MAGNESIUM IONS AND THE CONTROL OF THE CELL CYCLE IN YEAST

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SUMMARY
A study has been made of the role of magnesium ions in cell division cycle control in the fission yeast, Schizosaccharomyces pombe, and the budding yeast, Kluyveromyces fragilis. Synchronization of cell division in these organisms can be induced by restoring magnesium to magnesium-exhausted cultures. In S. pombe, a correlation exists between the time taken for cells to enter the first synchronous division and the period of magnesium exhaustion. During short-term incubation in magnesium-deficient media, S. pombe cells are observed to continue growth in length, but they fail to make a cell plate and divide; long-term magnesium deficiency results in the production of aberrant cell forms, and a reduction in viability. Analysis of total cell magnesium in cultures of both S. pombe and K. fragilis, synchronized by various induction and selection procedures, revealed that there is a fairly steady fall in magnesium concentration as cells grow, terminating in a rapid influx of magnesium just before cell division. This leads to the hypothesis that falling magnesium concentration may act as a transducer of cell size, eventually triggering spindle formation and a membrane change which permits rapid uptake of magnesium to a concentration which brings about spindle breakdown. The hypothesis was tested directly using the divalent cation ionophore, A23187, in the absence of calcium ions; the results obtained showed that a short pulse of A23187, very late in the cell cycle, accelerated cells into division and shortened the subsequent cycle. The hypothesis is discussed in relation to current models of cell cycle regulation.

INTRODUCTION
Over the past years, there has been great interest in the role of calcium ions in the control of growth and cell division (Heilbrunn & Daugherty, 1931; Mazia, 1937; Heilbrunn, 1952; Durham, 1974; Berridge, 1975; Mazia, 1977). Very few studies of dividing cells, however, have been concerned with the role of magnesium ions, despite the fact that magnesium is known to be of widespread importance in metabolic and physiological control systems (see e.g. Vernon & Wacker, 1978). Magnesium and calcium act antagonistically towards each other in many biological processes. This is due to differences in the structural, thermodynamic, and kinetic parameters of these ions (Williams, 1975). Calcium and magnesium exhibit differential binding affinities for naturally occurring chelatable ligands. In calcium complexes, bond distances are flexible, bond angles adjustable, and co-ordination numbers variable from 6 to 10; magnesium, on the other hand, has a much more restricted range of structures, with a fixed co-ordination number of 6. Thus, calcium can displace magnesium

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from most binding sites composed of complex sets of multidentate ligands (Williams, 1974). Because of this, cross-linking of biopolymers is effectively achieved by calcium, but magnesium may antagonize this action by binding to certain groups and making them unavailable. Rubin (1975) has postulated that the effects observed in numerous cell types in response to varying calcium concentrations are produced by calcium competing with magnesium for membrane sites. Certainly, many enzymes show variable activity depending on the magnesium/calcium ratio in their environment (Dixon & Webb, 1964; Bygrave, 1976). More significantly for the work to be described in this paper, calcium inhibits polymerization of tubulin in a way which is regulated by the magnesium concentration (Rosenfeld, Zackroff & Weisenberg, 1976). Thus, many workers have suggested that control of the calcium/magnesium ratio helps to govern microtubule assembly in vivo (Fuller et al. 1975; Harris, 1975; Hepler, 1976; Schliwa, 1976). If this is so, the calcium/magnesium ratio must also control spindle formation, which depends on microtubule assembly, and hence control the onset of cell division.

There are very few data regarding metal ion fluxes during the cell cycle. Magnesium and calcium levels in synchronized yeast cultures were studied by Duffus & Reid (1973) but the present paper represents the first detailed investigation of magnesium flux. It was initiated following the discovery that agents which were capable of altering magnesium levels in yeast cells, such as the ionophore A23187, and various magnesium-chelating agents, were also capable of controlling progress through the cell cycle (Duffus & Paterson, 1974a, b; Penman & Duffus, 1975; Ahluwalia, Duffus, Paterson & Walker, 1978; Walker & Duffus, 1979). The object was to determine whether cell magnesium concentration varied in any way which could be related to cell division. This paper describes the results obtained, the hypothesis that has been formed to explain these results, and an experiment with the ionophore, A23187, which appears to support the hypothesis.

