A REVISION OF THE *DICTYOSTELIUM DISCOIDEUM* CELL CYCLE

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SUMMARY

We have reinvestigated the *Dictyostelium discoideum* cell cycle using fluorometric determinations of cellular and nuclear DNA contents in exponentially growing cultures and in synchronized cultures. Almost all cells are in G_2 during both growth and development. There is no G_1 period, S phase is less than 0·5 h, and G_2 has an average length of 6·5 h in axenically grown cells. Mitochondrial DNA, which constitutes about half of the total DNA, is replicated throughout the cell cycle. There is no difference in the nuclear DNA contents of axenically grown and bacterially grown cells. Thus the long cell cycle in axenically grown cells is due to a lengthening of the G_2 phase.

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* normally grows on bacteria and multiplies by binary fission. Under these conditions the cells double over a 3- to 4-h period. Most biochemical work has been done with axenic mutants that can grow in an axenic medium (Watts & Ashworth, 1970). These cells show a doubling time of 8 h. All analysis of the *Dictyostelium* cell cycle has been done with axenic cells (Katz & Bourgignon, 1974; Zada-Hames & Ashworth, 1978). The results indicate that axenically growing *Dictyostelium* cells have a cell cycle with a G_1 phase of 1·5 h, an S phase of 2·5 h and a G_2 phase of 4 h (Zada-Hames & Ashworth, 1978).

In the course of investigations relating cell cycle phase to developmental fate, we found discrepancies between our findings concerning the distribution of cells over the various cell cycle phases and published observations on the cell cycle. Therefore, we found it necessary to reinvestigate the *Dictyostelium* cell cycle and have obtained results that are at variance with earlier work. In particular we find that the G_1 period is absent and that the duration of S phase is short, 30 min or less. Most of the cell cycle consists of a G_2 phase of 6·5 h.

The significant lengthening of the cell cycle in axenically grown cells compared to bacterially grown cells is due to a lengthening of the G_2 phase in axenically grown cells.

These findings place the *Dictyostelium* cell cycle among those of other primitive eukaryotic organism like *Physarum* (Mohberg & Rusch, 1971) and *Hydra* (David & Campbell, 1972; Campbell & David, 1974), in the sense that they also do not contain a G_1 period of any significant length.

MATERIALS AND METHODS

Strains and growth conditions

All experiments were performed with *Dictyostelium discoideum* strain Ax2, grown axenically according to standard procedures in axenic medium (Watts & Ashworth, 1970) at 23 °C. Under these conditions the cells had a doubling time of $7 \cdot 0 \pm 0 \cdot 5$ h in the concentration range of 2×10^6 to 6×10^6/ml.

Synchronization of cells

To induce synchrony cells were grown to stationary phase and then diluted out into fresh medium. After a lag-phase such cells exhibit a relatively synchronous cell doubling (Yarger, Stults & Soll, 1974). Since growth slows down when cells approach stationary phase the change in cell number is a relatively insensitive parameter of how long the cells have been in stationary phase. Therefore, to obtain standard stationary-phase cells, we diluted exponentially growing cells to 10^6/ml in fresh medium and allowed them to grow for 40 h at 23 °C, at which point the cells had reached a density of $1 \cdot 2 \times 10^7$/ml. These cells were then diluted into fresh medium at a density of 10^6/ml and the increase in cell number over time was followed. Under these conditions the cells generally showed a cell doubling over a 2- to 3-h period after a lag-phase of 2 h.

Cell counting

Cell counts were made with an electronic cell counter (Phywe), using a counting chamber with a 100μm orifice. Cells were counted in a $1/250$ dilution in $0 \cdot 9 \%$ NaCl. Duplicate cell number determinations never showed more than 2% variation.

Preparation of nuclei

Nuclei were prepared by the Triton lysis method (Charlesworth & Parish, 1977). One millilitre of cells (1×10^6 to 4×10^6/ml) was pelleted by centrifugation for 8 s in an Eppendorf microcentrifuge and resuspended in 300μl deionized water. After 5 min 600μl of a solution containing $2 \cdot 5 \%$ Ficoll, $0 \cdot 5$ M-sorbitol, $0 \cdot 5$ mM-$CaCl_2$, 1 mM-$MgCl_2$ in 20 mM-Tris·HCl buffer (pH $7 \cdot 5$) was added mixed and then 9μl of a 10% Triton X-100 were added. The mixture was inverted twice and left to stand for a minimum of 5 min, after which more than 99% of the cells were lysed. Counts of nuclei before and after cell lysis indicated that less than 2% of the nuclei were lysed.

Bulk DNA determinations

Bulk DNA was determined fluorometrically with the DNA-specific fluorochrome Hoechst 33258 (Labarca & Paigen, 1980) and calf thymus DNA as the standard. Owing to the difference in A+T content of *Dictyostelium* and calf thymus DNA, these measurements yield relative and not absolute values of DNA. Frozen pellets of cells or nuclei were resuspended in a solution containing 2 M-NaCl and 2 mM-EDTA in 50 mM-phosphate buffer (pH $7 \cdot 4$); 50μl of this suspension was then added to 3 ml of the same salt/EDTA solution containing 100 ng/ml of Hoechst dye. The fluorescence emission of the sample was measured at 450 nm in a Kontron SFM 19 fluorometer using an excitation wavelength of 350 nm (10 nm slit width). The DNA standard was prepared from a stock solution of calf thymus DNA (Serva no. 18560) dissolved in 5 mM-NaOH at 1 mg/ml, which was kept refrigerated. A calibration curve was constructed in the range of $0 \cdot 1$ to 3μg calf thymus DNA/sample; the curve is linear over this range. Using this assay, DNA-specific fluorescence increases linearly with increasing amounts of *Dictyostelium* extract.

Determination of DNA content of cells and nuclei by flow cytometry

DNA was measured by flow cytometry with a FACS IV cell sorter using Mithramycin as the fluorochrome (Crissman & Tobey, 1974) and the 450 nm line of the argon laser as the excitation wave length. Cells were fixed in 70% (v/v) ethanol and stained in a 50 mM-phosphate buffer (pH $7 \cdot 2$) containing 100μg/ml Mithramycin and 100 mM-$MgCl_2$. Cell densities did not exceed 5×10^6/ml; under these conditions the DNA-dependent fluorescence was independent of cell density. Nuclei

were prepared as described above, diluted without fixation in the Mithramycin solution and measured directly. There is no difference in the fluorescent yield between fixed and unfixed nuclei. However, unfixed nuclei do not clump and were therefore used in all the experiments shown here. Preparations of nuclei could be kept on ice in the Mithramycin staining solution over the course of a day without a detectable change in the fluorescence, indicating the absence of nuclease activity. In most experiments at least 100 000 cells or nuclei were measured.

The nuclear DNA distributions determined by flow cytometry have relatively large coefficients of variation. This is due principally to the low amount of DNA in *Dictyostelium* nuclei, which leads to a low signal-to-noise ratio. This phenomenon is more pronounced with nuclei than cells, which have twice as much DNA (see Results).

Microfluorometric determination of DNA content of cells and nuclei

Cells were fixed in a mixture of methanol and acetic acid (3:1, v/v) and air dried on cleaned microscope slides. The slides were incubated for 10 min in 200 mM-KCl and then stained for at least 60 min in a solution containing 0·5 μg/ml of the DNA-specific fluorophore DAPI (Leeman & Ruch, 1982) in McIlvain's buffer (pH 7·0) (Coleman & Maguire, 1982). After this the slides were washed once quickly in the same buffer without DAPI and, after application of a drop of a 20 % glycerol solution in McIlvain's buffer, a coverslip was added. The slides were kept in the dark until they were measured. There was no detectable change in fluorescence over the course of 2 days.

