did not multiply for 1.5 hours while the PGD amoebae had a 2.5 hour lag.

It was impossible to determine from the experiment just described whether PGD acanthamoebae must replicate their DNA before being able to divide when they are placed in fresh medium or are diluted to LM cell concentrations. There may have been a burst of DNA synthesis in the PGD cells prior to multiplication which did not show up
Figure 12. Growth and incorporation of radioisotope by LM A. castellanii transferred to fresh medium. Each point of the curves is an average of 3 measurements. A- Uptake of thymidine-H$^3$ by LM A. castellanii as a function of culture age. B- Growth curve of LM A. castellanii during the radioisotope experiment.
Figure 13. Growth and incorporation of radioisotope by PGD A. castellanii transferred to fresh medium. Each point of the curves is an average of 3 measurements. A- Uptake of thymidine-H$^3$ by PGD A. castellanii as a function of culture age. B- Growth curve of PGD A. castellanii during the radioisotope experiment.
because the isotope was slow to equilibrate with the intracellular precursors during DNA synthesis. It is also possible that incorporation of thymidine-H³ may have occurred at the time of division, or immediately before, if there is DNA synthesis just prior to division. Lastly, there may be no DNA replication before multiplication and, thus, uptake occurred just after division.

If PGD cells actually do require DNA synthesis in order to multiply, then inhibition of DNA replication should prevent cell division in PGD cultures but allow LM amoebae to complete at least one cell doubling. In accordance with this hypothesis, several known inhibitors of DNA synthesis were administered at various concentrations to unlabeled PGD cells growing in cell-free recovered medium. By using the original medium for each age cells, a lag phase of uniform length was obtained (Figures 2A and 2B). Cytosine arabinoside (1-5 x 10⁻⁵ M), mitomycin C (0.1 - 10.0 μg/ml) and hydroxyurea (2-30 mM) were added to the cultures and growth followed during a six hour incubation time to test the effectiveness of each compound in preventing cell division. Both cytosine arabinoside and mitomycin C, although reported to cause encystment in these organisms (Neff and Neff, 1966), had little effect on cell growth at the concentrations employed in these studies. Mitomycin C, while producing little variation in cell growth from that of the control, appeared to slightly stimulate division at the highest concentration tested, thus corroborating a similar observation made on growth of mammalian tissue culture cells under the influence of this antibiotic (Magee and
Miller, 1962). Of the three inhibitors of DNA synthesis tried, hydroxyurea seemed to be the most successful in preventing cell multiplication. Thirty mM concentrations of the drug effectively stopped cell division for at least 6 hours.

Having found a means to limit cell growth in PGD cells, the following experiment was designed to determine if PGD amoebae require DNA synthesis in order to multiply and to make sure that hydroxyurea was inhibiting only DNA replication while leaving unaffected RNA and protein synthesis. Accordingly, three sets of cultures containing LM and PGD amoebae in cell-free recovered medium were labeled continuously for nine hours with one of the following radioisotopes: thymidine-$^3$H (2 μC/ml; sp.act. 0.36 μC/mM), uracil-$^3$H (2 μC/ml; sp.act. 0.022 mC/mM) or proline-$^{14}$C (0.25 μC/ml; sp. act. 1.19 mC/mM). One half of each set of cultures contained 30 mM hydroxyurea, while the remaining half served as controls. In addition, one set of cultures without radioisotope was maintained to follow growth of LM and PGD cells with or without hydroxyurea during the labeling period.