**MATERIALS AND METHODS**

**Organisms, media and growth conditions**

*S. pombe* NCYC132 (ATCC24751) and *Kluyveromyces fragilis* NCYC100 (formerly *Saccharomyces fragilis* (Van der Walt, 1970)) were originally obtained from the National Collection of Yeast Cultures at Nutfield, Surrey, England. *S. pombe* haploid strain 972h~ was originally obtained from Dr Jorgen Friis, University of Odense, Denmark. The growth medium used throughout is Edinburgh Minimal Medium No. 2 (EMM2) (Mitchison, 1970). Medium referred to as ‘magnesium-free’ or ‘calcium-free’ is prepared by omitting the MgCl₂ or CaCl₂ components of EMM2 and ensuring that only AnalaR grade reagents (BDH, Poole, Dorset, England) and thrice glass-distilled water constitute the remainder of the medium, which is autoclaved in scrupulously-clean Pyrex culture vessels. Viable stock cultures of both yeast species were stored on EMM2/agar plates at 4 °C. Monthly subculturing was carried out by growing cells for 3–4 days at 30 °C prior to cold storage. Weekly subculturing of the cells was carried out by inoculating stationary phase cells into 10 ml EMM2 in McCartney bottles. Small volumes (generally 1–0 ml) of actively dividing precultures were used to initiate experimental cultures which were aerobically propagated in cotton-plugged Erlenmeyer flasks in an orbital incubator operating at 160 rev/min. *S. pombe* was grown at 32 °C and *K. fragilis* at 30 °C.
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Cell number determination

Cell numbers were determined using an improved Neubauer haemocytometer (Weber, England). Three to four counts of 150–200 cells were made on each sample and the mean calculated. For S. pombe, the criterion employed by Mitchison (1970) was adopted, in that cells were not taken as having divided until the constriction or ‘notch’ appearing between 2 daughter cells was observed. The cell plate index is the percentage of cells of the total population which show a cell plate, and is equivalent to the mitotic index of higher eukaryotes. For cell counting in K. fragilis cultures, the criterion employed by Penman (1974) was adopted in that cytokinesis is not regarded as complete until the daughter buds reach two-thirds or more the size of the mother cells. To counteract cell clumping for accurate number determination in S. pombe cultures, 1–0 ml samples were routinely sonicated at an amplitude of 16 μm (peak to peak) for 30 s using the exponential microprobe of an MSE 150-W ultrasonic disintegrator.

Nuclear staining

Yeast nuclei were stained using the following Giemsa method, modified from Nurse, Thuriaux & Nasmyth (1976). Samples (50 ml) from experimental cultures were centrifuged and the pellet immediately frozen in an ethanol cold bath (—45 °C). After thawing at room temperature, the cells were suspended in distilled water, briefly (5 s) sonicated and heat fixed onto glass slides. The smears were washed with tapwater and postfixed in Carnoy’s fixative (60 ml ethanol, 30 ml chloroform, 10 ml acetic acid) for 30 min. After further washing, the RNA content of the cells was digested by incubating at 30 °C with bovine pancreatic ribonuclease B (1 mg/ml in sodium phosphate buffer, pH 6.8) for 3 h. The cells were washed again and extracted for 30 min with 1 N HCl at 60 °C. A final rinsing was followed by staining with 0.05 M sodium phosphate buffer (pH 6.8) containing 4 % freshly added Giemsa stain.

Establishment of synchronous cultures: induction methods

Magnesium exhaustion-synchronization. Exponentially-dividing cultures of yeast were harvested by Millipore membrane (1.2 μm pore size) filtration and washed thrice with glass-distilled water at room temperature. Washed cells were then reincubated into prewarmed magnesium-free EMM2 and propagated as described above. After set periods of time, sufficient AnalaR grade MgCl2 was added back to the culture to restore the magnesium level to that in normal EMM2 (0.3 mM).

EDTA pulse synchronization. Cell division synchrony was induced in asynchronous yeast cultures by EDTA using a modification of the method described by Ahluwalia, Duffus, Paterson & Walker (1978). AnalaR grade EDTA (disodium salt) was added to exponentially growing cultures to give a final concentration of 50 mM. After 1-h exposure to the chelator, cells were harvested by Millipore membrane filtration, washed with glass-distilled water at room temperature and finally reincubated into fresh, prewarmed EMM2 (which lacked EDTA). The time taken for this procedure varied with culture volume and cell density but generally took about 5 min to complete. Control cultures, without EDTA treatment, were treated identically.

Deoxyadenosine pulse synchronization. Synchrony was established in S. pombe and K. fragilis cultures using the DNA synthesis inhibitor, 2′-deoxyadenosine (Adr) in modifications of the methods employed by Mitchison & Creanor (1971) (S. pombe) and Penman & Duffus (1975) (K. fragilis). Adr (Sigma Chemical Co. Ltd., London) was added to exponentially-dividing cultures to a final concentration of 20 mM. Cells were harvested by Millipore membrane filtration, washed with glass-distilled water at room temperature and finally reincubated into fresh, prewarmed EMM2. Control cultures, without Adr treatment, were treated identically.