The fluorescence of cells and nuclei was measured with a Leitz Dialux microscope fitted with a Leitz epifluorescence attachment using filter block A. The light of the 50 W mercury lamp was reduced 16 times with neutral density filters in order to reduce bleaching of the DAPI fluorescence. Cells were examined with a 100× Fluortar oil-immersion objective, under these conditions the bleaching half-time is about 30 min. Fluorescence intensity was measured with a Leitz MPV microscope photometer equipped with a variable diaphragm. The diaphragm was adjusted in size so that it contained either a nucleus or a whole cell. The photometer was connected to a Kontron PSI 80 micro computer, which performed all data collection and handling.

[³H]thymidine labelling of exponentially growing cells

Cells were labelled with [³H]thymidine according to the procedure described by Zada-Hames & Ashworth (1978). Exponentially growing cells at a density of 4×10^6/ml were labelled with [methyl-³H]thymidine 50 μCi/ml (44 Ci/mmol, Amersham) in axenic medium for 30 min. Cells were then washed four times in 20 mM-potassium phosphate buffer (pH 6·8) and a final wash in distilled water, fixed in methanol/acetic acid (3:1, v/v) and air dried on cleaned glass slides. The slides were treated for 2 min with bovine pancreatic RNase type A (Serva no. 34388) at 37 °C in phosphate buffer and dried. The slides were then dipped in Kodak nuclear track emulsion NTB2 and exposed for 4 weeks at 4 °C. The slides were developed for 5 min in Kodak D19 developer, fixed and washed. The nuclei stained through the film layer with a 10 % Giemsa solution in phosphate buffer. The slides were mounted in 20 % glycerol/phosphate buffer and the number of labelled nuclei scored. At least 400 cells/sample were counted.

RESULTS

Flow fluorometric measurements of the distribution of total and nuclear DNA content of vegetative cells

The DNA content of exponentially growing cells was measured in a FACS IV flow fluorometer, after staining with the DNA-specific fluorochrome Mithramycin. The results in Fig. 1A show two populations of cells. The mean DNA content of the cells with the most DNA is exactly double the amount of the lower distribution. We have sorted cells from the higher distribution and found that all these cells were binucleate. The distribution of DNA contents per cell of mononucleate cells is unimodal. Although the distribution was rather wide ($cv = 20$ %), there was not much sign of
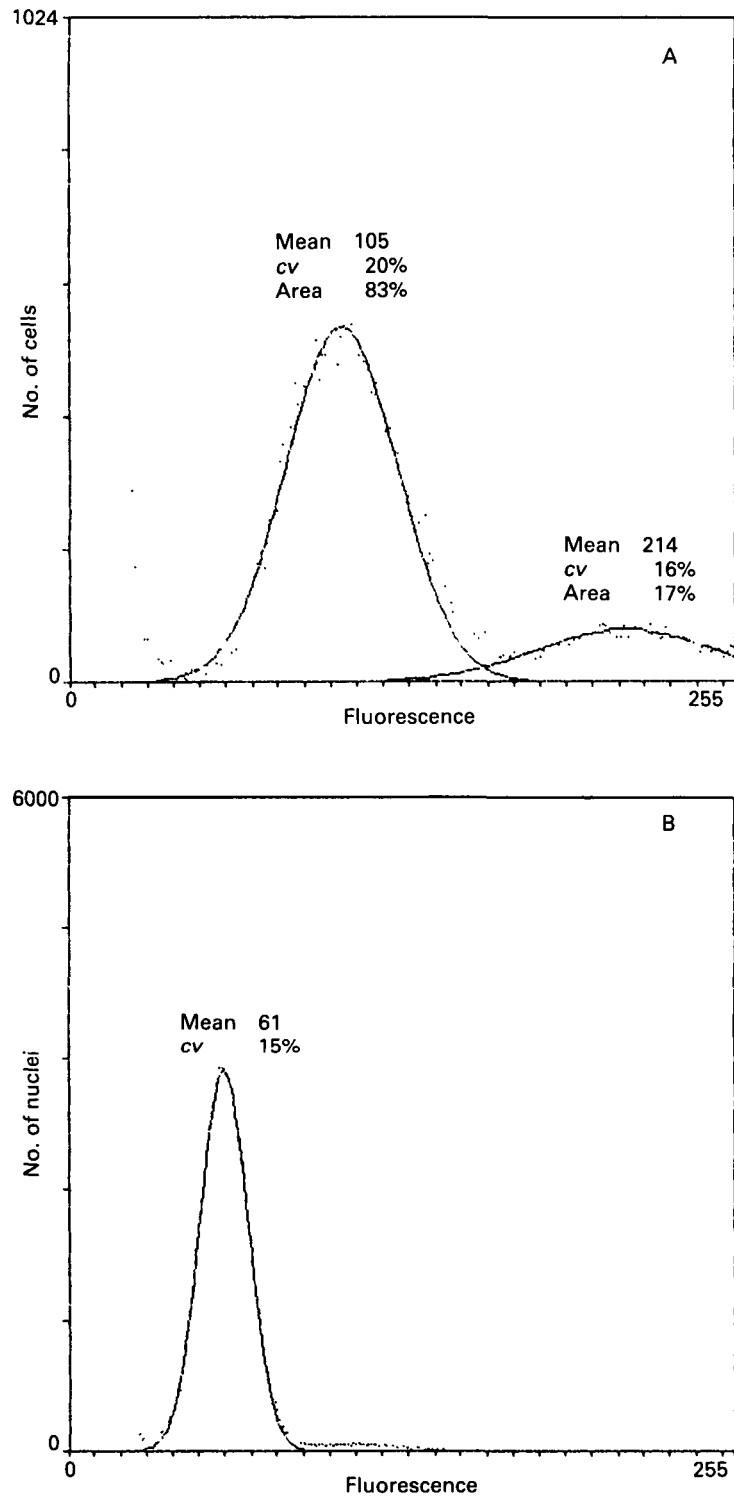
Fig. 1

assymetry. A series of independent measurements on different populations of growing cells always yielded the same unimodal distribution.

The above results are striking since all cells appear to have the same DNA content although cells with G_1, S and G_2 DNA contents would be expected in an exponentially growing population. Since it is known that in *Dictyostelium* a substantial amount of the total DNA is represented by mitochondrial DNA (Sussman & Rayner, 1971; Firtel & Bonner, 1972), one possible explanation for the results would be that the DNA content of cells is determined primarily by the number of mitochondria in the cells. If this were true, a typical bimodal distribution of nuclear DNA contents might occur in exponentially growing cells but be masked by mitochondrial DNA. To test this possibility we measured the DNA content of isolated nuclei.

The results in Fig. 1B indicate that nuclei isolated from exponentially growing cells also show a unimodal distribution of DNA contents and also that almost all cells must be in one particular phase of the cell cycle. The results show further that the nuclear DNA content (Fig. 1B) is about half the cellular DNA content (Fig. 1A), confirming the presence of large amounts of mitochondrial DNA in *Dictyostelium* cells.

The results in Fig. 2 indicate that the nuclear DNA contents of axenic and bacterially grown cells are identical. Both nuclear populations have the same unimodal distribution with little evidence of asymmetry. Thus bacterially grown cells must also be in one phase of the cell cycle. Fig. 3 also shows that the nuclear DNA content of slug cells is identical to that of growing cells. Since previous results suggested that aggregating cells and slug cells accumulate in G_2 (Katz & Bourguignon, 1974; Weijer, Duschl & David, 1984a), it seemed likely that the unimodal peak in our experiments represents cells in G_2. In order to confirm this interpretation we performed microfluorometric measurements to compare the nuclear and cellular DNA contents of interphase cells and mitotic cells.