Figures 14-17 present the data from the radioisotope experiments and cell growth studies in the presence and absence of inhibitor. Cell numbers were normalized to a starting concentration of $1 \times 10^4$ amoebae/ml in preparing the growth curves and the results obtained with thymidine-$^3$H labeling are from two separate experiments. All other data were gathered from a single experiment. LM and PGD cells grown in the absence of hydroxyurea (Figure 14) doubled within
Figure 14. Growth curves of A. castellanii during radioisotope experiment. Each point of the curves is an average of 3 measurements. □——□ Hydroxyurea added to the culture. ●●● Controls. A- LM cells transferred to cell-free recovered medium. B- PGD cells placed in cell-free recovered medium.
Figure 15. Uptake of thymidine-$H^3$ by A. castellanii as a function of culture age. Each point of the curves is an average of 3 measurements. $\square$--$\square$ Hydroxyurea added to the medium. $\bullet$--$\bullet$ Controls. A- LM cells transferred to cell-free recovered medium. B- PGD cells placed in cell-free recovered medium.
Figure 16. Uptake of uracil-$H^3$ by *A. castellanii* as a function of culture age. Each point of the curves is an average of 3 measurements. □——□ Hydroxyurea added to the culture. •——• Controls. A- LM cells placed in cell-free recovered medium. B- PGD cells transferred to cell-free recovered medium.
Figure 17. Uptake of proline-C\textsuperscript{14} by *A. castellanii* as a function of culture age. Each point of the curves is an average of 3 measurements. □□□ Hydroxyurea added to the medium. ••• Controls. A- LM cells transferred to cell-free recovered medium. B- PGD cells placed in cell-free recovered medium.
approximately 7.5 hours after a 1.5 hour lag phase. When LM amoebae were exposed to the inhibitor, there was a 60-65% increase in cell number (Figure 14A). At the time of inoculation, the LM cells were 30 hours old on the normalized growth scale and contained less than 2.1 pg DNA per cell. Therefore, 100% of the amoebae would not be expected to divide and the percent that actually did multiply (60-65%) was a reasonable number. The level of PGD cells remained relatively constant in the presence of hydroxyurea during the entire experiment (Figure 14B).

Thymidine-H³ uptake by LM and PGD cells is illustrated in Figure 15. The controls incorporated label into DNA as expected from previous experiments (Figures 12 and 13) but addition of hydroxyurea blocked DNA replication completely in amoebae of both ages.

Uracil-H³ and proline-C¹⁴ incorporation into macromolecules was relatively unaffected by the prevention of DNA replication in LM and PGD cells (Figures 16 and 17). The drug seemed to stimulate uptake of respective isotope into RNA and protein in both age amoebae. After a short lag, there was an increase in uracil-H³ incorporation, more so in PGD cells than in LM organisms, in inhibited as well as in control cultures. A similar pattern was evidenced in the uptake of proline-C¹⁴ except that incorporation of isotope began immediately after inoculation and was linear thereafter.
DISCUSSION

Volume, mass, and protein content of A. castellanii appeared to be relatively constant throughout LM, while RNA, DNA, and glycogen levels began to change before the termination of this phase. However, most of the cellular modifications which occurred as amoebae aged took place after the beginning of PGD. For means of comparison, a summary graph of the volume, mass, and macromolecule changes versus culture age has been compiled (Figure 18) and the molecular contents have been expressed as percentages of dry mass as read from the graphs (Results) at 20 hour intervals of population growth (Table 1). DNA content remained constant during early and mid-LM growth, then dropped about 50% during late LM and was finally reduced to a very slow rate of decrease during PGD. RNA dropped from its inoculum value during early LM to a minimum value during mid-log and then increased 1.9 times the minimum value during late LM and PGD. Volume changes were somewhat similar to the RNA pattern, with minimal volumes during LM and an increase of 1.7 times for average and 1.5 times for modal volumes during PGD. Values for average glycogen began to drop in mid-log phase reaching a minimum in early PGD, then increased 2.6 times and remained constant at that level until another drop occurred in late PGD. Dry mass was relatively constant until mid-PGD when it began to rise slightly.
Figure 18. Composite graph of volume, dry mass and macromolecule changes in *A. castellanii* as a function of culture age.
Table 1. Molecular contents of *A. castellanii* expressed as percentages of dry mass during population growth.

