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DISSERTATION
Presented in Partial Fulfillment of the Requirements for
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By
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* * * * * * *

The Ohio State University

1970

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LIST OF ABBREVIATIONS

Act D: Actinomycin D.
CH: Cycloheximide
COx: Cytochrome oxidase activity per cell
DNA: Deoxyribonucleic acid
HU: Hydroxyurea
LM: Logarithmic multiplication
mDNA: Mitochondrial DNA
N/C: Average number of nuclei per cell
PCA: Perchloric acid
PGD: Population growth deceleration
RNA: Ribonucleic acid
TCA: Trichloroacetic acid
Tg: Generation time
INTRODUCTION

It is well established that a cell can be induced to synthesize an enzyme or group of enzymes by specific environmental conditions. The enzymes within the inducible groups usually comprise a metabolic pathway, and so are functionally related (Vogel and Vogel, 1967). A cellular organelle can be considered to be a physically integrated and highly ordered complex of such functional groups of enzymes. A mitochondrion, for example, contains organized arrays of electron transport enzymes (cytochromes) as an integral part of its structure (Green and Perdue, 1966). The levels of these organelle specific enzymes are also regulated by the environment. In yeast grown under anaerobic conditions cytochrome synthesis is prevented and recognizable mitochondria disappear. Cytochrome synthesis and reappearance of mitochondria are induced by the presence of oxygen (Roodyn and Wilkie, 1968). The correlation between cytochrome levels and numbers of recognizable mitochondria suggests that the increased enzyme levels induced by oxygen are due to the biogenesis of the mitochondria of which the cytochromes are an integral part. Thus, the levels of cytochromes and number of mitochondria are interrelated and measurements of cellular cytochrome quantities are a way of indirectly determining relative numbers of mitochondria. By studying cytochrome levels as a function of time after aeration of
anaerobic yeast cultures, the regulation of organelle biogenesis has been examined (Polakis et al., 1964; Schatz, 1965; Tzagaloff et al., 1967). Since these cells possess a single nucleus, the number of mitochondria can vary independently from the number of nuclei. If mitochondria multiply by fission (Luck, 1963; Tandler et al., 1969), this means that mitochondrial division is separable from karyokinesis.

A similar separability, or "uncoupling" occurs between nuclear and cytoplasmic division in cultures of Acanthamoeba castellanii agitated by aeration (James and Byers, 1967). The quantity of multinucleated cells produced varies with culture age and can be controlled by the rate of aeration (James, 1966). Thus, Acanthamoeba seems to offer an opportunity to study the regulation of organelle biogenesis. In the present investigation variations in cytochrome oxidase activity were assumed to reflect changes in mitochondrial mass. An effort was made to answer the following questions: How are cytochrome oxidase activities related to the changes in oxygen tension that are known to occur in growth media during aging of cultures? Does the induction of multinuclearity by agitation cause a corresponding increase in the cytochrome oxidase activity? Are cytochrome oxidase activities directly proportional to nuclear numbers, or do they vary independently, as might be expected if mitochondrial biogenesis and nuclear replication were independent?
MATERIALS AND METHODS

The organism used in this study, *Acanthamoeba* sp., was originally obtained from R. J. Neff at Vanderbilt University. Page (1967), in redefining the genus *Acanthamoeba*, has identified Neff's strain as *Acanthamoeba castellanii* (Douglas, 1930).

Amoebae were grown in medium containing the following amounts of nutrients per liter (Neff, 1964): 7.5 g each of proteose peptone (Difco) and yeast extract (Difco), 246 mg MgSO$_4$·7H$_2$O, 7.4 mg CaCl$_2$·2H$_2$O, 272 mg KH$_2$PO$_4$, 30 mg ferric citrate, 0.5 µg vitamin B$_1$ hydrochloride, 0.1 µg biotin, 5 x 10$^{-4}$ µg vitamin B$_{12}$, and 1.0 N KOH to bring the pH to 7.0. The mixture was filtered as described by Byers et al. (1969). Thirty percent glucose was autoclaved separately and added to the medium aseptically at the time of inoculation to a final concentration of 1.5%.

Inoculations were performed in a hood under UV lamps using cells in logarithmic growth. Amoebae were grown at 30°C under two different conditions: in 50 ml amounts of medium contained in 250 ml Erlenmeyer flasks which were covered with aluminum foil or stainless steel caps and were placed in a dark incubator without aeration or agitation; and in 1300 ml amounts of medium contained in 1500 ml aspirator flasks sealed with aluminum foil and placed in a water bath. Figure 1 depicts the type of flask used for this latter growth condition.
Figure 1. Apparatus for growing agitated (aerated) cultures of A. castellanii. A culture sample is removed aseptically by opening the clamp and tipping the flask toward the sample receptacle.
Air passes first through a gas flow regulator, then a sterile 0.22 μm pore membrane filter (Millipore, Swinnex-25), and finally into the medium. An aeration rate of 870 ml/liter/min was used. A sample port is attached below the filter by means of a Pyrex T-tube and is clamped off during aeration of the medium. In order to take a cell sample aseptically, the sample port is unclamped, the rubber stopper in the receiving vessel is loosened, and the culture flask is tipped toward the receiving vessel. The air flow aids in pushing medium through the sample port. A constant aeration rate was maintained by readjusting the air flow after each sample was removed.

Cell Counting and Sizing

Amoebae were counted by an electronic particle counter (Celloscope 101-TN, Particle Data, Inc.) fitted with a 100 μm diameter orifice. Cells to be counted were fixed with 0.25 ml formalin per 50 ml of culture. After about 10 min the culture was swirled and cells collected by centrifugation at 600 x g for 3-4 min in a clinical centrifuge. The cells were resuspended with a vortex mixer in ultrafiltered counting medium containing 0.5% (v/v) formalin and 0.6% (w/v) NaCl. All glassware used for cell counting was rinsed with filtered distilled water.

Cell volume distributions were obtained simultaneously with cell counts with a 128-channel pulse-height analyzer (ND 110, Nuclear Data, Inc.) connected to the particle counter. When a particle goes through the counting orifice, a pulse is generated whose height is
proportional to the volume of the particle. The analyzer sorts these pulses according to amplitude into 128 linearly related classes (channels). Absolute volumes were assigned to the channels by using ragweed and mulberry pollen and glass beads of known sizes. The distribution was plotted on a chart recorder in the form of a histogram relating cell number to channel number, and mean cell volumes were calculated from the graph.

Macromolecule Assays

a. **Total protein.** Samples of 2-5 x 10^5 amoebae were collected by centrifugation at 600 x g for 3 min and washed twice with 0.15 M KCl. A 0.25 ml aliquot from the last wash was removed for a cell count. The remaining cells were centrifuged and the pellet was resuspended in 5% trichloroacetic acid (TCA). After being in the cold for 24 hours, acid precipitable material was collected by centrifugation at 2000 x g for 10 min.

