

STATIONARY PHASE AND THE CELL CYCLE OF *Dictyostelium discoideum* IN LIQUID NUTRIENT MEDIUM

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SUMMARY

Cells of the axenic strain of the cellular slime mould *Dictyostelium discoideum*, Ax-3, multiply in the liquid nutrient medium HL-5 with a doubling time of 12 h. When the cell concentration reaches approximately 1×10^7 per ml the rate of cell multiplication begins decreasing and after 20-30 h reaches zero, at a stationary phase cell concentration of 2 to 2.5×10^7 cells per ml. The intercept of the extrapolated log phase and stationary phase plots has arbitrarily been considered the onset of the stationary phase. We have found that after cells have been in stationary phase for 24-32 h, mean cell volume increases by 25 %, average dry weight by 37 %, and average protein content by 24 %. These values are close to the expected values for a cell population which is blocked at a point late in the cell cycle. Stationary phase cells also contain 25 % more nuclear DNA than log phase cells, indicating that the population of cells at stationary phase is blocked after the DNA replication phase. Finally, when stationary phase cells are washed free of stationary phase medium and reinoculated into fresh medium, they reinitiate cell division synchronously. In the light of the demonstrated relationship between stationary phase and the cell cycle, a possible role for the growth inhibitor produced at stationary phase is considered.

INTRODUCTION

When amoebae of the axenic strain of *Dictyostelium discoideum*, Ax-3, deplete nutrient medium of one or more growth-limiting components, the rate of cell multiplication decreases to zero. The cell concentration then remains stable for an extended period of time prior to cell lysis. This period is referred to as 'stationary phase' and is common for most cell populations grown in suspension in liquid nutrient medium. In the cellular slime mould, this phase is particularly interesting for several reasons. First, both a growth (Yarger, Stults & Soll, 1974) and a transcription (Yarger & Soll, 1975) inhibitor appear in the medium of stationary phase cultures. The functions of these inhibitors have not been demonstrated but it has been suggested that the growth inhibitor may play an autoregulatory role in the expression of a stationary phase 'phenotype' (Yarger *et al.* 1974). Second, there have been several reports that stationary phase cells are significantly different from either growing or developing cells with respect to specific phenotypic characteristics (see Weeks, 1973, for changes in sensitivity to Con A agglutination; Rossomando, Steffek, Mujwid & Alexander, 1974, for observable changes in the appearance of the cell surface under the scanning electron microscope; and Malkinson & Ashworth, 1973, for changes in the intracellular

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concentration of cAMP). Third, it was recently demonstrated that morphogenetic information specific for aggregation partially accumulates some time during early stationary phase, but that no information specific for later morphogenetic stages accumulates (Soll & Waddell, 1975). Therefore, stationary phase in liquid nutrient medium affords us with a method of distinguishing separate morphogenetic timers functioning in parallel.

In the work reported here, we have compared several cell parameters between log and stationary phase populations. We have found that the mean cell volume, average dry weight, average protein content, and average nuclear DNA content of stationary phase cell populations are significantly greater than those of log phase cell populations.

These increases are close to estimates for cell populations blocked at a stage late in the cell cycle. In addition, a stationary phase cell population reinitiates cell division synchronously when washed free of stationary phase medium and inoculated into fresh nutrient medium. Together, these observations are consistent with the conclusion that cells in stationary phase have accumulated at a stage late in the cell cycle. In the light of this conclusion, the relationships between the stationary phase growth inhibitor, the accumulation of limited aggregation 'information', and the cell cycle are considered.

METHODS

Maintenance of stocks

Basic stock cultures of Ax-3, a strain of *Dictyostelium discoideum* capable of growth in axenic culture, were maintained on lawns of *Aerobacter aerogenes* on nutrient agar (Sussman, 1966). For an axenic culture, a single sporangium was inoculated into 2 ml of the nutrient medium HL-5 (2 g proteose peptone, 1 g yeast extract, 2 g dextrose, 2 ml of 0.5 M phosphate buffer, pH 6.5, per 140 ml) containing 0.5 mg/ml streptomycin sulphate (Cocucci & Sussman, 1970) and incubated without agitation for 4 days. In turn this culture was inoculated into 140 ml of HL-5 medium in a 1-l. Erlenmeyer flask and rotated at 100 rev/min. When the cell concentration reached approximately 6×10^6 per ml it was diluted 15-fold into fresh medium. New cultures were initiated from sporangia every 2 months. Prior to use as a test culture, the kinetics of cell multiplication and morphogenesis were tested.