Studies with 23187 (Efrapeptin). Stock solutions of the ionophore (which was a generous gift from Dr R. L. Hammill, Eli Lilly and Co., Indianapolis, U.S.A.) were prepared by dissolving 0.5 mg in 0.5 ml AnalaR acetone and then adding 1.0 ml absolute ethanol (Prince, Rasmussen & Berridge, 1973). Small volumes of the stock solution were added directly to exponentially dividing S. pombe cultures in Ca-free EMM2; equal volumes of acetone/ethanol...
mixtures were added to control cultures without effect on cell division. In pulse experiments, cultures were exposed to the ionophore for 5 min, after which cells were rapidly harvested by Millipore membrane filtration, washed twice with prewarmed Ca-free EMM2, and finally reinoculated into fresh prewarmed medium. These operations took about 2 min to complete.

Establishment of synchronous cultures: selection methods

Density gradient selection synchronization. S. pombe cells were selection-synchronized using a modification of the density gradient sedimentation procedure first described by Mitchison & Vincent (1965). Cells from an exponentially dividing culture were filtered and concentrated to about $5 \times 10^8$ cells/ml by resuspending in $5 \sim 10^7$ ml EMM2. This suspension was carefully layered on top of a linear lactose gradient (15–30 % w/v), prepared in EMM2 and centrifuged at 1000 rev/min in an MSE 6L centrifuge for 6 min. Using a long, thin syringe needle, about $2 \sim 10^6$ ml of culture (containing small cells at an early cell cycle stage) were removed from the uppermost layer of the gradient cell suspension and reinoculated into fresh EMM2 to initiate synchrony. As far as possible, all steps were performed at 32 °C to avoid major temperature shifts.

Continuous flow size-selection synchronization. Synchronous cultures of both S. pombe and K. fragilis were established using a modification of the continuous flow size-selection centrifugation method first described by Lloyd, John, Edwards & Chagala (1975). Exponentially-dividing cultures were siphoned at a set flow rate (monitored by a rotameter) into a continuous-action rotor (MSE) operating at constant revolution in the bowl of an MSE 18 centrifuge. Overflow effluent containing small cells was retained and allowed to grow as a synchronous culture. All operations prior to this were carried out at 30 °C. Operating conditions for S. pombe and K. fragilis are described in the legends to Figs. 11 and 12, respectively.

Analysis of total magnesium

Instrumentation, working conditions and sample preparation:

The total magnesium content of yeast cells has been determined using flameless (electrothermal) atomic absorption spectrophotometry (FAAS). The advantages of electrothermal atomization over the older flame technique include: a greater intrinsic sensitivity (less than 1 ng of element can be measured during each analytical sequence), a simplified sample preparation (see below) and a greatly reduced sample volume (typical determinations require 5 μl of sample, compared to the minimum requirement of the flame system of about 500 μl for one integrated reading). The electrothermal carbon-rod atomization technique as employed in this study is especially suited for the rapid analysis of the magnesium content of yeast cells since the cell suspension is dried, ashed and atomized automatically in a single preset programme. The instrumentation consisted of a Varian Techtron Model 1100 atomic absorption spectrophotometer, a Model 63 carbon-rod atomizer and a BC-6 Module background corrector. Absorption readings were recorded on an Oxford 3000 series recorder. The optimal working conditions and range of linear response for magnesium were determined and are shown in Table 1. The data represent modifications of the guidelines suggested by Helin & Slaughter (1977) for the determination of magnesium in brewing materials by FAAS.

Preparation of cells for magnesium analysis was undertaken by pipetting a 1.0-ml sample of culture into microcentrifuge tubes and immediately freezing the tubes in an ethanol cold bath at −45 °C. Samples were thawed at room temperature and quickly centrifuged at 14 000 g in a Quickfit microcentrifuge for 2 min, again at room temperature. The supernatant medium was decanted and the pellet resuspended in an equal volume of glass-distilled water, using a vortex mixer. The cells were washed a total of 4 times with glass-distilled water using the same technique. Five-microlitre samples of final-washed cells were injected into the carbon-rod atomizer using a platinum-tipped microsyringe. As a check on magnesium contamination from the syringe and other laboratory hardware, several injections of glass-distilled water were routinely carried out in order to establish a baseline.

Magnesium ion content of yeast cells is expressed as fg/cell. To obtain an accurate calculation of this, cell numbers in the final washed cell suspension were directly determined immediately preceding FAAS analysis. This counteracts error which may result from cell loss during the washing procedure.
Table 1. FAAS working conditions for Mg analysis of yeast cells

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>HCL (^1)</th>
<th>(\text{H}_2\text{L} (^2)</th>
<th>Low (^3)</th>
<th>High (^4)</th>
<th>Ash (^5)</th>
<th>Atomize (^6)</th>
<th>Standard (^7)</th>
</tr>
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<tbody>
<tr>
<td>202.5 nm</td>
<td>5.0</td>
<td>5.0</td>
<td>0.5</td>
<td>3.0</td>
<td>6.2</td>
<td>15</td>
<td>6.6</td>
</tr>
</tbody>
</table>