A modification of the Lowry method (Oyama and Eagle, 1956) was used for estimating total protein. One ml of water and 4.0 ml of freshly prepared C reagent (50 parts of reagent A, containing 20 g Na$_2$CO$_3$, 4 g NaOH, 0.2 g NaK tartrate in 1.0 l of water, and one part of reagent B, containing 5 g CuSO$_4$·5H$_2$O in 1.0 l of water) were added to the TCA precipitate. This was followed by the addition of 0.5 ml of standardized Folin-Ciocalteau reagent with thorough mixing. Optical density at a wavelength of 660 nm was measured after one hour of incubation at room temperature. Samples of crystalline
bovine serum albumen (10-150 μg) were similarly treated as standards.

Values for protein/cell obtained in this study differed significantly from those of Byers et al. (1969), who reported 445 pg/cell compared with about 250 pg/cell in the present study. The basic difference in procedure was that overnight extraction in TCA was used in the present study, whereas 20 min extraction was used by Byers et al. Attempts made to find the source of disagreement included using several different concentrations of TCA (5, 7.5, 10, 15, and 20%) for precipitation and extraction of the cells and then assaying for protein at 0, 0.15, 0.3, 3.0, and 24 hours after introduction of the TCA. Also, the number of cells precipitated by 5% TCA and assayed for protein after 24 hours of extraction was varied from $10^4$ to $5 \times 10^5$. Precipitations were carried out either by placing 5% TCA directly on the pelleted cells or by adding an equal volume of 10% TCA to cells resuspended in 0.15 M KCl. No difference in the protein values was obtained when any of these methods were used. It was concluded that optimum conditions for preparation of the cells for protein assay were those already being used (24-hour extraction with 5% TCA at 0-4°C), and that the values reported in this paper had to be accepted. The difference between these and earlier values remains unexplained.

b. RNA. Samples of $5 \times 10^5$ amoebae were collected and washed twice in 0.15 M KCl. The final wash suspension was transferred into 9 x 75 mm Pyrex tubes and 0.1 ml was removed for a cell count.
Unless otherwise noted, the following steps were carried out at 0-4 C. RNA extraction was performed with a modified method of Fleck and Munro (1966) and Fleck and Begg (1965). To the transferred cell suspension was added 0.6 N perchloric acid (PCA) to a final concentration of 0.2 N PCA. After 10 min this was centrifuged at 4000 x g for 5 min, the pellet washed twice with cold 0.2 N PCA, and the supernatants discarded. Excess supernatant was drained by inverting the tube over filter paper and then wiping the inside walls with absorbent paper, avoiding disturbance of the pellet. To this final pellet 0.2 ml of 0.3 N KOH was added and the mixture was incubated at 37 C for one hour. The hydrolysate was cooled to 0 C and 0.125 ml of 1.2 N PCA was added to precipitate protein and DNA. After 10 min the precipitate was sedimented by centrifugation at 4000 x g for 5 min, and the resulting supernatant removed with a Pasteur pipette and saved. The pellet was washed twice with 0.5 ml of 0.2 N PCA and the washes combined with the original supernatant. The volume of the combined washes was brought to 2.7 ml with 0.2 N PCA and the optical density read at 260 nm. Yeast sRNA was treated similarly and used as a standard.

c. DNA. Samples of $3 \times 10^5$ amoebae were collected, washed twice with 0.15 M KCl and placed into 9 x 75 mm Pyrex tubes. A 0.1 ml aliquot was removed for a cell count.

DNA was estimated fluorimetrically according to the method of Kissane and Robbins (1959), as modified by Santoianni and Ayala (1965). The cell suspension was centrifuged at 1000 x g for 5 min
and the resulting pellet resuspended in 0.5 ml of cold 0.3 M TCA with mixing. After standing 24 hours at 0-4 C, the precipitate was sedimented by centrifugation at 4000 x g for 5 min and the supernatant was discarded. One ml of 0.1 M alcoholic potassium acetate was added to the pellet with gentle mixing. After standing 5-10 min at 0-4 C, the mixture was centrifuged at 4000 x g for 5 min and the resulting supernatant was discarded. One ml of absolute ethanol at room temperature was added to the pellet. The tube was capped with parafilm and kept at 60 C for 15 min. After centrifugation at 4000 x g for 5 min the supernatant was discarded and the precipitate was vacuum desiccated.

One-tenth ml of a freshly prepared 338 mg/ml solution of 2,4-diaminobenzoic acid hydrochloride was added to the alcoholic precipitate with vigorous mixing. The tube was capped with parafilm and after incubation of this mixture at 60 C for 30 min, the reaction was stopped by the addition of 2.0 ml of 0.6 N PCA. This mixture was transferred to a 12 x 78 mm Pyrex tube and 0.5 ml more of 0.6 N PCA was added. Fluorescence was measured with a Turner Fluorometer Model 110 (G.K. Turner Associates, Palo Alto, Cal.) using a straight type blue lamp (F4T5-B, General Electric) with a 405 nm primary and a 520 nm secondary optical filter. The range selector was set at XI.

Salmon sperm DNA dissolved in 0.1 N NH₄OH was treated similarly for use as a standard. Aliquots of 0.25 ml of suitable dilutions of the DNA standard were vacuum desiccated, so that the tubes contained 0-2.0 µg of DNA.
All glassware used in this assay procedure was washed with Alconox detergent and rinsed with distilled water, followed by immersion in cleaning solution (sulfuric acid-dichromate) and a thorough distilled water rinse. Care was taken not to allow the distilled water to pass through rubber tubing. These precautions were taken to avoid fluorescent compounds that might interfere with the assay.

Determination of Number of Nuclei Per Cell

When a coverslip is placed over a drop of a suspension of amoebae on a slide, the smaller cells (mostly mononucleates) spread out further than the larger cells (mostly multinucleates), so that a non-random distribution of amoebae is obtained. To compensate for this, the number of nuclei in intact amoebae was counted with a glass slide into which was etched five concentric circles separated into quadrants (Dolphin, 1968).

Cell samples were collected and fixed with formalin, as described above. A small drop of a suitably concentrated sample was placed in the center of the concentric circles and a coverslip gently placed over the drop. After allowing the suspension to spread out under the coverslip and the cells to flatten, the number of mononucleates and the number of cells belonging to each class of multinucleates in one quadrant were scored. From 700 to 1200 cells per sample were thus counted.
Respiration Rates

A YSI Biological Oxygen Monitor Model 53 (Yellow Springs Instrument Co., Ohio) was used for respiration rate determinations. Samples of about $10^6$ amoebae were harvested by centrifugation at 1000 x g and resuspended in 1.2 ml of the medium from which they were collected (homologous medium). A 0.1 ml aliquot was removed for a cell count. One ml of the suspension was added to 3.0 ml of homologous medium which had been preincubated at 30 C in a cuvette. The oxygen electrode was standardized using 4.0 ml of homologous medium and the oxygen content of the medium was determined by comparison with 4.0 ml of O$_2$ saturated distilled water.

Respiration rates were plotted on a Heath recorder at a chart speed of 0.5 inch/min.