Measurements of cell volume, average dry weight, average protein content, and average DNA content

Cell volume. A droplet of cells was removed from a growth flask and placed on a glass slide. The droplet was covered by a glass coverslip raised from the slide by small clay feet at the corners. Two diameters at 90° angles were measured for each cell with a calibrated ocular micrometer inserted into a 10X microscope ocular. Measurements were made at 400×. The average of the 2 diameters for each cell was converted to volume using the formula for a sphere. In each droplet removed from a flask, no more than 20 cells were measured within a few minutes. A total of 510 cells was measured for each distribution.

Average dry weight. 10^8 cells were pelleted, washed once with ice-cold 0.02 M phosphate buffer, pH 7.6, and collected on tared, prewashed Millipore filters (#HAWPO4700). The filter and collected cells were dried at 60° C for 1 h and weighed.

Average protein content. Protein determinations were made according to the methods formulated by Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as a standard.

Average DNA content. Determination of DNA content was performed according to the method described by Burton (1956) after treatment of each sample with RNase and pronase.

Without RNase and pronase digestion, DNA determinations were lower and extremely variable. The method of digestion is described below: 2×10^8 whole cells or nuclei isolated from 2×10^8 cells (Soll & Sussman, 1973; Yarger & Soll, 1975) were washed with ice-cold water. Pellets were then resuspended in 2 ml of buffer solution (0.02 M Tris-HCl, pH 8.2; 0.15 M NaCl; 0.01 M EDTA), and 0.2 ml of 20% sodium dodecylsulphate added. The sample was vortexed for 5 s. A 0.04-ml sample of 25 mg/ml self-digested pronase was added and the suspension incubated for 0.5 h at 50 °C. An additional 0.04 ml of self-digested pronase was added and the suspension incubated for an additional hour. Then 0.025 ml of 5 mg/ml pancreatic RNase was added and the suspension incubated 45 min at 37 °C. Five millilitres of ethanol were then gently mixed in and the solution stored at -20 °C. The precipitate was then pelleted, air dried, and tested for DNA content by the diphenylamine reaction described by Burton (1956).

RESULTS

Growth kinetics

Amoebae of the axenic strain of *Dictyostelium discoideum* Ax-3 grow and divide in the nutrient medium HL-5 (Cocucci & Sussman, 1969) with a population doubling time of 12 h (Fig. 1). When the cell concentration reaches approximately 1×10^7 per ml, the rate of cell multiplication begins decreasing so that after 20–30 h the rate approaches zero at a cell concentration of 2 to 2.5×10^7 per ml. The final cell concentration remains relatively constant for an additional 60–80 h and then decreases as cells lyse.

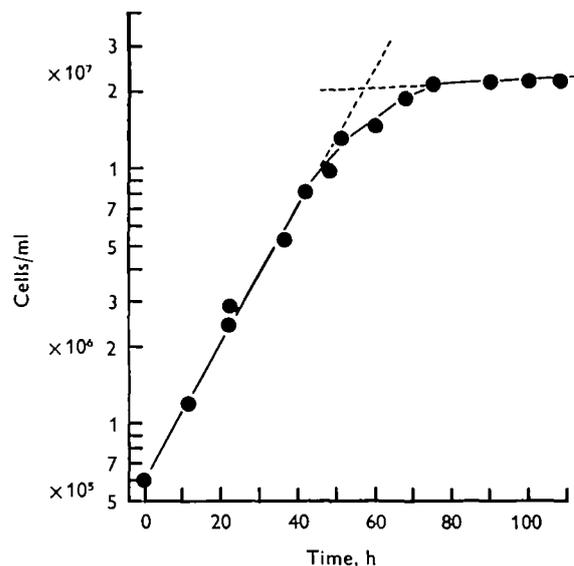


Fig. 1. Growth kinetics of a population of *Dictyostelium discoideum* strain Ax-3 in suspension in the axenic nutrient medium HL-5. The dashed lines represent extrapolations of the logarithmic and stationary phase plots. Intersection of these plots represents the onset of stationary phase.