Microfluorometric determination of cellular and nuclear DNA content

To measure the DNA content of single cells and nuclei, *Dictyostelium* cells were stained with a DNA-specific fluorochrome and the fluorescence of individual cells was determined using a fluorescence microscope equipped with a photomultiplier (see Materials and Methods). Exponentially growing cells were fixed in a methanol/acetic acid mixture, which yielded good metaphase spreads (Zada-Hames & Ashworth,

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Fig. 1. DNA distributions of exponentially growing cells and nuclei. A. Distribution of DNA contents of single cells measured flow fluorometrically in a FACS IV cell sorter after ethanol fixation and staining with the DNA specific dye Mithramycin. Abscissa: DNA content in arbitrary fluorescence units. Ordinate: number of cells. Dots represent the number of cells in each channel. The curves are the best fitting Gaussian distributions, with indicated mean and coefficient of variation (*cv*). The two curves represent the populations of mono and binucleate cells, and the areas under the two curves the percentage of cells with one or two nuclei. B. Distribution of DNA contents of isolated nuclei prepared from the same population of cells as shown in A. The mean value of the distribution of nuclei is 58 % of the mean of the DNA distribution of mononucleate cells. The 42 % difference represents the average mitochondrial DNA content of cells.
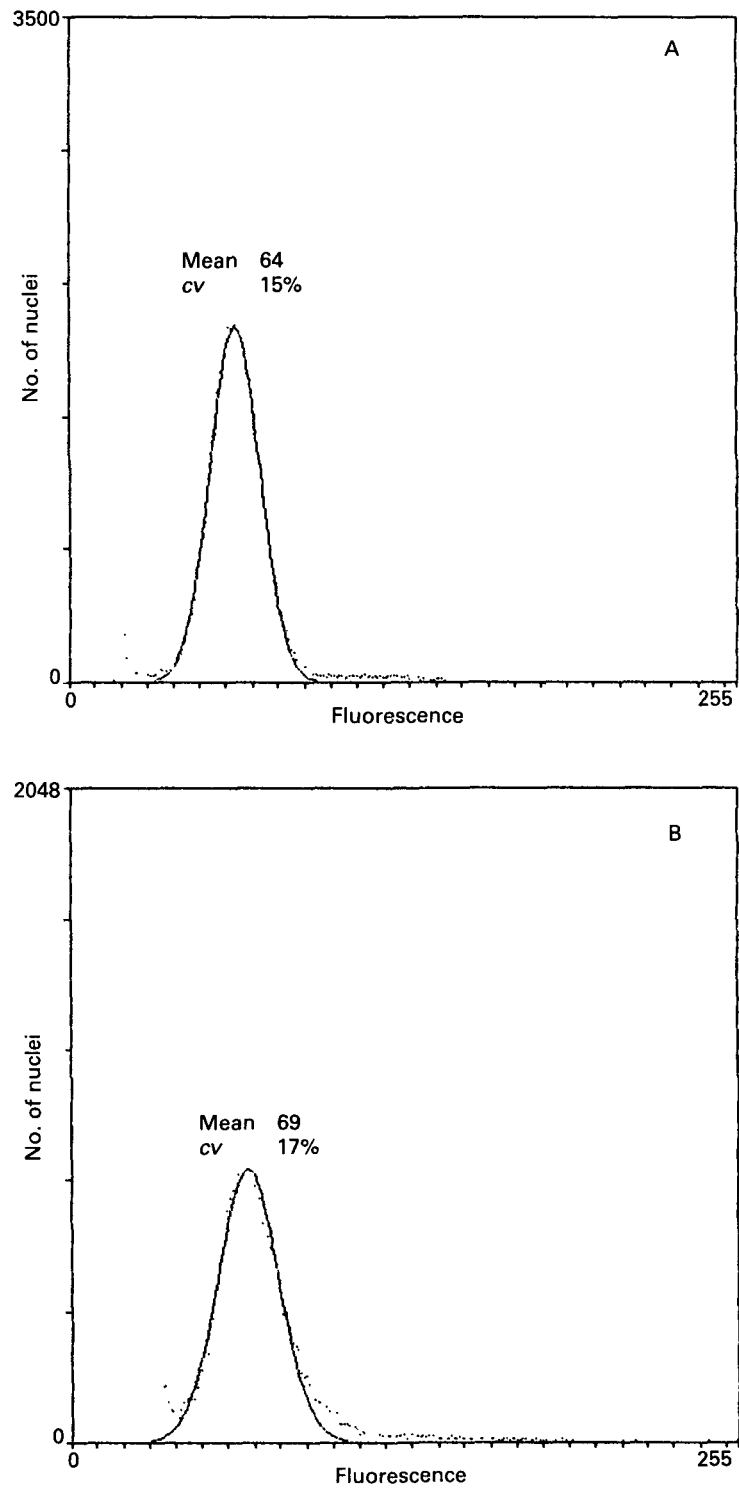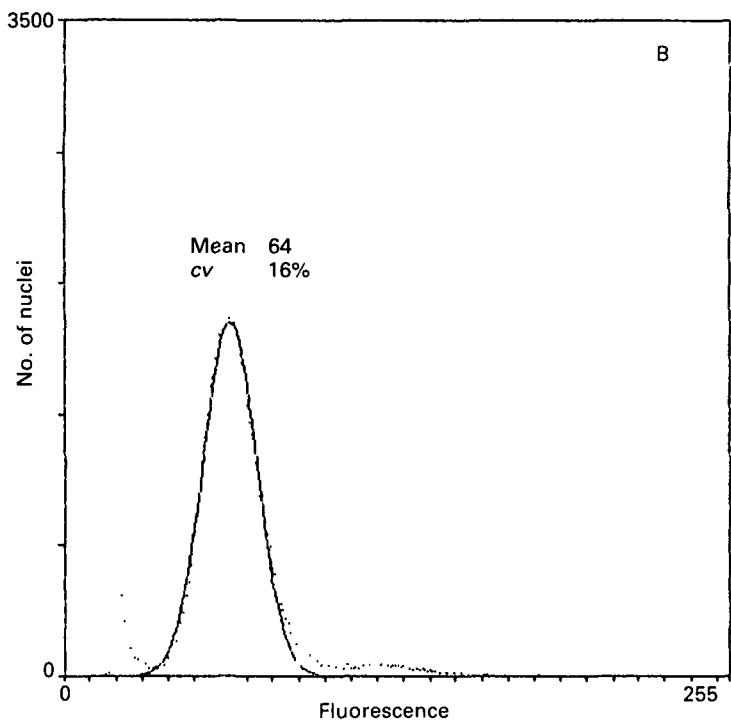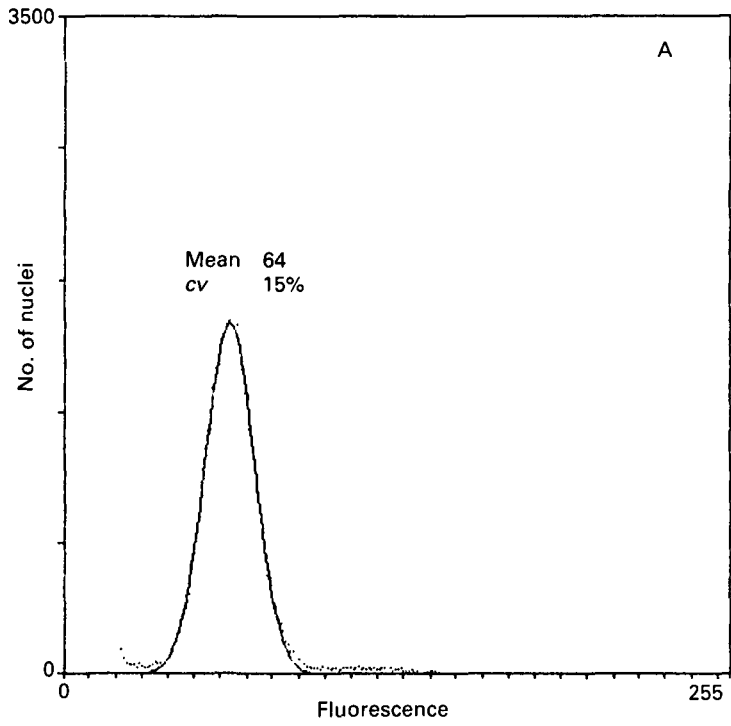
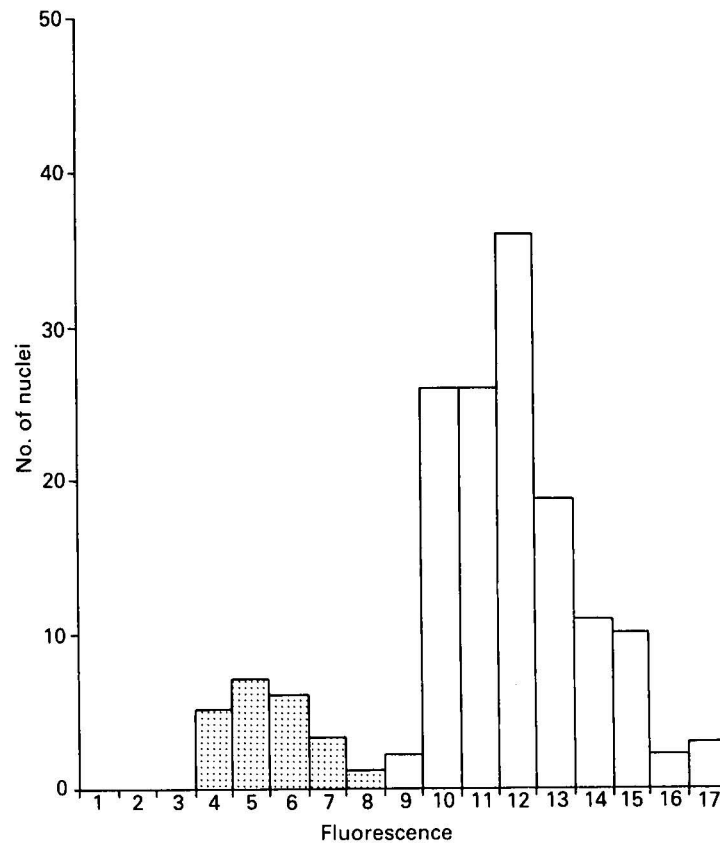Fig. 2. For legend see p. 118