Cytochrome Oxidase Assay

A modified method of Smith (1955) was used for the cytochrome oxidase assay. Cytochrome c (horse heart, type II, Sigma Chemical Co.) was dissolved in 29.9 ml of 0.01 M potassium phosphate buffer to a final concentration of 2.34 mg/ml. The cytochrome c was reduced by adding 0.1 ml of 100 mg/ml sodium dithionite and stirring until the excess hydrosulfite was removed. This solution was poured into serum bottles and gassed with nitrogen through the rubber bottle caps for 10 min. By thus replacing any dissolved oxygen the cytochrome c stayed in the reduced form for as long as two weeks.
However, this cytochrome c preparation was not used unless the OD$_{550}$/OD$_{565}$ ratio was greater than 6.0 (Smith, 1955).

For the cytochrome oxidase assay the enzyme preparation (homogenate) in 0.1 M potassium phosphate buffer (pH 7.0) was pipetted into a cuvette and the volume brought up to 2.6 ml with the same buffer. The reaction was started with the addition of 0.4 ml of reduced cytochrome c. The decrease in absorbance at 550 nm was followed with a Gilford Model 2000 spectrophotometer and the rate of oxidation of cytochrome c was calculated using an extinction coefficient of 19.6 mM$^{-1}$cm$^{-1}$ (Yonetani, 1965). Under these conditions cytochrome oxidase activity was completely inhibited by $10^{-4}$ M KCN, signifying that cytochrome oxidase is the enzyme being assayed.

All cytochrome oxidase activity (COx) was found to be associated with particulates and sedimentable from crude homogenates at 10,000 x g. In addition, it was found that the activity of resuspended 10,000 x g pellets exceeded that of the total homogenate and, therefore, the former were used as estimates of total cellular COx.

Cell Homogenization

For the determination of enzyme activities on a cellular basis, about $10^6$ amoebae were harvested and washed twice with 0.15 M KCl. After the final washing the cells were resuspended in 3.0 ml of an homogenizing medium (HM), which contains 0.3 M mannitol, 0.01 M KCl, 0.01 M tris, 5 mM K$_3$PO$_4$, 0.2 mM EDTA, pH 6.9 (Lloyd and Griffiths, 1968). Two-tenths ml was removed for a cell count. Then, two
homogenization methods were used. The cells either were ground in a glass-Teflon Potter Elvejhem homogenizer for 1.5 min, or disrupted in a tight-fitting Dounce homogenizer with 20 strokes. The homogenate was centrifuged at 10,000 \( \times \) g for 10 min, the pellet resuspended in HM and the centrifugation repeated. The last pellet was resuspended in 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.0) and used for the cytochrome oxidase assay.

Inhibition of DNA, RNA, and Protein Synthesis

Rudick (1969) has determined that 30 mM hydroxyurea inhibits DNA synthesis in *A. castellanii* and Mattar (1970) has shown that 5 \( \mu \)g/ml actinomycin D inhibits RNA synthesis. Cycloheximide (CH) at final concentrations of 50, 100, 300, and 500 \( \mu \)g/ml was used in these studies to determine the effective level for the inhibition of protein synthesis. Cells were grown unagitated up to \( 2 \times 10^4 \) amoebae/ml, policed, and 2.0 ml aliquots placed into vials containing the inhibitor and tritiated protein hydrolysate (final concentration 1.0 \( \mu \)C/ml), such that there were six vials for each concentration of inhibitor. After one hour of incubation at 30 C, three vials for each CH concentration were fixed by adding an equal volume of 10\% TCA. The remaining vials were similarly fixed after six hours of incubation. Two vials containing everything but cells were used as controls and as an indication of the background level of radiation. After overnight precipitation in the cold, the contents of each vial were filtered through a 0.45 \( \mu \)pore Millipore membrane filter which was
then dried and placed into a scintillation vial containing 10 ml of toluene scintillation fluid (4 g PPO and 150 mg dimethyl POPOP in 1.0 liter toluene). Ten min counts were made in a Tricarb Liquid Scintillation Spectrometer (Packard Instrument Co.) and background subtracted.

All of the concentrations of CH tested completely inhibited incorporation of the labelled amino acids into protein, and so the lowest concentration (50 µg/ml), which inhibits reversibly, was chosen for the inhibition experiments.
RESULTS

Cellular Growth, Respiration Rate, Cytochrome Oxidase Activity, and Nuclear Number in Long-Term Cultures

a. Unagitated cultures. Figure 2 shows the average growth curve obtained from 7 experiments. The values making up this curve were obtained by averaging individual growth curves at 20-hour intervals. All cultures were inoculated at $10^3$ amoebae/ml with logarithmically multiplying cells ($5 \times 10^4$ amoebae/ml) and no lag in multiplication was observed. For this reason, and since growth was terminated prior to maximum stationary phase, the growth curve may be divided into two regions: logarithmic multiplication (LM), lasting for the first 50 hours with a generation time (Tg) of $7.35 \pm 0.37$ hours, and population growth deceleration (PGD), during which the cell density slowly increases 7-fold in 130 hours.

Respiration rates (figure 3) increase slightly from 23.7 to 29.0 µl/hr/10⁶ cells during 180 hours of growth according to a line fit to the data by the least squares method.

Total COx is illustrated in figure 4. This value falls in late LM from about 800 to 570 µmoles cytochrome c oxidized/min/10⁵ cells and then returns during PGD almost to the original value of 800. Between 150 and 190 hours, COx approximately doubles to 1200, remaining at this level for the rest of PGD.
Figure 2. An average population growth curve for unagitated cultures. The data are from 7 experiments. Each point was obtained by averaging individual growth curves at 20-hour intervals.
Figure 3. Changes in average respiration rate in unagitated cultures as a function of culture age. The data are from 6 experiments. Each point represents an average of 6 measurements.
Figure 4. Changes in cytochrome oxidase activity in unagitated cultures as a function of culture age. The data are from 6 experiments and each point represents an average of 3 measurements.
A detailed examination of the late LM decrease in COx is depicted in figure 5. As the cell concentration increases from $5 \times 10^3$ to $3 \times 10^4$ amoebae/ml, the enzyme activity falls with a decelerating rate from 730 to 225. COx is steady at the latter value until the cell concentration reaches about $8-9 \times 10^4$ amoebae/ml, when COx rises to 440. This pattern was obtained when cultures were inoculated at $10^3$ amoebae/ml with inocula varying in age from $3 \times 10^4$ to $7 \times 10^4$ amoebae/ml.

When the logarithm of relative COx is plotted against the age of the cells assayed (figure 6), beginning at $5 \times 10^3$ and up to $3 \times 10^4$ amoebae/ml, a linear relationship is obtained by a least squares analysis which indicates that the decrease in cytochrome oxidase has a half-time of about 1.85 Tg's.