We have arbitrarily taken the intersection of the extrapolated log phase growth plot and the extrapolated stationary phase plot as the onset of stationary phase (Fig. 1). For analyses of the log phase phenotype, we have systematically employed cells removed from growth cultures at a concentration of 2×10^6 per ml. For analyses of the

stationary phase phenotype, we have systematically employed cells 24–32 h after the intersection of extrapolated plots.

Measured differences between cells in log and stationary phase

Mean cell volume. In Fig. 2 the distributions of diameters for a single population of log phase and a single population of stationary phase cells are presented. A total of 4 independent log phase cultures and 8 independent stationary phase cultures were

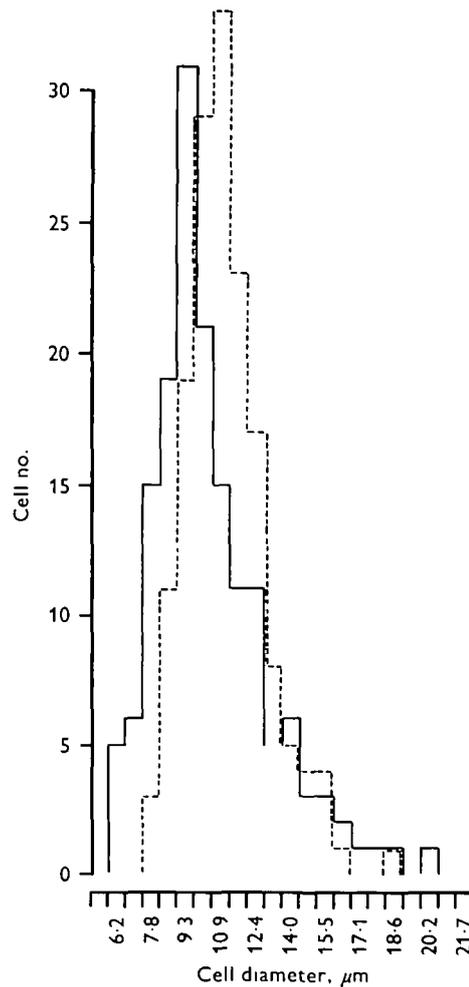


Fig. 2. Cell diameter distributions for log phase (—) and stationary phase (---) cell populations.

measured, 150 cells in each culture. By averaging the mean cell diameters of the 4 log phase culture distributions, we obtain an average mean cell diameter of $10.25 \mu\text{m}$. The standard error of the average of mean diameters is 0.06 . Consideration of the sampling variance of these means suggests that the range 10.08 – $10.41 \mu\text{m}$ contains the true mean, with 95% confidence. The mean cell volume, calculated from the

average mean cell diameter, is $565 \mu\text{m}^3$. The diameter of the smallest cells in a log phase cell population is $6.2 \mu\text{m}$ and the volume of these cells is $125 \mu\text{m}^3$. This is the minimum volume of a multiplying Ax-3 amoeba in HL-5 nutrient medium, since volumes smaller than this have never been observed. The diameter of the largest cells in a distribution of log phase cells is about $20.2 \mu\text{m}$ and the volume of these cells is $4000 \mu\text{m}^3$. This high level of diameter heterogeneity within a single cell population exists in freshly cloned populations as well as in populations maintained for several