1 HCL – Hollow cathode lamp current in mA.
2 \(\text{H}_2\text{L}\) – Hydrogen continuum lamp intensity (in arbitrary units), used to check for non-atomic absorption.
3 Low – Scale expansion setting.
4 High – Curve linearization setting.
5 Ash – Ashing conditions in volts (arbitrary units) and time (seconds).
6 Atomize – Atomization conditions in volts (arbitrary units) and time (seconds).
7 The concentration (in \(\mu\)g/ml) of lowest standard represents the effective sensitivity of the method. Standard MgCl\(_2\) solutions (BDH chemicals Ltd.) were prepared by appropriate dilution using thrice glass-distilled water from a Sorek all-glass still and the instrument was calibrated with fresh standards each time an analysis was performed.

RESULTS

Studies on yeast cell division in magnesium-deficient media; magnesium exhaustion-synchronization

When washed cells of *S. pombe* are inoculated into a minimal medium devoid of magnesium ions (or rather containing an irreducible minimum level of magnesium), after a lag phase, cell division commences normally then ceases. This cessation of cell division occurs after about 3 h and cells are permanently prevented from dividing in this environment, even after 24-h periods of incubation (Fig. 1). One of the most striking microscopic observations to be made during prolonged incubation of *S. pombe* in magnesium-deficient media is that cells continue lengthwise growth with a constant cell diameter. Giemsa nuclear staining reveals that these elongated cells are mononucleate implying that in the absence of magnesium ions *S. pombe* cells fail to carry out nuclear division, synthesize a new cell plate, and constrict their cytoplasm. After 24 h in magnesium-free EMM\(_2\), the majority of cells appear 3 or 4 times longer than those in control cultures grown with magnesium for similar periods (Figs. 2, 3). The latter appear small and rounded, characteristic of stationary phase cells of *S. pombe* (Mitchison, 1970). Fig. 3 shows that the nuclei in elongated cells are asymmetrically elongated, suggesting that some nuclear changes have occurred preparatory to division, but that complete chromosome segregation has failed to occur. The elongation process observed in magnesium-starved cultures of fission yeast does not continue indefinitely since it has been observed that the majority of cells attain a maximum constant length after 24 h, and that they fail to elongate further after extended incubation periods. In fact, extended periods of magnesium-deficiency result in the production of aberrant cell forms, with some cells showing signs of hyphal-type filamentous morphology. A ‘granularization’ of the cytoplasm and enlarged vacuoles are also observed when examined with phase contrast. The long periods of magnesium deficiency are associated with a reduction in viability, as judged by the granular
appearance of the cytoplasm, and also by a failure of cells to recover on the restoration of normal magnesium levels.

Concerning *K. fragilis*, incubation for a few days in Mg-free EMM2 produces cells which characteristically form buds which fail to separate from the mother cells. There is also a high incidence of bilateral budding (and, occasionally, multilateral budding), but again with a failure of cell separation. Giemsa staining of magnesium-starved *K. fragilis* revealed a high frequency of mononucleate cells showing incomplete nuclear migration, as with *S. pombe*.

![Graph](image)

**Fig. 1.** The Mg ion dependence of cell division in *S. pombe*. At zero time, washed cells of *S. pombe* 132 were inoculated into EMM2 which contained: (■) the normal complement of Mg (0.3 M), and (●) no added Mg (trace contaminant level of 0.16 M).

If magnesium ions are restored to starved cultures, cell division recommences synchronously. This phenomenon is referred to as magnesium exhaustion synchronization, and the effect is shown for *S. pombe* in Fig. 4 B–H, and for *K. fragilis* in Fig. 5. The addition of an excess supply of magnesium ions to a normally dividing fission yeast culture (one containing the normal magnesium complement) has no effect of phasing cell division, as seen in Fig. 4A. Further, increases in cell number occurring after transfer to Mg-deficient EMM2 (Fig. 4B, C) are still asynchronous, as can be seen from the cell plate index and the slope of increase in cell number, neither of which is significantly different from those in Fig. 4A. Figs. 4 B–H show that in *S. pombe* the period of magnesium exhaustion prior to magnesium replenishment has the effect of varying both the timing and degree of the resulting synchrony patterns. For example, a proportionality exists between the time of magnesium exhaustion and the
time taken for cells to commence their first synchronous division. Generally, the longer a cell is deprived of magnesium, the longer it takes to divide. In addition, after the first division in cultures synchronized by relatively short periods of magnesium exhaustion, cells rapidly enter a second synchronous division. This occurs in only a fraction of the normal cell cycle time, approximately $1.5$ h compared to $2.5$ h.
Fig. 4. Mg ion exhaustion synchronization of cell division in S. pombe. Washed cells of S. pombe 132 were inoculated (at zero time) into Mg-deficient EMM2 and incubated for various periods of time (9, 14, 5, 6 h, 12, 14, 16, 24 and 42 h respectively), after which the Mg level in the cultures was restored to normal by the addition of MgCl₂ (indicated by the arrows). A shows the effect of adding the same concentration of MgCl₂ to a normally-dividing culture (i.e. one already containing magnesium ions).