During these fluctuations in COx, the average number of nuclei/cell (N/C) remains at 1.01 for about 120 hours and rises during the next 30 hours to a plateau value of 1.04.

b. Agitated cultures. Figure 7 depicts the type of growth curve obtained when cells are agitated by aeration. This curve represents the averages of individual curves from 6 experiments at 20-hour intervals. These cultures were inoculated at about $4 \times 10^3$ amoebae/ml with logarithmically growing unagitated cells. A relatively short lag appears at the very beginning of agitation and lasts for 10-15 hours. Growth is then logarithmic for approximately 90 hours with a Tg of about 14 hours. This phase is followed by a relatively rapid deceleration of the rate of cell division. The cell density finally
Figure 5. Changes in cytochrome oxidase activity in unagitated cultures as a function of culture age. The data are from 5 experiments. Each point represents an average of 2 measurements. All cultures were inoculated at $10^3$ amoebae/ml.
Figure 6. A semilogarithmic plot of changes in cytochrome oxidase activity in unagitated cultures as a function of culture age. The data are the same as in figure 5.
Figure 7. An average population growth curve for agitated cultures. The data are from 6 experiments. Each point represents the mean cell concentration for a 20-hour interval.
reaches a plateau at about 10^6 amoebae/ml. In referring to the phases of agitated growth, the same conventions as are used for unagitated growth phases will be employed, i.e., the period of logarithmic multiplication (20-90 hours) is LM; population growth deceleration phase (90-180 hours) is PGD.

Figure 8 shows the respiration rates of agitated cells as a function of culture age. After a lag of 20-30 hours, the level of respiration rises from the unagitated cell value of the inoculum (22.0 μl O_2/hr/10^6 cells) to about 48 μl O_2/hr/10^6 cells and then remains constant from 60 to 100 hours. The rate then increases during PGD to a new plateau value of about 72 μl O_2/hr/10^6 cells. This step-wise pattern of increase in respiration rate seems to be reproducible.

Figure 9 depicts COx in agitated cultures. During LM it rises from the inoculum level of 400 to a plateau at about 960. This latter value substantially exceeds the highest value observed during LM in unagitated cultures (figures 4 and 5). At the end of LM and beginning of PGD this level rises to a plateau of 2400, a little more than a doubling of the LM value.

N/C increases (figure 10) from the unagitated cell level (1.01) to reach a LM plateau of about 1.24. The level then rises during late LM and early PGD to finally plateau by 140 hours at 1.73.

The relative values of N/C, COx and respiration rates in agitated cultures are shown in figure 11. Increases of N/C seem to slightly precede those of respiration rate. However, relative increases in
Figure 8. Changes in average respiration rate in agitated cultures as a function of culture age. The data are from 5 experiments. Each point represents an average of two measurements. The culture was inoculated at $4 \times 10^3$ amoebae/ml.
Figure 9. Changes in cytochrome oxidase activity in agitated cultures as a function of culture age. The data are from 6 experiments and each point represents an average of 3 measurements. The curve has been extrapolated to a zero time value typical of the unagitated cells of the inoculum.
Figure 10. Changes in the average number of nuclei per cell in agitated cultures as a function of culture age. The data are from five experiments. Each point represents an average of 3-5 measurements.
Figure 11. Relative changes in respiration rate and average number of nuclei per cell in agitated cultures as a function of culture age. o - Respiration rate; o - Average number of nuclei per cell; Δ - Cytochrome oxidase activity per cell. The data used are from figures 9, 10, and 11.
respiration rates in each case exceed those of N/C, the values being 1.8-fold and 1.4-fold for LM and 2.9-fold and 1.7-fold for PGD, respectively. COx rises 2.5-fold in LM and 3.75-fold in PGD, higher than either N/C and respiration rates.

Cellular Growth, Average Volume, COx, DNA, RNA, and Protein During Short Term Experiments

Since such significant changes in respiration rates and COx were induced by aeration, a more extensive examination was made of the initial cellular responses to this type of agitation. The process of inoculation requires that cells from unagitated cultures be suspended by policing and further disturbed by stirring. Therefore, it was first necessary to assess the effects of this type of agitation, in contrast to the agitation by aeration. Thus, freshly inoculated unagitated cultures were analyzed for one generation after policing.

a. Unagitated control cultures. Figure 12 shows that the control cultures exhibited a 1-2 hour lag in cell division followed by a rapid increase in cell number which suggested some synchrony in division. Cell number slightly more than doubled during the 8-hour period.

Unagitated cultures grown to $1 \times 10^4$ amoebae/ml and to $3.7 \times 10^4$ amoebae/ml, respectively, were treated as described in the legend of figure 12 and the subsequent changes in COx were then followed. COx (figure 13) in the young cultures gradually dropped 40% from about 360 to about 190, as the cells grew from $1 \times 10^4$ to $2 \times 10^4$ amoebae/ml.
Figure 12. An average relative growth curve for unagitated cultures over an 8-hour period. The data are from 6 experiments. Each point represents a mean cell concentration. Cultures were grown unagitated up to about $2 \times 10^4$ amoebae/ml (30 hours) from a starting concentration of $10^3$ amoebae/ml. Cells were then aseptically scraped from the flask sides with a rubber policeman and then allowed to settle and remain in their original flasks for further unagitated growth. Cells were collected for assay at intervals over an 8-hour period from the time of policing.
Figure 13. Changes in cytochrome oxidase in unagitated cultures over an 8-hour period. The data are from 2 experiments. Each point represents an average of 6 measurements. • - Initial cell concentration $10^4$ amoebae/ml. ○ - Initial cell concentration $3.7 \times 10^4$ amoebae/ml. Cultures were grown and treated as described in the legend of figure 12.
In contrast, in the older culture COx increased 2.3-fold from a value of about 180 to 420, as the cells grew from $3.7 \times 10^4$ to $7.4 \times 10^4$ amoebae/ml.

Changes in cellular protein, DNA, and RNA levels during the 8-hour period are depicted in figure 14. Protein is relatively constant at about 250 pg/cell. DNA decreases slightly from 1.3 to 1.1 pg/cell during the first 4 hours, and subsequently rises at the sixth hour to a final value of 1.6 pg/cell. RNA/cell decreases gradually during the first 4 hours from 54.0 to 51.5 pg, drops sharply to a minimum of 44.0 pg at 6 hours, then rises to 52.5 pg at the end of 8 hours.

No change in the usual N/C (1.01) was found. Average cellular volume (figure 14) at zero time is 3000 $\mu^3$. It increases about 15% during the lag period and then drops quickly during the early increase in cell number, continuing to drop more slowly below the zero time value to about 2900 $\mu^3$ at 8 hours.

**b. Agitated cultures.** In the continuously agitated cultures cell number (figure 15) remains constant for about 4 hours. This lag is followed by an increase in cell number of about 1.9-fold with an average doubling time of 4 hours, indicating a substantial synchrony in cell division.

COx (figure 16) increases from about 156 to 480 during the first 6 hours, remaining at that level for the next 2 hours.