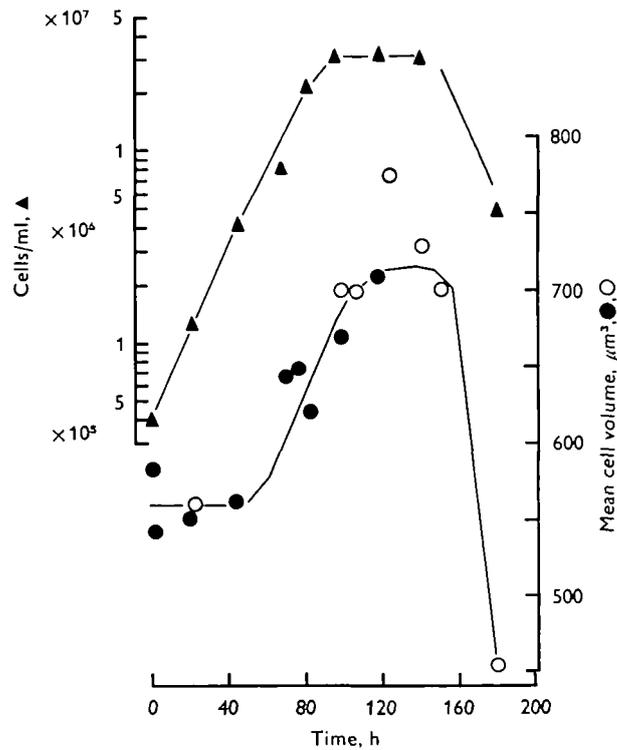


Fig. 3. Mean cell volume as a function of cell density. Each mean cell volume point was calculated from the mean cell diameter of 150 cells; all cell distributions were unimodal. Filled and empty circles for mean cell volume represent separate experiments.

months. This heterogeneity cannot be explained by volume changes during the cell cycle, since a strict cell cycle distribution would vary from $1X$ for cells just after cytokinesis to $2X$ for cells just prior to cytokinesis. The largest cells in a standard log phase cell population are 32 times larger than the smallest cells. Therefore, cells at all stages of the cell cycle will be found throughout the log phase distribution of cell diameters. With this in mind, one would predict that if the entire cell population were to accumulate at a point late in the cell cycle, then the entire distribution would shift to larger diameters without a drastic change in the standard deviation from the mean. The mean would increase, the minimum diameter value would increase slightly and the largest diameters would increase slightly. This prediction was borne out when stationary phase populations were measured.

By averaging the mean cell diameters of 8 independent stationary phase cultures, we obtained an average mean cell diameter of $11.06 \mu\text{m}$. The standard error of the average of mean diameters was 0.04 . Consideration of the sampling variance of these means suggests that the range 10.94 – 11.17 contains the true mean, with 95% confidence. The mean cell volume, calculated from the average mean cell diameter, is $711 \mu\text{m}^3$ (Fig. 3). This represents an increase of 26% over the mean cell volume of a log phase population. The difference between the log phase mean and stationary phase

Table 1. *Determination of whole cell and nuclear DNA content for log phase and stationary phase cell populations. The method of determination is presented in detail in Methods section*

	Experiment	No. of determinations	Whole cell DNA, $\mu\text{g}/10^8$ cells	Nuclear DNA, $\mu\text{g}/10^8$ cells	Mitochondrial DNA, whole cell DNA – nuclear, $\mu\text{g}/10^8$ cells
Log phase population	1	4	25.3	19.3	5.0
	2	4	24.3	17.3	7.0
	3	4	23.5	19.2	4.3
		Mean	24.4 ± 0.52	18.6 ± 0.64	5.4 ± 0.81
		% whole cell	—	76.0	24.0
Stationary phase population	1	2	32.5	23.0	9.5
	2	4	30.2	22.5	7.7
	3	4	32.0	24.1	7.9
		Mean	31.6 ± 0.69	23.2 ± 0.46	8.4 ± 0.57
		% whole cell	—	73.4	26.6

Mean values are stated \pm S.E.

mean is indeed significant, since ranges for the true means do not overlap. In addition, one can see in Fig. 1 that the minimum cell diameter has increased in the stationary phase distribution to approximately $7.8 \mu\text{m}$, although it is more difficult to see slight increases at the larger end of the distribution, which is skewed. Finally, the standard deviation of a single stationary phase cell population is, on the average, quite similar to that of a log phase cell population. The average of the standard deviations for the stationary phase distributions analysed above is $2.3 \mu\text{m}$ and the average for the log phase distributions is $2.3 \mu\text{m}$.

Average cell dry weight and average cell protein content. The dry weight of 10^8 log phase cells is $16.9 \text{ mg} \pm 0.66$ S.E. The dry weight of 10^8 stationary phase cells is $23.1 \text{ mg} \pm 0.18$, an increase of 37% over log phase cells.

The increase in dry weight is partially due to an increase in protein content: 10^8 log phase cells contain $9.3 \text{ mg} \pm 0.33$ of protein, approximately 55% of the dry weight; and 10^8 stationary phase cells contain $12.5 \text{ mg} \pm 0.27$ of protein, again approximately 55% of the dry weight. Since the dry weight of 10^8 log phase cells increases by 3.2 mg

when cells enter stationary phase, the increase in protein content accounts for approximately 50% of the total increase in dry weight.