Fig. 6 illustrates this by summarizing the timing of cell plate index peaks in cultures of S. pombe synchronized by various periods of magnesium exhaustion. The pattern of magnesium exhaustion synchrony in K. fragilis (Fig. 5) differs from that in S. pombe. K. fragilis is synchronized at 2 different points in the cell cycle, so that two synchronous divisions are required before doubling of cell number is attained.
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Fig. 4, cont'd.
Changes in cell magnesium levels during synchronous yeast growth

Regarding the preparation of yeast cells for magnesium ion analysis, the validity of the water wash technique (see Materials and methods) was checked by measuring cell magnesium levels in cells subjected to a series of multiple washes. Fig. 7 shows that, after the first resuspension in glass-distilled water, cell magnesium falls, and reaches a fairly constant value, which remains at this level, irrespective of prolonged washing. The routine technique of sample preparation for magnesium analysis involves 4 water washes. Although some intracellular magnesium may be lost during the first wash, there can be no doubt that a substantial fraction is retained, and that this fraction is unaffected by subsequent washing. It may be that the magnesium lost in the first wash is the intracellular pool component, but we believe that this fraction represents magnesium loosely retained within the cell wall.

![Diagram showing changes in cell magnesium levels during synchronous yeast growth.](image)
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Fig. 6. Schematic representation of the timing of cell plate index peaks in magnesium exhaustion synchronized cultures of S. pombe. CP₁, CP₂, and CP₃ with arrows indicate the appearance of the first, second and third cell plate index peaks, respectively.

Fig. 7. The effect of prolonged water-washing on the Mg ion content of stationary phase cells of S. pombe. Cells were repeatedly washed by microcentrifugation and resuspended by vortex mixing in glass-distilled water.

Changes in cell magnesium throughout the growth cycle of S. pombe were monitored as described, and, from Fig. 8, it is evident that during true exponential and asynchronous cell division, magnesium levels remain constant at about 10 fg per cell. Fluctuations in cell magnesium were observed during the lag phase of growth; one distinct peak occurred at the transition between lag phase and log phase, at the point corresponding to the onset of cell division. Concerning cell cycle-dependent changes in
magnesium, it is apparent that in cultures of both *S. pombe* and *K. fragilis*, synchronized by various induction and selection methods, qualitative similarities in the patterns of change in total cell magnesium exist (Figs. 9–12 and Fig. 14). Because of the discontinuous sampling regime, precise timing of the magnesium and cell plate peaks and quantitation of the maxima are impossible. The apparent timing of the peaks could vary considerably, say ± 10 min, from the true value. Hence, seeming discrepancies in their relationship to other cell cycle events, in Figs. 9A, B, 10A, and 11B, are readily explained. The general trend is one in which total cell magnesium increases sharply at a time just prior to cell division, and then falls equally dramatically. This has been schematically summarized in cell cycle maps (Figs. 15, 16). With *S. pombe*, the correlation between magnesium and cell division also extends to the timing of the appearance of cell plate index peaks, total magnesium levels exhibiting maxima just prior to this cell cycle stage (Fig. 15). Apart from this short-lived spike of total magnesium, the amount remains fairly constant through the cell cycle, resulting in a gradual fall in concentration as the cell volume increases. Quantitatively, the largest values of magnesium per cell are found in cultures to which magnesium has been restored after relatively short periods of magnesium exhaustion (Fig. 9A, B). Fig. 9A shows
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that, during incubation in magnesium-deficient medium, cell magnesium remains at a low and fairly constant level. *S. pombe* continues lengthwise growth in such conditions, and so again the intracellular concentration of magnesium will drop as cell water volume, by way of an increase in cell length, increases. By the time the magnesium complement is restored to initiate synchrony, therefore, the effective magnesium concentration in cells will be very low, or exhausted.

Some points of detail in the results shown in Figs. 9C and 11A deserve special mention. In Fig. 9C there is a peak in magnesium between 25 and 26 h, which precedes an increase in cell plate index, without any immediate increase in cell numbers. This suggests that there are 2 magnesium-dependent processes in division of *S. pombe*, the formation of the cell plate and the separation of the daughter cells, which are distinguishable here because of the long period of magnesium exhaustion (24 h). In the same figure, the absence of a magnesium peak before the second round of division can be explained only on the premise that it has been missed in sampling. In Fig. 11A, the magnesium peaks are much smaller than in Fig. 9A, where the degree of synchrony is similar. This must reflect a difference in cells subjected to magnesium exhaustion (Fig. 9A) and 2'-deoxyadenosine treatment (Fig. 11A).