Total protein, DNA, and RNA are depicted in figure 17. Protein rises from 240 to 310 pg/cell in the first 3 hours and, then, 2 hours
Figure 14. Composite graph of volume and macromolecule changes in unagitated cultures over an 8-hour period. Each curve is from 2 experiments and each point represents an average of 6-9 measurements. Cultures were grown and treated as described in the legend of figure 12.
Figure 15. An average relative growth curve for agitated cultures over an 8-hour period. The data are from 8 experiments. Each point represents a mean cell concentration for a one-hour interval. Cultures to be agitated were grown as described in the legend of figure 12. The policed cultures were then poured into aspirator flasks to a final volume of 650 ml. These cells were agitated as described in Materials and Methods and samples of cells were removed for assay during an 8-hour period after the initiation of agitation.
Figure 16. Changes in cytochrome oxidase in agitated cultures over an 8-hour period. The data are from 2 experiments. Each point represents an average of 6-12 measurements. Cultures were grown and treated as described in the legend of figure 15.
Figure 17. Composite graph of volume and macromolecule changes in agitated cultures over an 8-hour period. Each curve is from 2 experiments and each point represents an average of 6-9 measurements. Cultures were grown and treated as described in the legend of figure 15.
later rises again to a final level of 380 pg/cell, 50% above the zero time value. DNA/cell is constant for the first 2 hours at 1.80 pg and then rises continuously to 2.55 pg, a 41% increase. RNA/cell increases from 52.5 pg to 59.0 pg in the first 4 hours, decreases to 50.5 pg after 6 hours and then rises again to a maximum of 63.0 pg, an overall increment of 20%.

The number of multinucleates/1000 amoebae (figure 18), almost all of which are binucleates, increases continuously from 10 (N/C = 1.01) typical of unagitated cultures to a value of about 150 (N/C = 1.15) during the 8-hour period.

Figure 17 shows that average cellular volume increases 16% during the first 2.5 hours from 3170 μ³ to 4240 μ³ and remains at the latter level for the next 4 hours with perhaps a slight decrease at the end of this period to a value of 4140 μ³. In the final 1.5 hours average cellular volume rises to 4530 μ³, an overall increase in volume of 43%.

Effects of Inhibitors on COx in Agitated Cultures

Experiments were performed in order to determine whether the increase of COx after initiation of agitation is dependent upon DNA, RNA, or protein synthesis. Because of limited supplies of inhibitors, 500 ml Erlenmeyer flasks were adapted for agitation of cells by aeration at the standard rate. Cells were grown unagitated and collected for experimentation as described above for the 8-hour experiments. Each aspirator flask contained 350 ml of culture initially, the
Figure 18. Changes in the number of multinucleates per 1000 cells in agitated cultures over an 8-hour period. The data are from one experiment. Each point represents an average of 3 measurements. Cultures were grown and treated as described in the legend of figure 15.
inhibitor was added at zero time, and samples were periodically removed for assay of COX.

There is only a slight difference in enzyme activity between control and HU-treated cells (figure 19). Whereas control cell COX increases about 1.8-fold, that of treated cells reaches about 1.6-fold. Control cells do not divide for 3-4 hours, but then cell number rises about 1.4-fold. Treated cultures have a lag of about 5 hours and then cell number rises 1.2-fold. There is a clear inhibition of COX increase by actinomycin D and by CH. COX rises only slightly in actinomycin D-treated cells (figure 20) during the 8-hour period, while the lag in cell division is longer with actinomycin D than in the controls. CH likewise prevents an increase in COX and almost completely inhibits cell division (figure 21).
Figure 19. The influence of hydroxyurea on changes in cytochrome oxidase activity and cell concentration in agitated cultures over an 8-hour period. The data are from 2 experiments and each point represents an average of 4 measurements. o - Hydroxyurea added to the culture; e - Controls; (A) Relative cytochrome oxidase activity per cell; (B) Cell concentration. The initial cell concentrations were $2 \times 10^4$ amoebae/ml.
Figure 20. The influence of Actinomycin D on changes in cytochrome oxidase activity and cell concentration in agitated cultures over an 8-hour period. The data are from 2 experiments. Each point represents an average of 4 measurements. o – Actinomycin D added to the culture; © – Controls; (A) Relative cytochrome oxidase activity per cell; (B) Relative cell concentration. The initial cell concentrations were $2 \times 10^4$ amoebae/ml.
Figure 21. The influence of cycloheximide on changes in cytochrome oxidase activity and cell concentration in agitated cultures over an 8-hour period. The data are from 2 experiments. Each point represents an average of 4 measurements. o - Cycloheximide added to the culture; o - Controls; (A) Relative cytochrome oxidase activity per cell; (B) Relative cell concentration. The initial cell concentrations were $2 \times 10^4$ amoebae/ml.
DISCUSSION

A number of the growth characteristics of unagitated cultures of Acanthamoeba castellanii grown under conditions identical to those used in the present study have been reported by Byers et al. (1969). These authors propose that exponential multiplication continues until the level of dissolved oxygen in the medium becomes limiting and they show that the oxygen tension begins to drop at a cell density of $5 \times 10^3$ amoebae/ml and that it reaches a plateau value at $10^5$ amoebae/ml of about 50% of saturation. During LM, A. castellanii adheres to the bottom of culture flasks forming what appear to be uniform cell layers, but during PGD the cells form multi-layered clumps. If the cells in a PGD culture, in which the oxygen tension is at the 50% plateau, are suspended by scraping the bottom of the flask with a rubber policeman, the oxygen tension falls rapidly to zero. This indicates that cells normally clumped on the bottom do not respire at maximum rates unless they are suspended in the medium. The rates that can actually be measured in an oxygen electrode (figure 3) are potential respiration rates, i.e., they represent the maximum respiration rates in optimal growth medium for cells of those culture ages. It is quite possible that near anaerobic conditions exist within the cell clumps during aging.
Although the data in figure 3 indicate only a slight increase in potential respiration rates as cultures age, studies of COx levels suggest that there is about a 2-fold increase of this enzyme in PGD.

One of the striking features of the cellular COx pattern in unagitated cultures is the observation that during late LM COx drops 65%, while the cell multiplication rate and N/C remain constant (figure 6). This drop is interpreted as being due to the onset of anaerobiosis. A comparison of the data in the present study with that published (Byers et al., 1969) indicates that COx begins to drop simultaneously with oxygen tension in these cultures and reaches a minimum at about $3 \times 10^4$ amoebae/ml (figure 6). The observed decrease in COx is logarithmic, as would be expected if cytochrome oxidase synthesis were inhibited while cell division continued. If the enzyme's synthesis were completely inhibited, the enzyme activity would be expected to halve with every doubling in cell number. The observed half time of the decrease is about 13.6 hours, compared with a cell doubling time of 7.35 hours, so that this is not simply a dilution of enzyme extant at the beginning of the decrease. Instead, since the rate of cell division is relatively constant at maximum LM rate, COx must be produced continuously throughout the period during which the enzyme activity drops, but at a rate reduced from that of the maximum LM rate (before cell density reaches $5 \times 10^3$ amoebae/ml). Furthermore, at the end of LM, COx rises again (figure 6), indicating that when the cell division rate is finally retarded, the enzyme can continue to be synthesized.
The rate of synthesis of cytochrome oxidase during the logarithmic decrease in COx can be calculated from the data. Cytochrome oxidase/ml (M) at any time (t) is obtained by multiplying cells/ml (C) by cytochrome oxidase/cell (M/C).

\[ C \times \frac{M}{C} = M \]  

(1)

C at any t is given by

\[ C = C_0 e^{kt} \]  

(2)

where \( C_0 \) is the cell concentration at \( t=0 \) and \( k \) is the cell growth rate constant. Similarly, figure 6 indicates that the M/C decrease is first order, and so M/C at any time t is given by

\[ \frac{M}{C} = \frac{M_0}{C_0} e^{-k't} \]  