Fig. 3. For legend see p. 118

Fig. 4. Distribution of DNA content of interphase nuclei and half-anaphases. Nuclei and half-anaphases are measured microfluorometrically as described in Materials and Methods. The sample was taken from an exponentially growing population of cells. The half-anaphases (see Fig. 5) are represented by dotted bars in the histogram, the open bars represent normal nuclei. The distribution does not reflect the normal proportions of anaphase to other cells since we especially selected anaphase in order to collect enough values to obtain a reasonable distribution for anaphase cells.

1978), and air dried on cleaned microscope slides. The cells were then stained with DAPI and their DNA-specific fluorescence was measured. The results in Fig. 4 show that almost all interphase nuclei contain twice the amount of DNA present in daughter nuclei of anaphase mitotic figures. This implies that almost all cells in an exponentially growing population are in the G_2 phase of the cell cycle.

Fig. 2. Distribution of nuclear DNA content of axenically grown exponential cells and bacterially grown exponential cells measured in a flow fluorometer. A. Isolated nuclei from axenically grown cells. The line represents the best fitting Gaussian to the data with given mean and *cv*. B. Isolated nuclei from bacterially grown cells. The nuclei in B were measured on the same day under the same machine settings as in A.

Fig. 3. Distribution of nuclear DNA of axenic vegetative cells and slug cells measured in a flow fluorometer. A. Isolated nuclei from vegetative cells. The line represents the best fitting Gaussian to the data with given mean and *cv*. B. Isolated nuclei from slug cells.
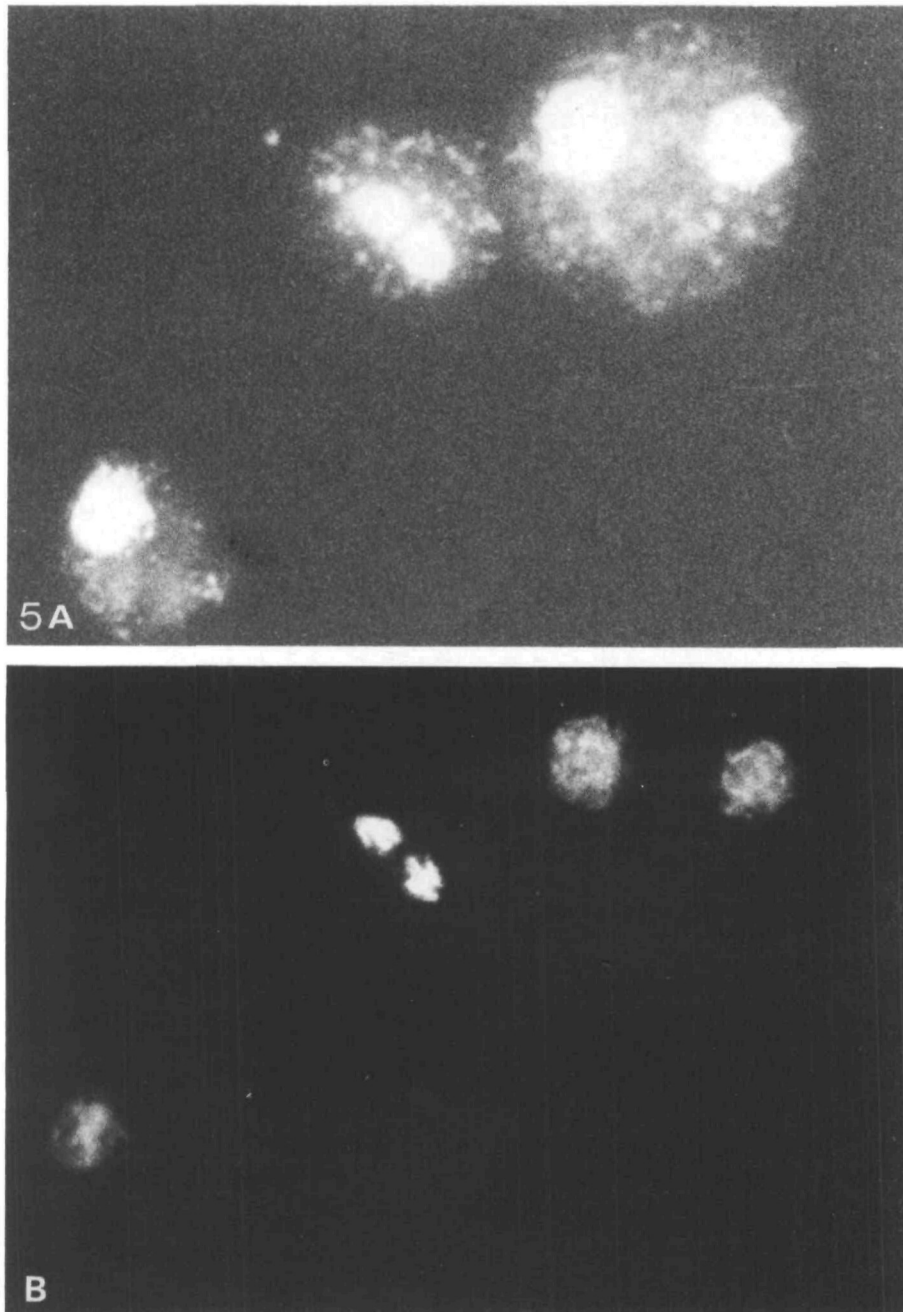
Fig. 5. Fluorescence photograph of cells from an exponentially growing axenic culture stained with Hoechst. A and B are photographs of the same field of cells, using two different exposure times to show the mitochondria (A) and the nuclear structure (B). In the upper right corner lies a binucleate cell, in the middle a cell in anaphase and in the lower left corner a mononucleate cell.

Parallel determinations of the DNA content of whole cells (data not shown) indicated that the total cellular DNA content is about twice that of the nucleus in agreement with the flow fluorometric determinations (Fig. 1a,b). At high magnification this non-nuclear DNA-specific fluorescence could be seen to occur as tiny bright dots throughout the cytoplasm (Fig. 5). Based on its localization in the cytoplasm and occurrence in small discrete units it appears to be mitochondrial DNA.