DNA content. The total DNA content of 10^8 log phase cells is $24.3 \mu\text{g} \pm 0.52$ (Table 1). This is a higher figure than previously reported by Sussman & Rayner (1971), but it is probably more accurate since previous determinations were performed on preparations not treated with RNase and pronase (see Methods). The nuclear DNA content of 10^8 log phase cells is $18.6 \mu\text{g} \pm 0.64$ (Table 1), 76% of the whole cell DNA value.

Both the whole cell DNA and nuclear DNA content of 10^8 stationary phase cells are approximately 27% higher than the values for log phase cells. The DNA content of 10^8 stationary phase cells is $31.6 \mu\text{g} \pm 0.69$ (Table 1) and the nuclear DNA content $23.2 \mu\text{g} \pm 0.46$ (Table 1).

Parameter changes in relation to the cell cycle

The increases in the measured parameters suggest that cells in stationary phase have accumulated at a stage late in the cell cycle. To evaluate this possibility further, we have compared the observed measurement of each parameter for a stationary phase cell population to an estimate of that parameter for a synchronous cell population late in the cell cycle.

Table 2. *The relationship of measured physical parameters and the cell cycle*

Parameter	Observed log phase value	Mode of increase (multiple representing log phase value)	Estimate of 1X value	Estimate of 2X value	Observed stationary phase value	Observed stationary phase value as multiple of estimated 1X value
Mean cell volume, μm^3	565	Logarithmic (1.46X)	387	774	711	1.84X
		Linear (1.44X)	392	785		1.81X
		Exponential (1.38X)	409	819		1.74X
Average dry weight, mg/ 10^8 cells	16.9	Logarithmic (1.46X)	11.6	23.2	23.1	2.00X
		Linear (1.44X)	11.7	23.5		1.97X
		Exponential (1.38X)	12.3	24.5		1.89X
Protein, mg/ 10^8 cells	10.0	Logarithmic (1.46X)	6.85	13.7	12.4	1.81X
		Linear (1.44X)	6.94	13.9		1.78X
		Exponential (1.38X)	7.25	14.5		1.71X
Nuclear DNA, $\mu\text{g}/10^8$ cells	18.6	Step (1.62X)	11.5	23.0	23.2	2.02X

Observed log phase and stationary phase values were obtained from the relevant sections of each parameter in the text. The multiples representing the log phase values are discussed and references in the text.

Mean cell volume, average cell dry weight, and average cell protein. Any value for a particular cell parameter may be expressed as a multiple of $1X$ the value for that parameter just after cytokinesis. If a particular parameter increases logarithmically, or linearly, or exponentially during the cell cycle, then a measure of this parameter for an asynchronous log phase cell population would represent multiples of $1.46X$, or $1.44X$, or $1.38X$ respectively (Cook & James, 1964). Assuming that mean cell volume, average cell dry weight, and average cell protein content increase continuously during the cell cycle of *Dictyostelium*, we have equated our log phase measurements of these parameters with the above multiples in order to estimate the $1X$ and $2X$ figures (Table 2). We have also calculated the equivalent multiples for the observed stationary phase values (Table 2). For logarithmic, or linear, or exponential rates of increase, the observed mean cell volume at stationary phase represents multiples of $1.84X$, or $1.81X$, or $1.74X$, respectively; the observed average dry weight at stationary phase represents multiples of $2.00X$, or $1.97X$, or $1.89X$, respectively; and the observed average cell protein content represents multiples of $1.81X$, or $1.78X$, or $1.71X$, respectively. These multiples are relatively close to $2.00X$, indicating that cells are accumulating at a stage late in the cell cycle.

Nuclear DNA. By assuming a linear increase in DNA for $1X$ to $2X$ during the *S*-phase of the cell cycle, by employing Katz & Bourguignon's (1974) estimates of the proportions of G_1 , *S*, G_2 , and *M* during the cell cycle, and by applying the equation described by Cook & James (1964) to correct for proportions of cells at different cell cycle stages, we have calculated the average nuclear DNA content in a log phase cell population to equal 1.62 times the unreplicated nuclear DNA content (Table 2). From this estimate, we have calculated the $2X$ figure. The observed stationary phase measure of nuclear DNA is almost identical to the estimated $2X$ figure and equals 2.02 times the estimated unreplicated value. This is consistent with the conclusion that the entire stationary phase cell population has accumulated at a stage following the DNA replication phase of the cell cycle.