(3)

where \( M_0/C_0 \) is the cytochrome oxidase/cell at \( t=0 \) and \( k' \) is the rate constant for M/C decay. Substituting equations (2) and (3) in equation (1) gives

\[ M = [C_0 e^{kt}] \left[ \frac{M_0}{C_0} e^{-k't} \right] = M_0 e^{(k-k')t} \]  

(4)

Equations (2), (3), and (4) can be converted to a linear equation with the general form,

\[ \ln\left(\frac{Y}{Y_0}\right) = kt \]  

(5)

Graphically, the slope of equation (4) may be determined by subtracting the curve in figure 6 from the growth curve (figure 2). However, the rate constants are most easily obtained by using the
cell doubling time \( (T_g) \) and the half-time \( (t_{1/2}) \) for the COx decrease.

\[
k = \frac{\ln 2}{T_g} = \frac{0.693}{7.35} = 0.094/\text{hr}
\]

\[
k' = \frac{\ln 2}{t_{1/2}} = \frac{0.693}{13.6} = 0.050/\text{hr}
\]

Therefore, the doubling time for cytochrome oxidase \( (T_{g_{m}}) \) is

\[
T_{g_{m}} = \frac{\ln 2}{k''} = \frac{0.693}{0.044} = 15.75 \text{ hr}
\]

where \( k'' = k-k' \).

Unfortunately, it is impractical to measure COx in cultures with less than \( 5 \times 10^3 \) amoebae/ml and, since this is close to the cell concentration at which oxygen begins to drop, it is impossible to say whether COx establishes a steady state during early LM. If it does, then \( T_{g_{m}} = T_g \) before the decrease begins and after the drop the growth rate of M has decreased about 2-fold.

The drop in COx is probably precipitated by the simultaneous decrease of oxygen dissolved in the medium. As has been pointed out, most of the cells are on the bottom of the flask where the actual cell density is much higher than \( 5 \times 10^3 \) amoebae/ml and where conditions may, therefore, approach anoxia. In yeast it is well known that anoxia depresses the synthesis of cytochromes and biogenesis of mitochondria (Wilkie, 1964; Roodyn and Wilkie, 1968; Maroudas and Wilkie, 1968). In view of the fact that readmission of oxygen to anaerobically grown yeast cells induces the synthesis of cytochrome
oxidase in parallel with the formation of mitochondria (Roodyn and Wilkie, 1968), the logarithmic decrease in COx in A. castellanii may reflect a similar reduction in the number of mitochondria/cell.

An increase in COx is observed under two conditions in the unagitated cultures. First, late LM cells used as inocula have the minimum COx and, thus, there must be an increase to the level observed before the late LM drop. It is possible that this increase is due to mitochondrial biogenesis induced by oxygen. Second, there is an approximate doubling of COx in PGD when the cells stop dividing (figure 4). One possible interpretation of this observation is that mitochondria can undergo one round of replication after cytokinesis is blocked. Since the simultaneous increase in N/C is only from 1.01 to 1.04, this would mean that nuclear and mitochondrial replication are uncoupled from each other. A similar uncoupling of chloroplast and nuclear replication in aging culture of Euglena has recently been reported (Carell, 1969).

Figure 13 shows the changes in COx occurring during growth in the short term unagitated cultures. Even though these cultures have been policed, the enzyme displays the sort of pattern expected on the basis of the long term experiments. The younger cells which would be expected to have maximal COx activity, show a decrease in the enzyme activity and the older cells, which would be expected to have minimal COx activity, show increases in COx content with aging. Changes in other cellular parameters (figure 14), however, are probably an indirect result of disturbing the cells by policing.
Many of the curves are similar in pattern to each other, displaying a slight decrease after 2-3 hours, the time when cell number begins to increase. The first burst of cell division has a doubling time of about 6.5 hours, compared to the normal $T_g$ of 7.35 hours, so that there is a small degree of synchrony apparently due to inhibition of cell division by the disturbance of the cells, although the mechanism of inhibition is unknown. Because of this synchrony, an accumulation of older predivision cells is expected during the division lag. Compounds that are continuously synthesized during the cell cycle should increase in amount/cell until cell division occurs. This is most clearly observed in the protein and volume data. After division is reinitiated a high proportion of daughter cells in the population is expected, and, therefore, a transient decrease in values/cell.

DNA/cell is different, however, with a small initial drop, followed by a return to the zero time value. The drop occurs because of the initial synchronous burst of cell division, but unlike the other parameters, there is a subsequent rise. These cells synthesize DNA at the very beginning of the cell cycle (Neff and Neff, 1969) and spend a small fraction of the cycle in the DNA synthetic phase (S). Therefore, once the cells have divided, reducing the average value of DNA/cell, they enter S immediately and restore the original value of DNA/cell.

More insight into the control of COx synthesis is obtained when cultures of *A. castellanii* are agitated by aeration. The immediate effects of increasing the oxygen supply by aeration are to extend LM
to a final cell density of $4 \times 10^5$ amoebae/ml and to shorten the
time required for attainment of stationary phase after the beginning
of PGD (compare figures 2 and 7). James and Byers (1967) reported
a Tg of about 19 hours for agitated cultures of *A. castellanii* and
observed no initial lag in cell division. In the present study a Tg
of 14 hours was obtained and a 10-15 hour lag phase observed (fig­
ure 7). The discrepancies in Tg and shape of the curves may be a
result of differences in methods of culture, especially since dif­
ferent volumes of medium were used and the relationship between agita­
tion intensity and culture volume is not clear. In this study
inoculations were made from late LM unagitated cultures into 1200 ml
of medium to a final concentration of $2 \times 10^4$ amoebae/ml. James
and Byers (1967) used post-LM unagitated cells ($5-9 \times 10^5$ amoebae/
ml) as inocula for 1500 ml cultures with a final concentration of
$2 \times 10^4$ amoebae/ml. The rate of aeration used was the same as
employed in the present study. Byers (1969) has plotted agitation
rate vs Tg for cultures grown in a reciprocating water-bath shaker
and shown that Tg is very sensitive to the intensity of agitation
in the range used in these studies. As noted earlier (James and
Byers, 1967), the induction of multinuclearity is a primary effect
of aeration. Values reported by these authors for N/C in LM and
PGD are 1.30 and 1.75, respectively. These values are higher than
the ones reported here, 1.25 in LM and 1.70 in PGD, but this is to
be expected since James (1966) and Byers (1969) have shown that N/C
increases with the generation time. They proposed that multinucleates
form as a result of karyokinesis in the absence of cytokinesis and, thus, conclude that nuclear and cell division are uncoupled.