<table>
<thead>
<tr>
<th>Norm. Culture Age (Hrs)</th>
<th>Protein</th>
<th>RNA</th>
<th>Glycogen</th>
<th>DNA</th>
<th>Others (lipid cellulose, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>71.4</td>
<td>6.7</td>
<td>18.0</td>
<td>0.3</td>
<td>4.1</td>
</tr>
<tr>
<td>40</td>
<td>71.4</td>
<td>7.2</td>
<td>16.1</td>
<td>0.3</td>
<td>5.0</td>
</tr>
<tr>
<td>60</td>
<td>71.4</td>
<td>9.6</td>
<td>8.0</td>
<td>0.2</td>
<td>10.8</td>
</tr>
<tr>
<td>80</td>
<td>71.4</td>
<td>10.8</td>
<td>15.7</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td>100</td>
<td>71.4</td>
<td>11.2</td>
<td>20.9</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>120</td>
<td>67.7</td>
<td>11.5</td>
<td>19.9</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>140</td>
<td>65.1</td>
<td>11.4</td>
<td>19.8</td>
<td>0.2</td>
<td>3.5</td>
</tr>
<tr>
<td>160</td>
<td>62.8</td>
<td>11.0</td>
<td>19.0</td>
<td>0.2</td>
<td>7.0</td>
</tr>
<tr>
<td>180</td>
<td>62.7</td>
<td>10.6</td>
<td>18.3</td>
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</tr>
<tr>
<td>200</td>
<td>60.3</td>
<td>10.2</td>
<td>14.2</td>
<td>0.1</td>
<td>15.2</td>
</tr>
</tbody>
</table>
By summing all of the macromolecule weights and subtracting them from total dry mass, other molecules appear to make up from 2-15% of the dry mass (Table 1). These other molecules are probably mostly lipid although cellulose may be present in encysting cells (Griffiths and Hughes, 1969).

In order to understand the compositional changes, it is helpful to look first at the morphology of the amoebae and, secondly, at the factors which lead to the termination of growth and which may necessitate encystment if the population is to survive (Halvorson and Sussman, 1966). Byers et al. (1969) determined the proportion of cells in cultures of *A. castellanii* to form cysts in OGM under conditions identical to those used here (Figure 19). Cysts constituted 0.2% or less of the total population during LM and then increased to several percent in late PGD. Additional encystment seems to be prevented by the lack of oxygen at this culture age. Neff et al. (1964) and Band (1961; 1963) have demonstrated that *Acanthamoeba* requires vigorous aeration in order to complete, but not initiate, encystment, and Byers (1969) has recently found that if *A. castellanii* in OGM are allowed to grow undisturbed until PGD, and are then aerated, the numbers of cysts obtained are about 10-100 times greater than in unaerated control cultures. Therefore, although the amoebae may be prepared to encyst in unaerated cultures, few will actually be able to complete the process.

Various suggestions are made in the literature as to the reasons for the cessation of active growth (Dean and Hinshelwood, 1966) and
Figure 19. Proportion of *A. castellanii* trophozoites to form cysts during culture aging. Reproduced from Byers et al. (1969).
the initiation of encystment (Hanson, 1967). One obvious possibility is the exhaustion of necessary nutrient material and/or the build-up of toxic products in the medium. Byers et al. (1969) have observed, however, that PGD medium from which A. castellanii had been removed would support vigorous growth upon reinoculation with fresh cells at LM concentrations and have reported that similar total cell numbers were obtained in 25, 50, and 100 ml volumes of medium. Thus, post-LM yields were not dependent on nutrient availability unless such a deficiency were located in the vicinity of the cell layer on the bottom of the culture flasks. Furthermore, in the present study both LM and PGD amoebae reinoculated into cell-free recovered medium at log phase cell concentrations had the same generation times as they did in fresh medium, at least through one doubling (Figure 2). For these reasons, it appears unlikely that nutrient deficiencies and/or toxic accumulations are the terminators of further exponential multiplication.