Stationary phase cells reinitiate cell division synchronously

One further observation reinforces our interpretation that stationary phase cells have accumulated at a particular stage in the cell cycle. When a population of cells in stationary phase for 24 h is washed free of stationary phase medium and inoculated into fresh nutrient medium at a lower cell density, cell number remains constant for 4 h and then increases synchronously by 70–90% within the next 2 h (Fig. 4). The T_{50} for the first synchronous division of the three independent populations plotted in Fig. 4 is $5.3 \text{ h} \pm 0.34$. After the first division, cell number remains relatively stable for approximately seven subsequent hours, and then again increases semisynchronously by 90–100% in the next 4–5 h. The interval between the T_{50} 's of the synchronous divisions is approximately 11 h, close to the normal 12-h generation time.

Cell volume and dry weight during the reinitiation of cell division. When stationary phase cells are reinoculated into fresh nutrient medium, the distribution of cell diameters becomes far more heterogeneous and, just prior to cell division, appears bimodal (Fig. 5). After the first synchronous division, cell diameters revert to the

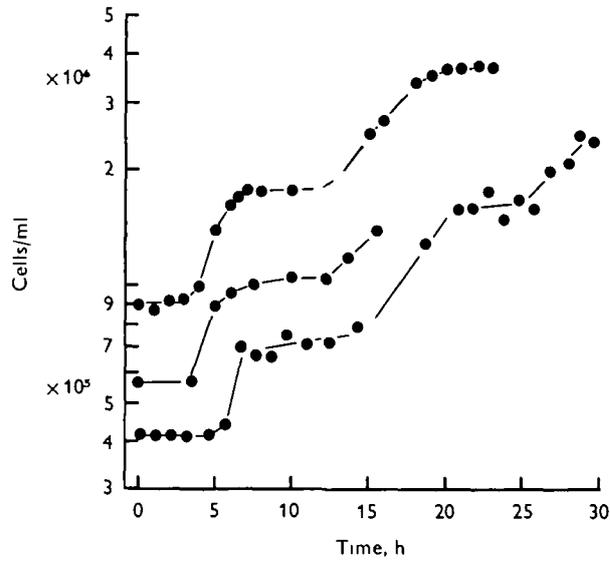


Fig. 4. Synchronous divisions of 3 independent stationary phase cell populations washed free of stationary phase medium and reinoculated into fresh nutrient medium.

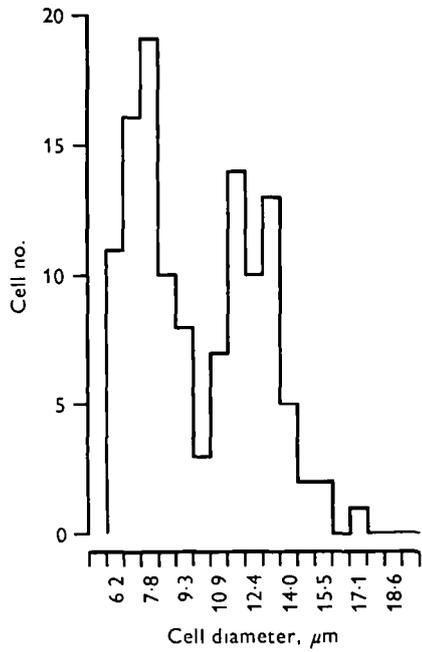


Fig. 5

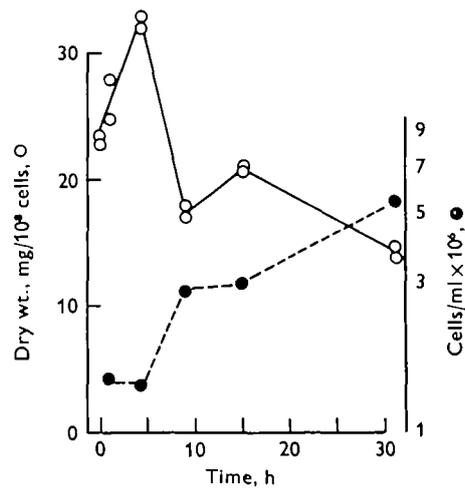


Fig. 6

Fig. 5. Bimodal cell diameter distribution for a stationary phase cell population 4 h after removal of stationary phase medium and reinoculation into fresh nutrient medium. Synchronous cell division occurred at 5 h in this particular experiment.