In the present studies agitation induces respiration rates and COx to increase to a plateau simultaneously with N/C (figure 11). Therefore, there is probably a similar mechanism controlling the levels of all three. However, the increments in respiration rate and COx exceed those of N/C, indicating that agitation has a differential effect upon respiration rates or COx and production of multinucleates. Since almost all cellular respiratory capacity and all COx are localized in mitochondria, COx and respiration rate should be related to numbers or mass of mitochondria, although other factors such as availability and type of substrate also affect respiration rate. Because of the similarity in pattern in agitated cultures between N/C, respiration rates and COx (figure 11), and because the changes in N/C indicate that "uncoupling" of nuclear and cytoplasmic division occurs, it seems to be a good possibility that the observed changes in respiration rate and COx signify a similar uncoupling of mitochondrial reproduction and cytoplasmic division.

If nuclear and mitochondrial reproduction are uncoupled both from cytokinesis and from each other, agitated cultures would, then, provide good examples of the independent replication capabilities of different organelles. The PGD doubling of COx in agitated cultures seems to occur when the cells stop dividing (figure 9), as is also the case for unagitated cultures. In late PGD N/C rises
from 1.25 to 1.70 in agitated cultures simultaneously with the increase in COx. However, both LM and PGD increases in COx are higher than the corresponding increases in N/C.

Perhaps this possible separability of mitochondrial and nuclear reproduction is not surprising considering the evidence below favoring partial mitochondrial autonomy in the cell. Mitochondria have been shown to be capable of synthesizing in vitro their unique DNA (Nass, 1969; Meyer and Simpson, 1969; Gross and Rabinowitz, 1969), RNA (South, 1968; South and Mahler, 1968), and protein (Kroon et al., 1967; Neupert et al., 1967) by mitochondria-specific enzymes (Barnett et al., 1967; Kalf and Ch'ih, 1968). Of special interest in the present study is the suggested role of mitochondrial genes in the synthesis of the cytochromes. The most convincing evidence for mitochondrial genetic autonomy in cytochrome synthesis is from studies of cytoplasmic inheritance in yeast and Neurospora reviewed by Roodyn and Wilkie (1968). There are classes of mutants of these organisms which are deficient in respiratory capacity, and lack cytochromes a, a3, and b. These mutants are produced in high yield by treatment of cells with acridine dyes, whose site of action has been shown to be DNA replication and/or transcription. Respiratory mutations of this type are inherited maternally, suggesting that the genetic determinant is located in the cytoplasm in general, the mitochondrion in particular. COx (cytochrome a+a3) as assayed in this study, therefore, may be an indication of mitochondrial gene expression, as well as a measure of mitochondrial numbers.
In yeast anoxia causes repression of the synthesis of electron carriers comprising the respiratory chain, and this effect can be reversed by exposing the cells to oxygen, which derepresses the formation of cytochromes (Wilkie, 1964; Roodyn and Wilkie, 1968). A similar phenomenon is observed when unagitated cultures of *A. castellanii* are agitated by aeration. In the inocula for these cultures the COx activity is depressed due to the age of the inocula, but aeration causes the COx to rise again to levels that are near maximal for unagitated cultures in LM (figure 16). Furthermore, this increase is prevented by act D and cycloheximide (figures 20 and 21), suggesting that de novo RNA and protein synthesis are required for the increase in COx to occur, and that, therefore, the increase is a true induction of enzyme synthesis.

Act D is a known inhibitor of DNA-dependent RNA synthesis in mitochondria (Kroon, 1963; Wheeldon and Lehninger, 1966; Wintersberger and Tuppy, 1965). Cycloheximide (CH) has been found not to affect the formation of cytochromes a and b in yeast, while inhibiting microsomal protein synthesis (Clark-Walker and Linnane, 1966; Wilkie, 1968). This has been interpreted to mean that the cell has two protein synthesizing systems, one cytoplasmic (sensitive to CH) and the other mitochondrial (insensitive to CH), although brain mitochondrial protein synthesis has been found to be CH sensitive (Cunningham and Bridgers, 1970). As mentioned above, the increase in COx after initiation of agitation of cultures of *A. castellanii* may also signify an induction of organelle biosynthesis. On the
basis of the foregoing discussion, it is probable that some of the proteins incorporated into growing cells and dividing mitochondria are synthesized on cytoplasmic ribosomes and are therefore inhibited by CH.

Hydroxyurea (HU) does not seem to affect this induction of cytochrome oxidase synthesis (figure 19). This suggests that either mitochondrial DNA (mDNA) synthesis is not affected by the drug or an increase in COx does not require mDNA synthesis. The latter possibility may imply that increased COx represents augmented mitochondrial size without mitochondrial replication, but an increase in mitochondrial numbers without accompanying mDNA synthesis could occur if most of the mitochondria had already replicated their genomes before the addition of the inhibitor, enabling them to divide once.

In order to more carefully examine the initial responses of the cells to agitation, the kinetics of increase of cellular parameters were followed for 8 hours after the initiation of agitation and are summarized in figure 22. The results are plotted in relative quantities as a function of time after initiation of agitation. Average volume and protein/cell have somewhat similar patterns, consisting of an initial increase in the first 3 hours of 1.25-1.30, followed by a plateau for about the next 2.5 hours and a final rise to 1.45-1.50. Since protein is the most abundant macromolecule in this cell, it is not surprising that cellular volume should follow protein so closely, being largely determined by protein content. RNA/cell also rises initially (the first 4 hours) to 1.09, drops off
Figure 22. Relative changes in average number of nuclei cytochrome oxidase activity, volume, and macromolecule content in agitated cultures over 8-hour period.
to a minimum at 6 hours of 0.95, and then rises again to a maximum at 8 hours of 1.09. Thus, the patterns of increase of protein, RNA, and volume have some similarities. They exhibit an initial rise, then a leveling or slight drop, followed by another increase. The leveling takes place when cell number begins to increase following the initial lag. This indicates that there is enough synchrony of cell division to result in the production of a relatively large percentage of young, newly divided cells, and to cause the average values of the measured cellular parameters to be low. Also, one would expect the mitotic index to be higher than in logarithmically growing cells. Since it has been shown that RNA synthesis is greatly reduced or non-existent in cells undergoing mitosis (King and Barnhisel, 1967; Hodge et al., 1969), the drop in the level of RNA in these agitated cultures might be due to an increased fraction of cells in mitosis.

In addition, it has also been demonstrated that protein synthesis is somewhat lower during mitosis than during interphase (Konrad, 1963; Johnson and Holland, 1965). Thus, in A. castellanii a leveling of protein/cell, in contrast to the marked drop of RNA/cell, would be expected and is observed.

$CO_x$ increases fairly constantly to a little above 2.00 (figure 22). A cell density of $2 \times 10^6$ amoebae/ml was attained before the onset of agitation in these 8-hour experiments. At that concentration of unagitated cells, $CO_x$ is already about half of its maximum value (figure 6). It was suggested earlier that this drop is
caused by anoxia at the bottom of the culture flask where the cells adhere. Therefore, agitation by aeration probably restores the depleted oxygen supply and original number of mitochondria. In fact, COX does not actually achieve the maximum values observed in young unagitated cultures (figure 5).