Changes in pH might also lead to the cessation of logarithmic increase and subsequent encystment (Hanson, 1967). Although data on pH variations in these cultures are limited, it seems as if the pH's in the range normally found during aging were not inhibitory to growth since PGD cells were able to multiply at LM rates when diluted in cell-free recovered medium (Figure 2).

Information on the effects of crowding on these organisms is practically nonexistent. Upon inoculation, acanthamoebae settle to the bottom of the flask and adhere tenaciously to the glass. As the
culture ages, the cells continue to attach to the bottom and walls of the flask, but they also form clumps which are found free in the medium. Band and Mohrlok (1969) have analyzed clumping in the soil amoeba Mayorella palestinenensis which is closely related to A. castellanii (Page, 1967). The exact nature of the amoeba-to-glass or amoeba-to-amoeba adhesion was not discovered, although it was shown not to be associated with starvation or encystment. If acanthamoebae require an inert surface to divide on, then crowding would reduce the availability of the glass and clumping would result from the increased number of amoeba-to-amoeba contacts. As adhesion to glass became less possible, the division rate would be expected to slow down as in the change from LM to PGD.

There are several lines of evidence to suggest that low oxygen tension plays a role in terminating logarithmic multiplication and may affect preparation for encystment. Oxygen has been shown to be essential for growth of A. castellanii (Neff et al., 1958). Furthermore, Byers et al. (1969) demonstrated that a decrease in the dissolved oxygen levels preceded the beginning of PGD in A. castellanii cultures and that aeration prolonged LM. Many of the changes in morphology and macromolecular composition observed in the present study during the transition from LM to PGD may be tied to the organism's response to the decrease in oxygen tension, and the necessity, in this unfavorable environment, to prepare for encystment.

Variations in cell volume during aging have been reported for several protozoans. The pattern of change illustrated here is similar
to that previously described for A. castellani under different culture conditions by Dolphin (1968). Loefer (1952), Summers (1963) and Schmid (1967) have reported that cell volumes of Tetrahymena pyriformis either decrease slightly, or, in the work of the latter author, remain constant during logarithmic growth, but increase during stationary phase. T. pyriformis volumes have been shown to rise when oxygen is limiting and to decrease when it was not (Scherbaum, 1956). Byers et al. (1969) have demonstrated that the volume rise in A. castellani occurred when oxygen became limiting and that similar volume increases occurred when actively growing amoebae were subjected to a nitrogen atmosphere.

Schmid (1967) has suggested that a constant cell volume during logarithmic phase may be the result of a coupling of cell division to the synthetic processes in the cell. As the population enters stationary phase, the rate of cell division is uncoupled from the rates of syntheses and consequently, the cells begin to get larger. In the present study, although cellular volume changes were later accompanied by changes in dry mass, the latter only increased 15-20% compared to 60% in the former (Figures 1 and 18). Electron microscopy demonstrated that the additional increase in cell volume was probably caused by vacuolization as the cells aged (Plates I-IV). Similar observations have been made in other electron micrographic investigations of A. castellani. Both Bauer (1967) and Bowers and Korn (1968; 1969) have reported an increase in the number of membrane-bound vacuoles as the cells age and prepare for encystment. Vacuolization has also been described in encysting Naegleria gruberi, another
amoeba (Schuster, 1963) and in T. pyriformis, which does not encyst, when subjected to adverse environmental conditions (Levy and Elliott, 1968).

Aging acanthamoebae which are in the very early stages of encystment are reported to have an increase in the number of autolysosomes, as well as empty vacuoles (Bauer, 1967; Bowers and Korn, 1969). Autolysosomes may be distinguished cytchemically because they contain acid phosphatase and cytologically by their recognizable contents of cytoplasmic components (De Deuve and Wattiaux, 1966). Vacuoles containing membrane systems and cellular debris have been shown to accumulate in PGD cells in this study also (Plates III and V). Since autolysis and excretion of cytoplasmic constituents are consistent with decrease in dry weight, protein, phospholipid and glycogen which occurs in the mature cyst (Bowers and Korn, 1969), the presence of autolysosomes in these PGD cells helps to confirm the belief that they are actively preparing for encystment.