Changes in nuclear and cellular DNA contents during growth of synchronized cell populations

The results above indicate that most cells are in G_2, no cells are in G_1 and few cells are in S phase. The cell cycle, therefore, appears to consist of nuclear division followed immediately by a short S phase and a long G_2 phase. In order to support this
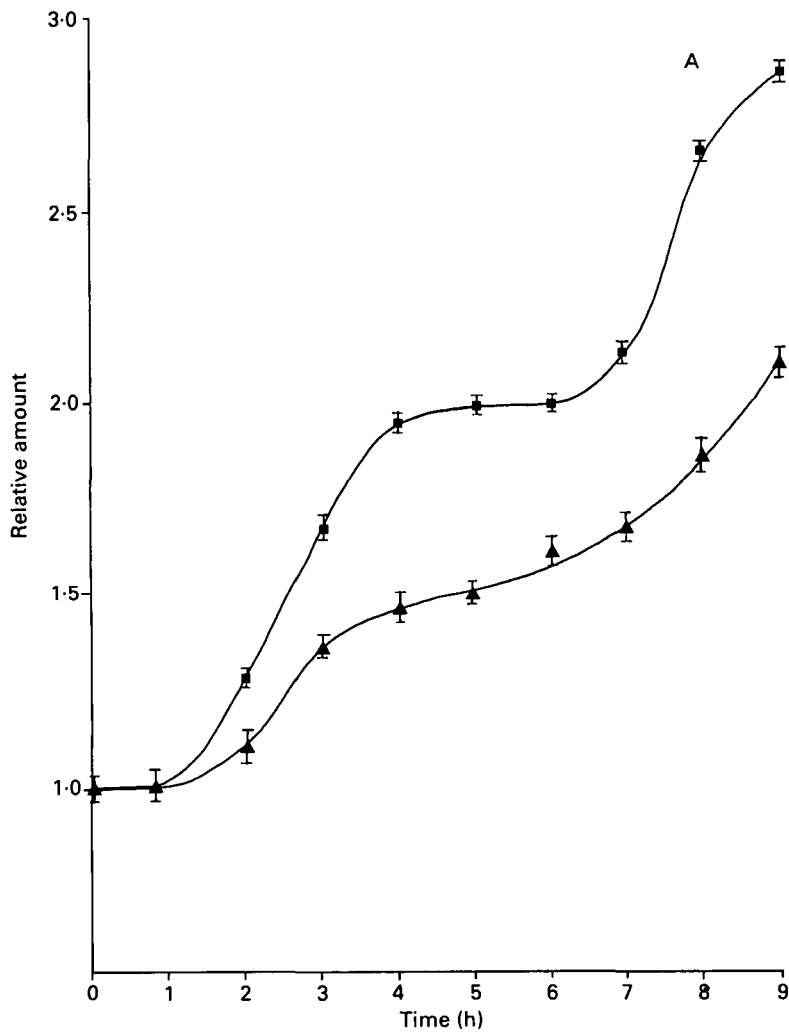

Fig. 6A. (legend is opposite)

interpretation of the cell cycle further, we have used synchronized cell populations to follow the progression of cells through the phases of the cycle.

Cells were synchronized by letting them grow into stationary phase for a defined period of time. Under these conditions the cells secrete an inhibitor and accumulate at a point late in the G_2 phase of the cell cycle (Zada-Hames & Ashworth, 1978; Yarger *et al.* 1974). After dilution into fresh medium the cells synchronously initiate a new cell cycle. Fig. 6A shows such a cell population, which doubled over a period of 3 h. The average DNA content per cell initially showed a rapid 50 % increase and then rose slowly to about 180 % of the initial value. At this point the cells have achieved the level of cellular DNA typical of exponentially growing populations (data not shown). Since stationary-phase cells accumulate an excess of mitochondrial DNA the increase after one cycle is only 1·8-fold relative to stationary-phase cells.

During the course of the synchronous doubling of cell number, the mean of the distributions of nuclear DNA content determined microfluorometrically remained
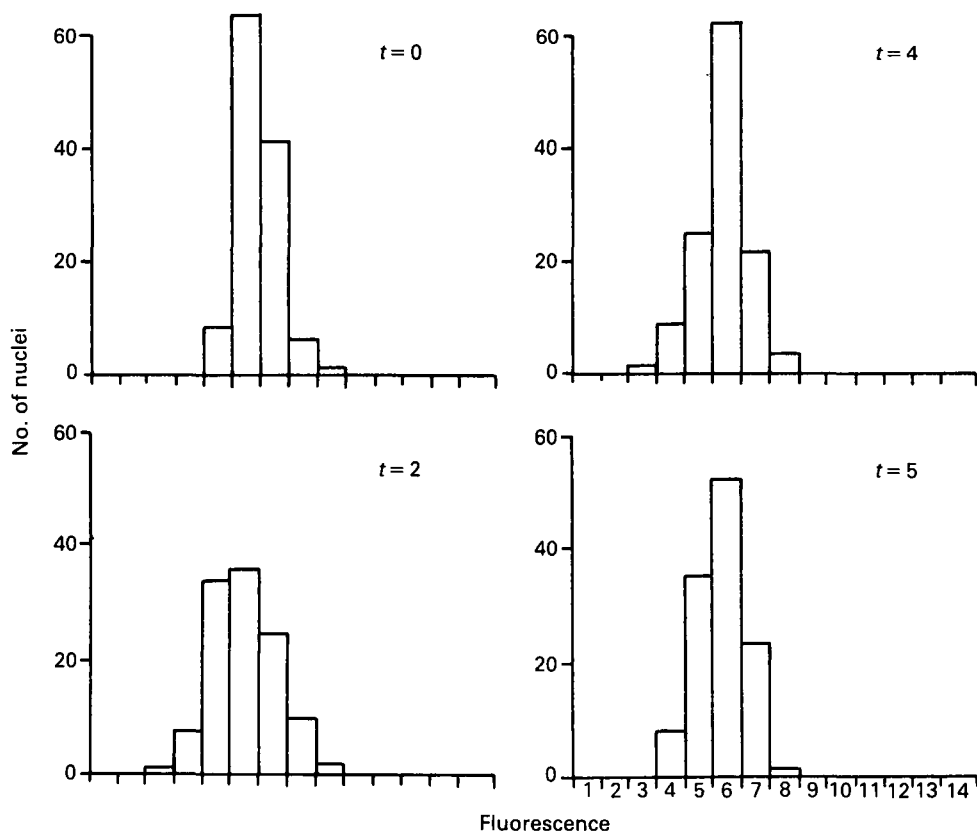

Fig. 6. A. The relative change in cell number (■) and total DNA content (▲) in a synchronized cell culture. B. The distribution of nuclear DNA contents from the synchrony experiment shown in A. Samples were taken at 0, 2, 4 and 5 h. The nuclear DNA content was measured microfluorometrically. The DNA content is expressed in arbitrary fluorescence units.
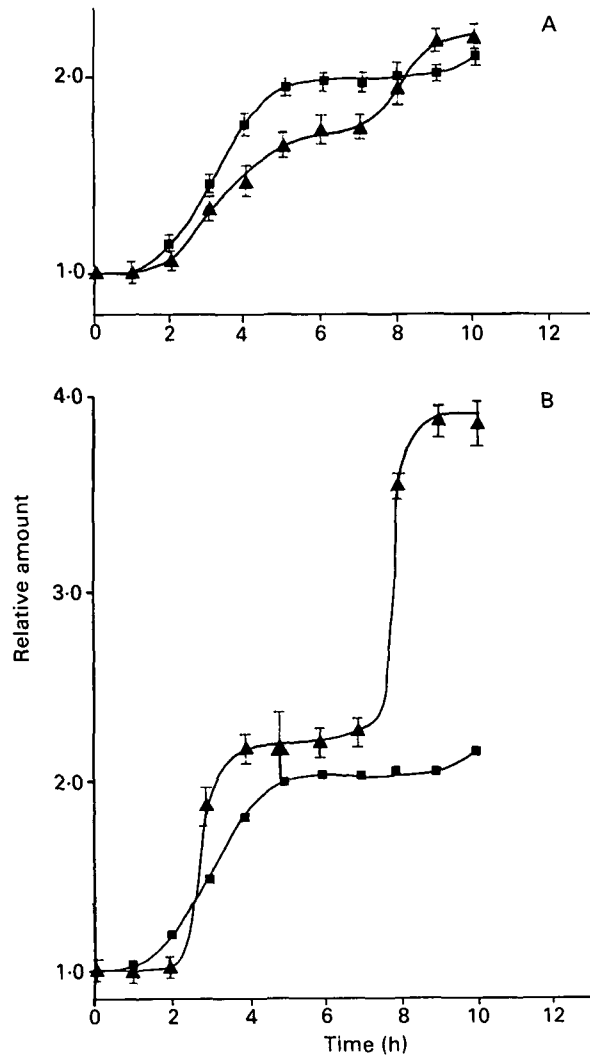
Fig. 7. Release of stationary-phase cells in fresh axenic medium. A. The relative increase in cell number (■) and total cellular DNA (▲). B. The relative increase in nuclear DNA (▲) for the same experiment as in A. The relative increase in cell number (■) shown in A has been redrawn.