With regard to cultures synchronized by continuous flow size selection, observable magnesium levels must be viewed in the context of proper controls. For *S. pombe*, Fig. 13A represents a control culture for this method. No significant differences in cell magnesium patterns between the asynchronous control and the partially synchronized culture (Fig. 13B) could be detected. However, the degree of synchrony attained was low, and any cell cycle-related peaks would, therefore, be difficult to detect.

Control of cell division with ionophore A23187 and magnesium ions

Studies on *S. pombe* cell division with the ionophore A23187 were carried out in calcium-free EMM2. This medium contained a trace contaminant level of 0.4 µM calcium, as detected by FAAS, and the magnesium to calcium concentration ratio was calculated as about 750:1. A23187 can act as an ionophore for both these ions, but in this environment it is assumed that its major effects lie in an alteration of the magnesium ion homeostasis. When cells of *S. pombe* were washed and inoculated into calcium-free EMM2 the rate of cell division did not differ from that of cells grown in complete EMM2 (Fig. 17A). When A23187 was applied to an exponentially dividing *S. pombe* culture at 0.5 µg/ml, and in the absence of calcium, cell division was immediately inhibited. Cells were apparently arrested late in the cell cycle, since the cell plate index increased about 4-fold after 2 h exposure to the ionophore; continued exposure at this concentration resulted in some residual cell division (Fig. 17B). Calcium ions added to 5 mM during ionophore arrest produced no effect (Fig. 17C), but magnesium ions at the same concentration reversed the ionophore-mediated arrest, since cells recovered quickly upon magnesium addition, displaying a reduction in cell plate index and a corresponding increase in cell numbers (Fig. 17D).

Concerning the effects of A23187 in selection-synchronized *S. pombe* cultures, Fig. 18A shows that, when applied in mid cell cycle, cell division is inhibited and about one third of the cells contain cell plates. A 5-min pulse of A23187 applied just
after the first cell plate index peak in a synchronous culture has the effect of accelerating cells through their second synchronous division (Fig. 19B) and approximately halving the cell cycle time.

**DISCUSSION**

*The magnesium ion dependence of cell division in yeast*

Magnesium ions are essential for cell division in yeast. This absolute requirement has been demonstrated in the present study using magnesium-deficient media, and in previous reports by exposing cells to magnesium-chelating agents (Ahluwalia et al. 1978; Walker & Duffus, 1979). The observation that cells of *S. pombe* complete about one doubling in numbers in magnesium-free EMM2 before cell division ceases (Fig. 1) may reflect a utilization of endogenous stores of magnesium known to occur in yeast (Lichko & Okorobov, 1976). Cell division is inhibited by limiting the magnesium...
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Fig. 9, cont'd
supply, but growth by way of continued protein synthesis is not inhibited, either by chelating agents like EDTA (Ahluwalia et al. 1978) or by specific magnesium-deficiency (Walker, 1978). DNA synthesis, however, is inhibited. Moreover, while cells of *S. pombe* continue to elongate in the absence of magnesium, nuclear division and subsequent cell plate formation is arrested (Fig. 3). This, together with other work using A23187 reported here (Figs. 17, 18) and elsewhere (Duffus & Paterson, 1974), showing that the ionophore prevents progress through the cell cycle at a point just before cell division, makes it likely that specific magnesium limitation in yeast arrests cells somewhere late in the $G_2$ phase of the cell cycle.
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Fig. 11. A. Fluctuations of cell Mg levels in a culture of S. pombe 132 synchronized by a 5-h pulse of 2 mM 2'-deoxyadenosine. Zero time marks the end of the inhibitor pulse by reinoculation into fresh EMM2. B. As in A, but the culture is K. fragilis 100 which was synchronized by a 2-h pulse of 2'-deoxyadenosine. Control cultures (not shown), which lacked inhibitor treatment, grew asynchronously. •, log cell no; ○, cell plate index; ■, fg Mg/cell.
The observation that cell division can be synchronized by periods of magnesium exhaustion has not previously been reported. Synchrony is only established by replenishment of magnesium to previously-exhausted cells, since addition of this ion to normally-dividing cultures (with sufficient magnesium) does not result in synchrony (Fig. 4A). The response to magnesium is also a specific one because it was found (Walker, 1978) that cations of similar ionic configuration (Ca, Sr and Be) and ones with similar effects on enzyme activation (Mn and Zn) all fail to substitute for magnesium in the induction of synchrony.

**Figure 12.** Fluctuations of cell Mg levels in a culture of *S. pombe* 972h− synchronized by size-selection. A concentrated suspension of asynchronously-dividing cells was centrifuged at low speed through a linear (15–30 % w/v) lactose gradient and small cells from the top layer were selected off and used to initiate the synchronous culture (zero time). ●, log cell no.; ○, cell plate index; ■, fg Mg/cell.