The increase in dry mass is partly due to the stimulated production of RNA and glycogen during PGD. However, the difference between dry weight and the summed macromolecule weights suggest that other molecules such as lipids and perhaps cellulose, while less than 5% of the total mass during LM, appear to increase during late PGD (Table 1). Lipid droplets have been shown to increase in Tetrahymena during early stationary growth (Allison and Ronkin, 1967) and Bowers and Korn (1969) reported that lipids in agitated populations of A. castellanii make up approximately 2% of the trophozoite dry mass
and increase to 18% in encysting amoebae. Although there is little information on cellulose levels in *A. castellanii*, this molecule has been shown to increase in encysting cells (Griffiths and Hughes, 1969) and may, therefore, contribute to the dry mass. On the basis of this cited information, the rise in other molecules for the amoebae studied here is probably reasonable.

Several workers have examined RNA content as a function of aging in other protozoans (Buetow and Levedahl, 1962; Wilson and Levedahl, 1965; Pogo *et al.*, 1966; Cook and Heinrich, 1968). However, in all cases stationary phase cells were used as inocula so that long lag phases ensued with macromolecule accumulation but no cell division. Under such conditions, Dean and Hinshelwood (1966) have shown that macromolecular synthesis will be overstimulated during the lag phase and, thus, the patterns of macromolecule content in subsequent phases will reflect the cell's attempts to recover from the lag phase events. Since *A. castellanii* cultures were always inoculated from LM stocks, the pattern of RNA level represented here is uncomplicated by such lag phase synthesis.

Cellular RNA has been shown to increase in actively multiplying bacteria, and then to level off prior to sporulation (Young and Fitz-James, 1959a). The RNA pattern is very similar in *A. castellanii* (Figures 8 and 18). 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 large rise in RNA level may be partly explained by the formation of these ribosomes which are thought to function in the translation of protein for cyst wall construction (Neff et al., 1964; Bauer, 1967).

More direct evidence that the RNA build-up is related to encystment was obtained in the actinomycin D experiments in this investigation. Actinomycin D was shown to interfere with cyst formation if administered before RNA accumulated in the cells, but if the amoebae were exposed to it at later times, the ability to form cysts became progressively insensitive to the inhibition of RNA synthesis (Figure 11). Maximum insensitivity corresponded with the beginning of the final plateau level reached by RNA in mid-PGD. Similar results have been obtained in sporulating bacteria. Aronson and del Valle (1964; del Valle and Aronson, 1962) demonstrated that the capacity of Bacillus cereus to sporulate became resistant to actinomycin D and base analogues if the inhibitors were added after the cells had stopped accumulating RNA. These results were interpreted as reflecting the formation of stable messenger RNA during vegetative growth which would later be required for the elaboration of spore components. Long-lived RNA (Tyler, 1966) 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. Further evidence for such long-lived RNA being present in A. castellanii has recently been obtained by Mattar (1969). With the use of radioisotopes he has shown that there is some carryover of RNA from trophozoite to cyst and that this RNA has a lifetime of at least two weeks in the cyst. Furthermore, there is no additional

Glycogen levels have been reported to increase in Tetrahymena from 3.6% of the dry weight in exponentially dividing cells to as high as 22% in stationary phase organisms (Scherbaum, 1964) and have risen to as much as 50% of the dry weight in E. gracilis (Cook, 1967). The changes in glycogen level in A. castellanii during population aging, were somewhat similar to the patterns described for other protozoa, and may also be related to encystment. The first decrease in glycogen, during late LM and early PGD, occurred during the build-up of RNA. Since the only polysaccharide reserve in A. castellanii is glycogen (Bowers and Korn, 1968), its breakdown at the time of RNA accumulation may have been necessary to supply energy for the stimulated RNA synthesis. The glycogen level was only depressed until the rate of RNA build-up began to slow down and then, it too began to rise to its previous level.