essentially constant (Fig. 6B). At no time during the doubling of the synchronous population was there evidence for the presence of G_1 cells. In the absence of a G_1 phase, daughter nuclei must enter S phase immediately following nuclear division, i.e. simultaneously with the increase in cell number. Indeed, the 2 h fraction does show a broadening towards lower DNA contents, possibly indicating the presence of some S phase cells at this time. The fact that more cells containing S-phase DNA contents were not observed indicates, in agreement with the measurements in Figs 1 and 2, that the S phase is short.

The observations that G_1 is absent and S phase very short suggest that the initial

rapid increase in the average DNA content per cell (Fig. 6A) is due to nuclear DNA replication. To test this we measured the average nuclear DNA content as well as the average cellular DNA content in an independent experiment with synchronized cells. The results in Fig. 7 show that the average nuclear DNA content doubled rapidly and in parallel with the doubling in cell number. The subsequent slow increase in the average DNA content per cell during the remainder of the cycle presumably represents the replication of mitochondrial DNA. The results also show that the second round of nuclear replication occurs before the second round of cell division. This effect occurred to a greater or lesser degree in all our experiments (see Discussion).
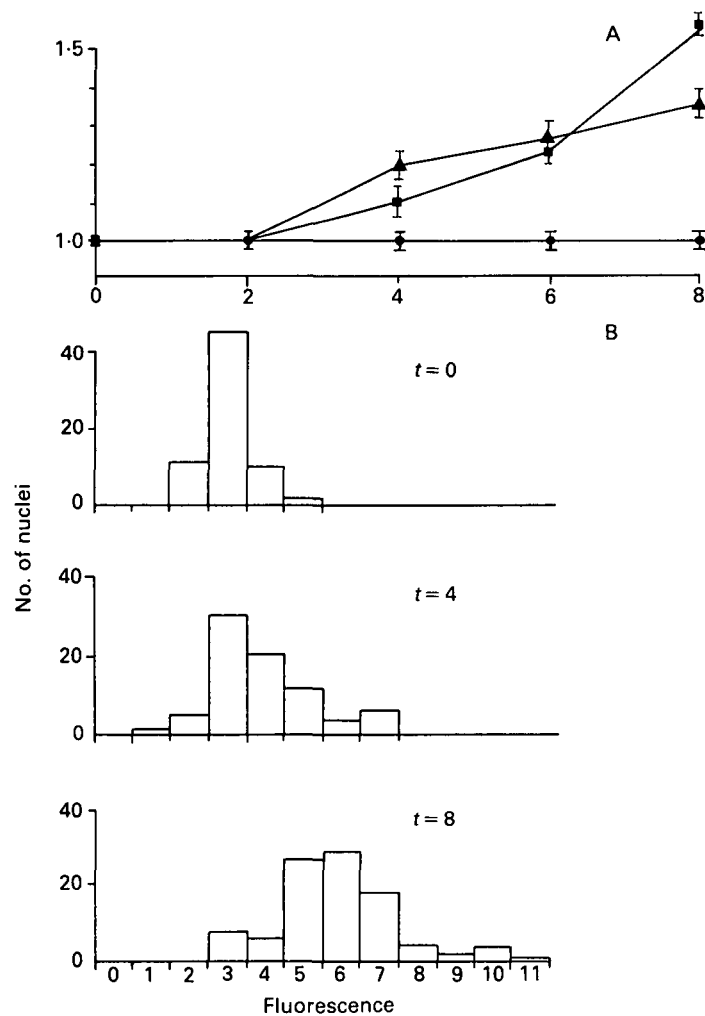

Fig. 8. Exponentially growing cells treated with 5 μg/ml CIPC. A. Relative increase in cell number (●), total cellular DNA (▲) and nuclear DNA (■). B. Distribution of nuclear DNA content in samples taken from the experiment shown in A. The DNA content was measured microfluorometrically as described in Materials and Methods. There is a shift from $2n$ to $4n$ DNA values (3–6 units of fluorescence) over the course of the experiment.

Response of exponentially growing cells to mitotic inhibitors

The interpretation of the *Dictyostelium* cell cycle presented above – no G_1, short S and long G_2 – can be tested by measuring how quickly nuclear DNA synthesis stops following the addition of mitotic inhibitors. After addition of a mitotic inhibitor, one expects cells that are past mitosis to continue cycling. After a period equivalent to G_1+S, the last cycling cell should pass out of S phase and nuclear DNA synthesis should cease. The results of treating exponentially growing cells with CIPC (isopropyl-N-3-chlorophenyl carbamate), a mitotic inhibitor for *Dictyostelium* (White, Scandella & Katz, 1981), are shown in Fig. 8A. CIPC caused an immediate block in cell division and nuclear DNA replication, as would be expected if the G_1 and S phases are negligible in duration.

The block in cell division continued for at least 8 h but mitotic figures accumulated only for the first 4 h, reaching a level of 30–40 %, after which they declined (data not shown). Similar behaviour has been reported in *Dictyostelium* cells treated with other mitotic inhibitors (Zada-Hames, 1977; Williams, 1980). Simultaneously with the decline in mitotic figures, a resumption of DNA synthesis was observed (Fig. 8A). This suggested that nuclei escape from the mitotic block and commence a new round of DNA synthesis. Measurements of nuclear DNA contents (Fig. 8B) support this interpretation: from 0 h–4 h the mean DNA content did not change. After DNA synthesis began again, nuclei appeared with $4n$ DNA content as well as values between $2n$ and $4n$. Such an endomitotic diploidization has previously been observed with other mitotic inhibitors and has been used in genetic studies to generate diploid strains (Welker & Williams, 1980).

Labelling with [³H]thymidine

The results in Figs 1 and 2 indicate that relatively few cells in an exponentially growing population are in S phase. To confirm this conclusion independently we labelled growing cells for a brief period with [³H]thymidine and scored the proportion of labelled cells by autoradiography. In agreement with the data on nuclear DNA content we observed only 7–8 % labelled nuclei.

Two other features of these labelled preparations were interesting in relation to the results above. First, a large fraction, 48 ± 4 % ($n = 200$), of the labelled nuclei occurred in pairs, i.e. in binucleate cells. Since the fraction of binucleate cells in this culture was only 35 ± 3 % ($n = 360$), this high proportion cannot be due simply to binucleate cells in S phase. Rather, the results provide independent support for the hypothesis (see Discussion) that S phase follows immediately upon nuclear division without an intervening G_1 phase. Frequently this S phase actually occurs before the daughter nuclei have been segregated to daughter cells by cytokinesis. It should be noted here that Zada-Hames & Ashworth (1978) also observed [³H]thymidine labelling in some telophase mitotic figures in synchronous cultures.