**Magnesium ions as transducers of size control of cell division**

In *S. pombe*, the cell cycle maps of Fig. 6 reveal that periods of magnesium exhaustion affect subsequent synchrony patterns, with a correlation existing between exhaustion time and the timing of the first synchronous division. This correlation also extends to cell size; *S. pombe* elongates in magnesium-free EMM2 and so, generally, the longer a cell is the longer it takes to divide after restoration of normal magnesium levels. This observation, together with the other that intervals between first and second synchronous divisions in magnesium-exhausted cells are shorter than normal cell cycle times (Fig. 6), is relevant to current ‘timer’ and ‘sizer’ models of cell cycle
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control in *S. pombe* (reviewed by Mitchison, 1977a). It has been suggested, for example, that in *S. pombe* there exists a homeostatic size control over cell division (Nurse, 1975; Fantes, 1977) where large daughters have short cycles and small daughters have long cycles. This inverse proportionality does not hold if one considers the present results, where the converse appears to be true; that is, the larger the cells, the longer they take to divide. However, magnesium exhaustion must be regarded as

![Graphs showing levels of cell Mg](image)

**Fig. 13.** A. Levels of cell Mg in an asynchronous culture of *S. pombe* 132. Cells were siphoned at 600 ml/min into a continuous-action centrifuge rotor operating at 3000 rev/min. The effluent culture (45% of the original cell density) was collected and propagated normally. Zero time marks the completion of centrifugation. The culture is taken as being representative of a "control" for other cultures which had been synchronized by modifications of the same technique. B. Levels of cell Mg in a culture of *S. pombe* 132 synchronized by continuous-flow size selection. Cells from an asynchronously dividing culture were siphoned at 400 ml/min into a continuous-action centrifuge rotor operating at 3000 rev/min. The effluent (24% of the original cell density) was collected and propagated as a synchronous culture after completion of centrifugation (zero time). •, log cell no.; O, cell plate index; ■, fg Mg/cell.
a special case in this instance, since distortions in cell size are not brought about by genetic lesions (as in the paper by Nurse), and it is possible that long periods of magnesium-deficiency produce an abnormal effect through a general depression of cell metabolism (Rubin, 1975). More significant in relation to normal division control are probably the abbreviated intermitotic times between first and second synchronous divisions. They represent only a fraction of the normal cell cycle time (about 60%). The cells produced by the first division are still exceptionally large, and thus the shortened intermitotic times conform with the observations of Fantes and Nurse cited above. This has interesting corollaries in experiments with *S. pombe* involving metabolic inhibitors (Mitchison & Creanor, 1971), nutritional shifts (Fantes & Nurse, 1977) and temperature shocks (Kramhøft, Nissen & Zeuthen, 1976) where cell size is also altered at the time of division. For example, Mitchison & Creanor (1971) found that 2'-deoxyadenosine inhibition of *S. pombe* produced oversized cells, and postulated that shortened intermitotic times observed following induction of synchrony with this inhibitor were due to accumulation of ‘division proteins’ during the induction period, in a manner similar to that postulated for heat-shocked *Tetrahymena* cells (Zeuthen, 1964). Perhaps a similar explanation applies here.

A size control model may closely correlate results from the division of *S. pombe* cells altered in size either by genetic lesions (Nurse, 1975) or by nutrient depletion.
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Fig. 15. Cell cycle maps of *S. pombe* from cultures synchronized by induction and selection. *D*₁ and *D*₂ indicate midpoints of synchronous divisions. Closed triangles above the lines show the time of the major peaks in cell magnesium and 'CP' with an arrow represents the time of appearance of cell plate index peaks. Zero time in each case indicates the end of the treatment employed to synchronize the cells.

Fig. 16. Cell cycle maps of *K. fragilis* from cultures synchronized by induction and selection. See legend of Fig. 15 for an explanation.
Fig. 17. Effect of ionophore A23187 with Ca and Mg ions during asynchronous growth of *S. pombe* in Ca-free EMM2. A. Ca-free growth of *S. pombe*. Where indicated by the arrow, normally-dividing cells were filtered, washed and reinoculated into Ca-free EMM2 (●). An untreated control culture (□) was grown throughout in complete EMM2. B. Effect of ionophore A23187 on Ca-free growth of *S. pombe*. Where indicated, A23187 was added (final concentration of 0.5 µg/ml) to an asynchronously-dividing culture in Ca-free EMM2. C. Effect of Ca on reversal of A23187 arrest. As in B, but where indicated, Ca ions were added to the culture (final concentration 50 mM). D. Effect of Mg on reversal of A23187 arrest. As in B, but where indicated, Mg ions were added to the culture (final concentration 5 mM). ●, log cell no.; O, cell plate index.
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(Fantes & Nurse, 1977), but it fails to indicate the underlying control mechanism. The observed influence of magnesium on cell elongation in *S. pombe* and also in bacterial cells (Webb, 1949; Brock, 1962; Kennel & Kotoulas, 1967) may mean that under normal steady-state conditions, changes in intracellular magnesium concentrations directly exert a size control over cell division. Further work is necessary in order to substantiate this.