DNA/cell rises fairly constantly after the first 2 hours up to 1.34, while N/C reaches 1.15. If the amount of DNA/nucleus did not change throughout the 8-hour period of agitation, the curve for N/C and DNA/cell would coincide. However, DNA/nucleus does increase. The cells of the inoculum, grown from $10^3$ amoebae/ml to $2 \times 10^4$ amoebae/ml, contain 1.75 pg DNA/cell, which is less than the 2.00 pg/cell found in the postsynthetic phase of the DNA growth-replication cycle (Byers et al., 1969). The lower value is due to the fact that DNA synthesis has been inhibited in cells of this age in G_1, the presynthetic phase of the DNA growth-replication cycle.

At the same time that DNA/cell is dropping, the dissolved oxygen content of the culture medium and COX are decreasing, as explained above. These changes may be interrelated such that when the cells are aerated (agitated) the oxygen level of the medium is replenished and the COX increase is induced, enabling the cells to resume DNA synthesis so that the G_2 amount of DNA/nucleus is restored. If DNA/nucleus increases from 1.75 to 2.00 pg during the 8-hour period of agitation, while N/C rises from 1.01 to 1.15, DNA/cell would then be expected to increase from 1.75 to 2.30, or 1.31-fold. The observed rise in DNA/cell is 1.34, which is only 10% higher than expected.
It is possible that some of the DNA is mitochondrial and that the increase in COx is due to mitochondrial replication accompanied by a synthesis of mDNA. The drop in DNA/cell begun at $10^4$ amoebae/ml in unagitated cells (Byers et al., 1969), might signify a lower rate not only of nuclear DNA synthesis, but also of mDNA synthesis. Perhaps the 10% excess of DNA/cell after 8 hours of agitation is mDNA. Cytoplasmic DNA in A. castellanii has been detected by Ito et al. (1969) and Adam et al. (1969). In the latter report the quantity of mDNA was found to vary depending upon the age of the cells, older cells containing a higher percentage of mDNA.

Moustacchi and Williamson (1966) observed that yeast mDNA is 3% of total DNA in log phase cells, and 20% of total DNA in stationary phase cells, and that this can be correlated with numbers of mitochondria/cell and respiration rate. COx doubles in late PGD cultures of agitated or unagitated cells (figures 4 and 9), which could be a result of the increase in mitochondrial genes per cell implicit in a rise in mDNA/cell. In synchronized yeast populations mDNA replicates discontinuously in the cell cycle at a time different from that of nuclear DNA (Tauro et al., 1969). A doubling of cytochrome oxidase also occurs at that point in the cell cycle (Catrell and Avers, 1970).
Summary and Conclusions

Populations of *Acanthamoeba castellanii* were grown in optimal growth medium either unagitated or agitated by aeration through phases of logarithmic multiplication (LM) and population growth deceleration (PGD).

In unagitated cultures the average number of nuclei/cell (N/C) and respiration rate change very little with culture age, but cytochrome oxidase activity (COx) drops logarithmically 65% in mid-LM. Byers *et al.* (1969) have shown that the level of oxygen dissolved in the medium also drops in mid-LM and it is probable that conditions approach anoxia at the bottom of the culture flask. Since these amoebae adhere to the bottoms of the culture flasks in unagitated cultures, the decrease in COx is probably a result of oxygen deficiency. The rate of COx decrease is less than would be expected if its synthesis were completely inhibited and its amounts halved with every cell doubling. It is concluded that enzyme synthesis continues throughout the logarithmic drop, but at a reduced rate compared to that before the drop. At the end of LM and beginning of PGD, COx returns to the level found in early LM before the drop and then subsequently rises again at the end of PGD until the early LM value is doubled. It is proposed that these increases are
due to progressive retardation of the rate of cytokinesis with continuing synthesis of cytochrome oxidase.

In agitated cultures, N/C, respiration rate, and COx rise from the unagitated cell level of the inoculum to a LM plateau level. Since N/C probably changes as a result of an "uncoupling" of karyo- and cytokinesis, COx and mitochondrial function may likewise increase because of uncoupled mitochondrial division and cytokinesis. However, respiration rate and COx increase more than N/C, and so mitochondrial and nuclear division may be somewhat independent. In late PGD all these variables further increase to a new plateau. One possible interpretation of this observation is that mitochondria and nuclei divide approximately once after cytokinesis ceases.

The kinetics of increase of COx, average cell volume, N/C, protein, RNA, and DNA content as a function of time after the initiation of agitation were followed. All parameters increased relative to unagitated controls. By using inhibitors of protein (cycloheximide) and RNA (actinomycin D) synthesis it was demonstrated that the rise in COx upon agitation requires enzyme synthesis rather than simple activation, and probably organelle biogenesis. However, inhibiting DNA synthesis with hydroxyurea (HU) had no effect upon COx synthesis. It is suggested that nuclear, but not mitochondrial, DNA synthesis is prevented by HU.

Perspectives

Several intriguing questions for future investigations arise as a result of this work. The decrease in COx and possibly
mitochondrial numbers in unagitated mid-LM cells suggest that the biogenesis of mitochondria is repressed during the period of the decrease. Since mitochondria may synthesize RNA (South and Mahler, 1968), DNA (Nass, 1969), and some of their own proteins (Roodyn and Wilkie, 1968), the mitochondria from those mid-LM cells should not be synthetically active. Therefore, the macromolecule synthesis in vitro of mitochondria isolated from mid-LM cells could be examined. Mitochondrial macromolecular synthesis may be controlled by extramitochondrial factors. Experiments analogous to those of Mattar (1970) could be performed by assaying for mitochondrial macromolecule synthesis in vitro in the presence or absence of cytoplasmic extracts from mid-LM amoebae and from cells whose mitochondria are synthetically active.

Only one electron carrier, cytochrome oxidase, has been analyzed in this study. Other cytochromes and mitochondrial oxidative enzymes in general could be examined to determine whether they are similarly regulated. Since some oxidative enzymes, such as malate dehydrogenase (Davidson and Cartner, 1967) isocitrate dehydrogenase (Tait, 1970), and cytochrome c (Tzagaloff, 1969), are products of nuclear genes, and others, such as cytochromes a and b (Roodyn and Wilkie, 1968), are thought to be encoded by mitochondrial genes, studying the levels of these enzymes in A. castellanii could indicate differences in their regulation which might be correlated with N/C and mitochondrial numbers/cell, suggesting differences in their sites of synthesis. It should be noted in this regard that in the
present study respiration rate rises only with increases in N/C plus COx (agitated cultures), not just with an increase in COx (unagitated PGD cultures), which suggests a nuclear role in the development of mitochondrial function, although other factors, such as type of substrate, etc., also determine respiration rate.

Tomlinson (1967) has shown that *A. castellanii* synthesizes enzymes of the glyoxylate pathway before encystment. In addition Sharpless and Butow (1970a,b) demonstrated a correlation between adaptation to glyoxylate metabolism and induction of a new terminal oxidase, besides cytochrome oxidase, in *Euglena*, causing an increase respiration rate. It would be interesting if there were qualitative as well as quantitative changes in cytochromes in *A. castellanii* preparing for encystment.
LITERATURE CITED


James, T.E. 1966. Changes in the number of nuclei per cell and their relation to ribonucleic acid and protein syntheses in aerated cultures of Acanthamoeba sp. Neff. M.A. Thesis. The Ohio State University, Columbus. 43 p.