Secondly, although cells containing labelled nuclei (S phase) were infrequent, the great majority of cells exhibited labelling over the cytoplasm. Assuming that this
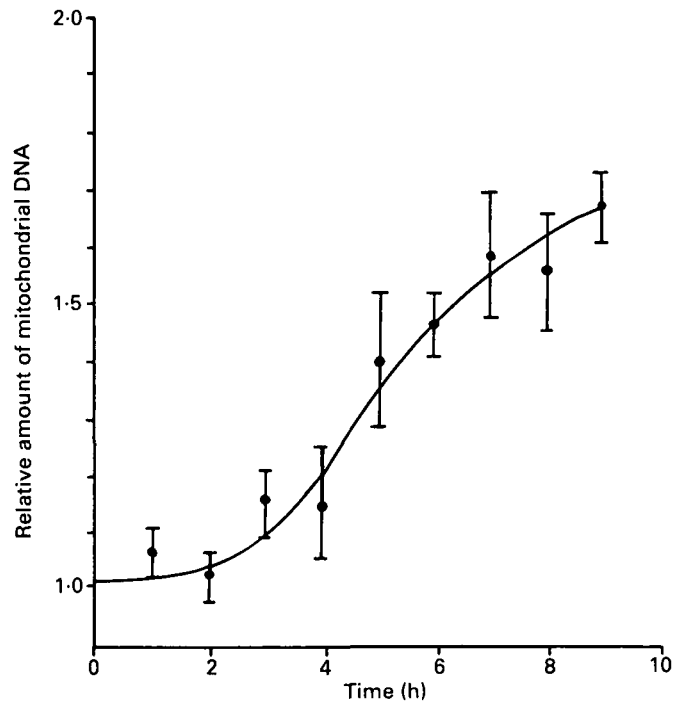
Fig. 9. Relative increase in mitochondrial DNA during release from stationary phase. Mitochondrial DNA was determined as the difference between total cellular DNA and nuclear DNA (see Materials and Methods). The data represent the mean values of five independent experiments and the error bars are the standard deviations of the mean. In these experiments a doubling of cell number occurred between 2 h and 5 h.

labelling represents mitochondrial DNA synthesis, the results indicate, as do the results in Fig. 9 (see below), that mitochondrial replication occurs throughout most of the cell cycle.

Replication of mitochondrial DNA

Since *Dictyostelium* cells contain so much mitochondrial DNA, it is of interest to know how this DNA replicates during the cell cycle. We have measured the kinetics of total cell DNA replication and also the replication of nuclear DNA in synchronized cells. The difference between these measurements represents the synthesis of mitochondrial DNA. The results in Fig. 9 show the increase in mitochondrial DNA during the cell cycle of synchronized cells. Although mitochondrial DNA increases continuously throughout the cell cycle, there appears to be a period of increased mitochondrial replication near the middle of the cycle. Comparison of Figs 7 and 9 indicates a clear temporal separation between nuclear DNA replication and mitochondrial DNA replication, as has been found for *Physarum* (Kuroiwa, Kawano & Hizume, 1977).

DISCUSSION

The results we have presented lead to an interpretation of the cell cycle in *Dictyostelium* that differs significantly from previous observations (Katz & Bourguignon, 1974; Zada-Hames & Ashworth, 1978). We interpret our data on the cell cycle of *Dictyostelium* in the following way. After nuclear division daughter nuclei directly enter *S* phase (in some cases even before cytokinesis). *S* phase is short, probably of the order of 30 min or less. The rest of the cell cycle is taken up by a G_2 period of about 6·5 h duration. In the following we discuss the evidence for each cell cycle phase individually.

G_1

The G_1 period is very short or absent in the normal cell cycle. The evidence for this comes from four observations.

(1) The distributions of nuclear DNA content as measured by flow cytometry (Fig. 1) and micro-fluorometrically (Fig. 2) do not show any evidence of cells with G_1 DNA content.

(2) The synchronization experiments show that nuclear DNA content increases simultaneously with or even slightly before cell division (Fig. 7).

(3) In the pulse-labelling experiment a number of cells that still appeared to be in telophase had labelled nuclei (see also Zada-Hames & Ashworth, 1978). Thus *S* phase follows directly after nuclear division.

(4) The mitotic inhibitor CIPC caused a rapid block in nuclear DNA synthesis (Fig. 8).

All the experiments above indicate that G_1 is absent from the *Dictyostelium* cell cycle. Nevertheless, it is not possible to exclude the existence of a very short G_1 period, having a duration of less than 15 min, due to the resolution of the experimental methods. Evidence for the absence of G_1 has also been observed in previous cell cycle studies on *Dictyostelium* (Woffendin & Griffiths, 1982; and see below). Thus *Dictyostelium* appears to be similar to other primitive organisms such as *Physarum* and *Hydra* in which G_1 is also absent.

There are two reports of G_1 based on observations of [^{3}H]thymidine incorporation in synchronized cells (Katz & Bourguignon, 1974; MacDonald & Durston, 1984). Although incorporation is low, there is a roughly twofold increase over background during a 2- to 3-h period in the middle of the cell cycle. If this incorporation represents nuclear replication, these experiments demonstrate the existence of a G_1 period before the *S* phase. Unfortunately neither group provided evidence that their incorporation was nuclear and not mitochondrial. Furthermore, recent experiments of Podgorski (1983), in which the relative amounts of [^{3}H]thymidine incorporation into nuclear and mitochondrial DNA were measured, indicated that most of the incorporation was into mitochondrial DNA. Hence it appears that [^{3}H]thymidine incorporation cannot be used to identify nuclear *S* phase. It seems likely, therefore, that these earlier reports identified a period of mitochondrial replication in the middle of the cell cycle, in agreement with our observations (Fig. 9).

S *phase*

The duration of S phase is very short, probably less than 30 min in a cell cycle of 7 h. The evidence for this comes from three observations.

(1) A [^{3}H]thymidine pulse labelled only 7–8 % of the nuclei in an exponentially growing population. Since the average cell generation time under these conditions is about 7 h, the duration of S phase can be estimated to be 0·5 h, assuming S phase occurs at the beginning of the cell cycle. The duration of S phase is calculated by integration of the age distribution for an exponentially growing population of cells, $f(x) = 2^{(1-x)}$, where x is the relative cell cycle duration, which varies between 0 and 1. This leads to $N(x) = 2(1-2^{-x})$, where $N(x)$ is the number of cells in a compartment of length x.

It should be noted that even shorter S-phase durations would also be compatible with the data, since an S-phase duration shorter than 30 min would not be distinguishable from a 0·5 h S phase when measured with a 0·5 h thymidine pulse.

(2) The distributions of nuclear DNA content in growing cells show a narrow peak around a mean that corresponds to the G_2 DNA content of nuclei. The data in Fig. 1B show a good fit to a single Gaussian distribution. Qualitatively, it is clear that there are few cells in the distribution with S phase DNA contents and thus the results of the DNA distributions are consistent with the pulse-labelling data.

(3) The synchrony experiment (Fig. 7) shows that, in a synchronous population of cells in which the cell number doubles over a 2-h period, nuclear DNA content also doubles in the same period. This can only be true if the duration of S phase is similar to the duration of cell division.

The length of the G_2 *period*

The length of the G_2 period was determined as the difference between the population doubling time and the length of the other phases of the cell cycle. Since most of the cell cycle consists of G_2 (Fig. 1), this is a relatively accurate estimate of the average duration of G_2. Under the culture conditions used here the average cell generation was 7·2 h (see Materials and Methods). The duration of S phase is 0·5 h and the duration of mitosis is 0·2 h (mitotic index, 2 %). Thus the average duration of G_2 is 6·5 h. Evidence for the fact that the G_2 phase is variable in length comes from experiments performed by Zada-Hames & Ashworth (1978), which will be discussed below.