The second decrease in glycogen, during late PGD, coincided with the relative increase in cyst numbers (Figures 10 and 18) and may be related to cyst wall synthesis, as a structural component and/or as an energy source. Supporting this suggestion are the observations of Bowers and Korn (1969) that glycogen levels in agitated cultures of A. castellanii are lower in the mature cyst than in the trophozoite. The chemical composition of the cyst wall in these amoebae has not been precisely defined. However, Tomlinson and Jones (1962) have identified cellulose in isolated cyst walls and this has been confirmed by Neff and Benton (1962) and by Page (1967). Since glycogen
indirectly supplies precursors for cellulose synthesis in plants 
(Mahler and Cordes, 1966), it is likely to do so in A. castellanii 
as well, and its level would be expected to drop as cellulose is 
synthesized. In fact, Griffiths and Hughes (1969) have reported 
such a drop in glycogen level coinciding with the rise in cellulose in 
agitated cultures of Hartmanella (Acanthamoeba) castellanii induced 
to encyst in minimal salts medium.

The decrease in DNA content found in A. castellanii during late 
LM and early PGD (Figures 9 and 18) has also been observed in sporu-
liating bacteria. Direct analysis of DNA content of B. cereus 
harvested during exponential growth and of the spores resulting from 
such growth demonstrated that each cell contained approximately 
double the amount of DNA as did the spore (Young and Fitz-James, 
1959a).

The drop in A. castellanii DNA level may be explained if it is 
assumed that most LM amoebae contain the G2 amount of this macromole-
cule and that the DNA synthetic cycle is inhibited in G1 during PGD, 
as was suggested in the Results. Goldstein and Prescott (1967) have 
shown that in growing Amoeba proteus the G2 phase may constitute over 
90% of the growth replication cycle, and, therefore, if A. proteus 
and A. castellanii are similar, most LM amoebae would have the dupli-
cated amount of DNA. Direct evidence for this hypothesis was obtained 
by exposing LM and PGD cells to hydroxyurea in the presence of radio-
active precursors. If 100% of the LM cells were in G2, then all of 
them would be expected to double, in spite of the fact that hydroxyurea
was inhibiting DNA synthesis. However, since the cellular DNA level had already begun to decrease by 30 hours, when the amoebae were harvested, and there was approximately 1.7 pg DNA per cell, only about 60% of the LM amoebae would be in G2 and able to multiply. The data corroborated the hypothesis since 60-65% of the LM cells divided in the presence of the inhibitor of DNA replication (Figure 14). In contrast, FGD amoebae, which were hypothesized to be in G1, were completely prevented from multiplying (Figure 14).

The reasons for a drop in DNA content and the inhibition of the DNA synthetic cycle in G1 are obscure, although these events should logically be affected by those factors which limit logarithmic cell increase and necessitate encystment, namely decreased oxygen tension and/or crowding. Information on the effects of the former on DNA synthesis is limited. However, there is some evidence to suggest that crowding, perhaps in conjunction with decreased oxygen availability, prevents uptake of thymidine-H3 into DNA. LM amoebae were shown to be unable to incorporate this isotope when inoculated at high cell concentrations, and Kohn (1963) has reported that the amount of thymidine-H3 taken up by monolayer cultures of chick fibroblast and various continuous lines of mammalian cells into acid-soluble pools and then incorporated into DNA decreased as cell concentrations increased.