Fig. 6. The dry weight of a stationary phase cell population after removal of stationary phase medium and reinoculation into fresh nutrient medium. Synchronous cell division occurred at 5.5 h in this particular experiment.

normal distribution observed for log phase cell populations (Fig. 2). Mean diameters of the separated distributions of the bimodal population are 7.67 and 12.5 μm , both significantly different from the means of either a log phase cell population, 10.25 μm , or a stationary phase cell population, 11.06 μm . A repeat experiment gave mean diameters of 7.3 and 13 μm for the separated distributions. An immediate explanation for the bimodal distribution is not at hand.

Upon reinoculation into fresh nutrient medium, the dry weight of stationary phase cells immediately begins increasing (Fig. 6). After 4.5 h, just prior to the first division, the dry weight of 10^8 cells reaches 33 mg, double that of 10^8 log phase cells. Immediately after cell division, the dry weight of 10^8 cells is halved to 17 mg, still far higher than estimates of 11.6–12.3 mg for 10^8 cells just after cytokinesis (Table 2). Prior to the second doubling, cells increase their dry weight at a reduced rate to 21 mg per 10^8 cells, close to the expected value of 23.2–24.5 mg for 10^8 cells just prior to cytokinesis. Probably, the rate of cell growth decreases after the first synchronous cell doubling in order to reduce average cell mass to the normal level without affecting generation time.

DISCUSSION

In this report we have demonstrated that when cultures of *Dictyostelium discoideum* Ax-3 enter stationary phase in axenic liquid nutrient medium, they exhibit changes in cell parameters consistent with the conclusion that stationary phase cells accumulate at a stage late in the cell cycle. In the light of this conclusion we may now reconsider possible relationships between the cessation of cell multiplication, the stationary phase growth and transcription inhibitors, the limited accumulation of aggregation information, and the cell cycle.

We previously demonstrated that a growth inhibitor (Yarger *et al.* 1974) and a transcription inhibitor (Yarger & Soll, 1974) accumulate in the medium of stationary phase cultures. A recent analysis of the kinetics of appearance of these inhibitory activities (Yarger, Ferguson & Soll, unpublished observations) indicated that both activities reach effective levels in the medium after cells have stopped multiplying, after the increase in cell volume, and after the limited accumulation of aggregation information. Therefore, if these inhibitors are causally related to the cessation of cell multiplication and the expression of the stationary phase phenotype, they must accumulate to effective intracellular levels prior to or early in the transition period to stationary phase. Alternatively, cells may accumulate at a stage late in the cell cycle due to the depletion of one or more growth-limiting components in the medium. When cells accumulate at this stage, they may continue to produce stage-specific factors involved in the regulation of the cell cycle and these factors would escape from the cells into the medium. Since stationary phase cultures reach extremely high cell concentrations, the titre of these factors in the medium would eventually reach effective levels. These alternative hypotheses are now under investigation.

Besides the physical parameters compared in this report, there have been many reports in the literature of cell and culture characteristics of log phase and stationary phase populations that differ. For instance: (a) the cell surface of stationary phase cells possesses far more filipodia than that of log phase cells (Rossomando *et al.* 1974);

(b) stationary phase cells require 10-fold more of the plant lectin concanavalin A for agglutination than log phase cells (Weeks, 1973); (c) the intracellular concentration of cAMP in stationary phase cells is approximately 50% higher than log phase cells, based on cAMP measurements of Malkinson & Ashworth (1973) and our volume measurements; (d) stationary phase cells are far more resistant to ionic detergents (Soll, unpublished observations); (e) transcription in stationary phase cells is not affected by the inhibitors present in stationary phase medium whereas transcription in log phase cells is inhibited drastically (Yarger & Soll, 1974), and stationary phase cells aggregate 3–4 h faster than log phase cells (Soll & Waddell, 1975). These differences may be specific to a new stationary phase phenotype or they may be specific to cells at a stage late in the cell cycle. Analyses of these parameters during synchronous cell division will distinguish between these alternatives.

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