The difference in cell cycle time between axenically grown cells and bacterially grown cells must be due to a lengthening of the G_2 period of the cell cycle in axenically grown cells, since the nuclear DNA distributions for both growth conditions are the same (Figs 1, 2). Our observations indicate further that differences in cellular DNA content between axenically and bacterially grown cells cannot be due to differences in the cell cycle phase of these two populations, as suggested by Leach & Ashworth (1972), but must be due to differences in non-nuclear, e.g. mitochondrial, DNA.

The Zada-Hames/Ashworth model for the Dictyostelium *cell cycle*

The most extensive study of the cell cycle of *Dictyostelium* was done by Zada-Hames & Ashworth (1978). They used both the labelled mitosis method and
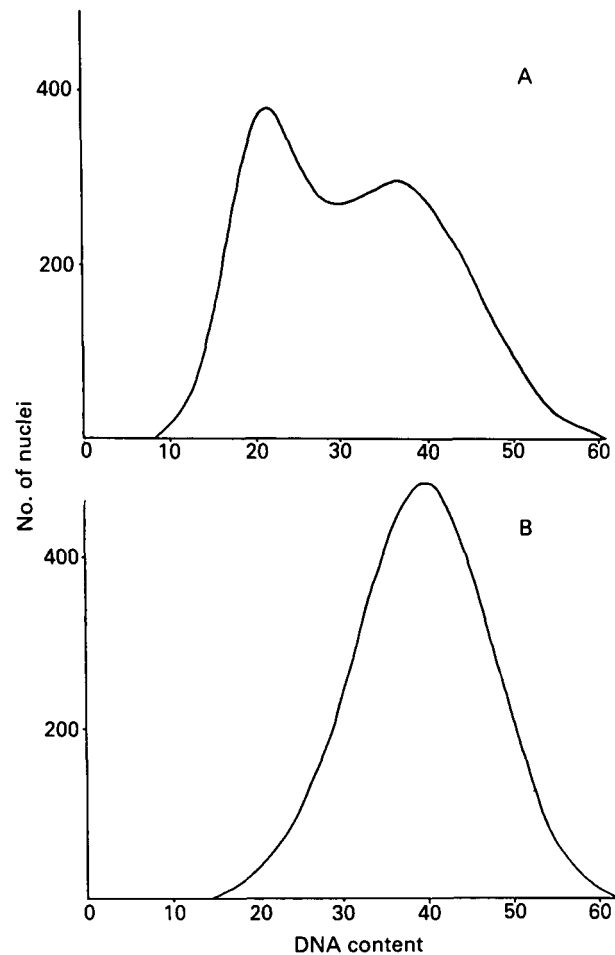
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Fig. 10. Distribution of nuclear DNA predicted by: A, the Zada-Hames & Ashworth values for the lengths of G_1, S and G_2 in the *D. discoideum* cell cycle; and B, according to the revised values for these parameters presented here. The curves were derived by summing a set of Gaussian distributions (Dean & Jett, 1974) whose means corresponded to the DNA content of various cell cycle stages and whose areas were proportional to the expected number of cells in each particular stage, calculated according to the age distribution (see Discussion). G_1 and G_2 were modelled as single Gaussian distributions; since the DNA content is not constant during the S phase, it was broken down into eight subclasses and a Gaussian constructed for each. All Gaussian distributions had the same coefficient of variation (20 %). A. Distribution of DNA/nucleus assuming $G_1 = 1\cdot5$ h, $S = 2\cdot5$ h and $G_2 = 4$ h. The G_1 and G_2 DNA values were set equal to 20 and 40, respectively. The distribution is shown for an assumed 10 000 cells. B. Distribution calculated assuming $G_1 = 0$, $S = 0\cdot5$ h and $G_2 = 7$ h. All other conditions are the same as in A. The curve in B clearly resembles the observed distribution of DNA/nucleus (Figs 1, 2); the curve in A clearly does not.

continuous labelling of synchronized cells to determine the length of the various cell cycle phases. In particular, they suggested the existence of a significant G_1 phase ($1\cdot5$ h), a longer S phase ($2\cdot5$ h) and a shorter G_2 phase than we find. The calculated distribution of DNA/nucleus based on their results (Fig. 10A), however, does not

agree with our observations of the distribution of DNA/nucleus (Figs 1, 2). Fig. 10b shows the results of a similar calculation based on our parameters for the cell cycle, which agrees closely with the observed distributions in Figs 1 and 2. In view of this discrepancy between their conclusions and ours we re-examined the results of Zada-Hames & Ashworth. In the following discussion we present a reinterpretation of their results that is entirely compatible with our conclusions on the cell cycle phases.

Zada-Hames & Ashworth estimated G_2 durations with the classical labelled mitosis procedure. A remarkable feature of their labelled mitosis data, however, was that the percentage of labelled mitoses never exceeded 35 % (fig. 4 of Zada-Hames & Ashworth, 1978), instead of the expected 100 %. This sort of behaviour has been observed in other systems (David & Campbell, 1972; Campbell & David, 1974) in situations where the S phase is short compared to a long and variable G_2. Under these conditions, the small cohort of cells labelled by a [^{3}H]thymidine pulse 'disperses' (due to the variable G_2) as it passes through G_2 so that when labelled cells pass through mitosis they tend to do so with unlabelled cells. The result is that the labelling index of mitotic figures never reaches 100 %. Based on the results of Zada-Hames & Ashworth (1978, fig. 4) G_2 appears to vary between 4 h and 8 h.

Zada-Hames & Ashworth estimated a G_1 period of 1·5 h, from the labelled mitosis experiment. However, because of the variability of G_2 (see above) it is not possible to estimate G_1 in this experiment. They also estimated G_1 from the labelling kinetics of synchronous cell populations. Careful comparison of the pulse-labelling index with the increase in cell number indicated that both parameters increased simultaneously (fig. 8 of Zada-Hames & Ashworth, 1978). There was no evidence for a lag ($= G_1$) between cell division and the onset of labelling ($= S$ phase). Indeed a remarkable feature of these results was that the increase in labelling index initially appeared to precede the increase in cell number. Although Zada-Hames & Ashworth discounted the importance of these observations, they are in complete agreement with our observations on nuclear DNA content.

Additional results in these synchrony experiments provide clear evidence for a short S phase. Despite quite synchronous cell doubling in 3 h, the pulse-labelling index never exceeded 20 % at any point during the cell doubling. Such a result can only occur if the duration of the S phase is significantly shorter than the 3-h period required for nuclear replication by the entire population. An estimate of 0·5 h for S phase is quite consistent with their observations. Furthermore, in their asynchronous exponentially growing cultures the [^{3}H]thymidine-labelling index was about 10 %, which is similar to our measurements and indicates that the S phase is about 6·5 % of the total cell cycle.

A possible role for the cell cycle in development

Takeuchi (1969) found that prespore and prestalk pieces of slugs, which are allowed to regulate and build fruiting bodies, form spores that sort out from each other following germination. The cells that come from spores formed by the regulated prestalk pieces sort to the prestalk region when mixed with germinated spores from prespore pieces. This difference in sorting properties disappears when the cells are

allowed to grow for a few generations (Takeuchi, 1969), indicating that there is a connection between the cell cycle and the stability of the acquired sorting properties.

Takeuchi (1969) also showed that vegetative cells can be fractionated on density gradients and that these fractions sort out from each other. More recently it has been shown that cells separated on density gradients are in different phases of the cell cycle (Weijer et al. 1984b) and hence the idea that cell cycle position controls sorting behaviour is an attractive one. Indeed, we will show in an accompanying paper that cells in different cell cycle phases from synchronized cultures sort out from each other (Weijer, Duschl & David, 1984a).

What correlates with cell cycle phase and could be related to later cell type differentiation? The observations presented in this paper indicate that Dictyostelium cells are primarily in the G_2 phase of the cell cycle and hence cell fate cannot be simply associated with G_1 and G_2, for example. Our results, however, also indicate that these G_2 cells do differ, among other things, in their mitochondrial DNA content, which varies continuously during the cell cycle. In this regard it is interesting to note that prestalk and prespore cells have differing levels of mitochondrial emzymes (Takeuchi, 1960).

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