Figure 20 summarizes the physiological and morphological events that have been shown to occur or have been suggested to occur during aging of A. castellanii. Crowding and/or low oxygen supply have
Figure 20. Events occurring during growth of *A. castellanii* in optimal growth medium cultures.
been indicated as terminators of active growth and inhibitors of DNA synthesis in $G_1$. The resultant unfavorable environment induces the cells to prepare for encystment and so RNA synthesis accelerates. Some cysts do appear in the culture and the glycogen level decreases as this macromolecule is used up during cellulose build-up in the cyst wall, but most trophozoites are unable to complete encystment because of the low oxygen level. If the oxygen supply is replenished, cyst numbers increase. The cycle is begun again when the cysts are allowed to excyst in fresh medium.
Summary and Conclusions

Populations of Acanthamoeba castellani grown in optimal growth medium were used to study cell differentiation during phases of logarithmic multiplication (LM) and population growth deceleration (PGD). Changes in cell size and macromolecule composition reflected varying culture conditions and the cell's preparation for encystment, a process which begins during mid-PGD. Crowding and/or low oxygen levels were suggested to prevent the continuance of active growth and to cause encystment.

Levels of RNA and DNA, in particular, changed considerably during growth. RNA dropped from its inoculum value in early LM, reached a minimum in mid-log and then increased about 75% in late LM to reach a plateau level in early PGD. By transferring cells at various times during growth to Neff's nutrient-free medium containing actinomycin D, the accumulation of RNA was shown to be required for encystment.

The level of DNA decreased about 50% from its inoculum value in mid-LM and then remained relatively constant until growth was terminated. In explanation of this observation, it was suggested and then demonstrated that the DNA synthetic cycle is inhibited in G₁ during
PGD. PGD cells which have been exposed to hydroxyurea, an inhibitor of DNA synthesis, are unable to divide upon transfer to PGD medium at log phase cell concentrations. In contrast, LM amoebae, the majority of which are hypothesized to be in G2, are able to complete one doubling even in the absence of DNA synthesis.

Perspectives

All of the events occurring during aging and encystment of A. castellanii suggest possibilities for future investigations. Separation and analysis of the RNA fraction into various species would be enlightening in order to determine if the accumulation of this nucleic acid is mostly ribosomal in nature or if it actually represents synthesis of new messengers, some of which may be long-lived and function in cyst protein formation.

The examination of several enzymes might yield information on the mechanism by which the DNA synthetic cycle is inhibited during PGD. DNA polymerase has not yet been satisfactorily isolated and characterized from protozoan preparations (Bollum, 1968), but it would be interesting to know if the inhibition of its activity is an allosteric effect or if its synthesis is being repressed in PGD. Other enzymes, not directly involved with DNA synthesis, may control levels of this macromolecule, too. Thymidine kinase, which catalyzes the phosphorylation of thymidine to thymidylic acid (Mahler and Cordes, 1966), is known to be absent during G1 in plants (Stern and Hotta, 1963) and both thymidine kinase and thymidylate
synthetase, which catalyzes the conversion of deoxyuridylic acid to thymidylic acid (Mahler and Cordes, 1966), cannot be formed in the absence of certain amino acids in Tetrahymena (Prescott and Stone, 1967). Thus, an investigation of the activity of these enzymes might help to explain regulation of DNA levels in *A. castellanii*.

Other aspects of encystment also suggest opportunities for exploration. Since it appears that higher numbers of cysts can be obtained by aerating PGD cultures (Byers, 1969), this system may be used for studying mechanisms of enzyme regulation in a eukaryotic organism. Some of the enzymes connected with changing glycogen levels, such as UDPG-cellulose transglucosylase which is necessary for cyst wall synthesis (Tomlinson, 1962), may be under an induction-repression type of control. Since few inducible enzymes are known for protozoans, it would be of interest to determine if *A. castellanii* possesses any.

Lastly, the effects of crowding per se on these cells has not been adequately elucidated. Since crowding may bring about changes in macromolecule levels, it would be worthwhile to attempt to examine its effects on growth in an environment in which oxygen tension were not also a factor.
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