![Fig. 18. Effect of continual exposure to ionophore A23187 during synchronous growth of *S. pombe* in Ca-free EMM2. A. Control culture synchronized by lactose gradient size-selection. B. As in A, but where indicated, A23187 was added (final concentration 0.5 μg/ml). •, log cell no.; ○, cell plate index.](image)

Magnesium ions are 'timers' of cell cycle progression

The qualitative similarities in the timing of magnesium influx in cultures of both fission and budding yeasts synchronized in various ways encourages us to believe that these are related to the fundamental cell division cycle. Quantitative differences in cell magnesium may be explained by the fact that different methods were used to synchronize the cells, or by the discontinuous sampling regime which would frequently miss short-lived peak values. The necessity of establishing suitable control cultures during cell cycle studies has recently been emphasized by Mitchison (1977b), and it is, therefore, important to note that the dramatic peaks in magnesium levels observed in the synchronous cultures were absent from corresponding controls (for example, Fig. 8). That they are also absent from the culture of *S. pombe* synchronized by continuous flow size-selection (Fig. 13B), probably reflects the low degree of synchrony attained. In successfully synchronized cultures, the relevant increases in magnesium
per cell occur transiently prior to division, and in most cases are too large (i.e. more than a doubling) to be simply due to the parallel increase in the volume of cell water which doubles during the cell cycle as the cells grow from one division to the next. In other words, the observable fluctuations represent genuine physiological responses by the cells, since, even if magnesium were to be expressed on a per ml of culture basis, or on a cell concentration basis, peaks would still occur at the time point indicated.

![Graph](image)

Fig. 19. Effect of a 5-min pulse of ionophore A23187 during synchronous growth of *S. pombe* in Ca-free EMM2. A. Control culture synchronized by lactose gradient size-selection. B. As in A, but where indicated, A23187 was added (final concentration 4.0 μg/ml) to the culture and pulsed for 5 min. Long arrow, beginning of pulse. Short arrow represents the time when cells were reinoculated into fresh Ca-free EMM2 after filtration and washing. ●, log cell no.; ○, cell plate index.

From the results obtained, it may be seen that the predominant pattern of change in magnesium concentration through the cell cycle is a fall (total cell magnesium constant, with cell volume increasing) followed by a sudden increase which precedes cell division, the concentration thereafter declining to a level characteristic for dividing cells under a given set of conditions. Although these results show the possibility of a cell cycle 'timer' based on fall in magnesium concentration, and a close temporal relationship between magnesium uptake and cell division, they do not, of themselves, allow us to distinguish between change in magnesium concentration as a cause of cell cycle events and as a response to them. Therefore, in an attempt to test the hypothesis that the fall in magnesium concentration with cell growth is the prime basis of both size and time control, we undertook some experiments with the ionophore, A23187. The effects of A23187 are assumed to be due mainly to an equilibration of magnesium ions between the cytosol and surrounding medium; calcium effects
are regarded as negligible in media containing very high magnesium to calcium concentration ratios. Support for this assumption comes from observations (Fig. 17c, d) where magnesium, but not calcium under the same conditions, rapidly reversed A23187 arrest of cell division. In the continuing presence of A23187 in a synchronized *S. pombe* culture, where cells lose magnesium in equilibrating with the medium (Duffus & Paterson, 1974b), cells accumulated at the end of the cell cycle with a fair percentage managing to form cell plates (Fig. 18). This is what one would expect if the end of progression through the cell cycle to cell plate formation were characterized by a minimal magnesium concentration. On the other hand, a 5-min pulse treatment of A23187 applied very late in the cell cycle of *S. pombe* (just before fission) results in an acceleration of cell division (Fig. 19b). Although it is not unknown for *S. pombe* cells to be accelerated into division (Smith & Mitchison, 1976; Kramheft, unpublished), it is unusual that so short a pulse treatment should produce such a dramatic response. In our opinion, what happens in this experiment is that the ionophore, applied at a time when the intracellular magnesium concentration is high, accelerates the normal subsequent fall in magnesium concentration and this prematurely generates the conditions necessary for the next division. In other words, the normal relationship between magnesium concentration and cell growth has been upset in such a way as to shorten the cell cycle. This effect is reminiscent of the retiming of the cell cycle observed in the synchronization of cell division using chelating agents (Ahluwalia *et al.* 1978; Walker & Duffus, 1979).

**CONCLUSION**

The results reported in this paper and discussed above all support the previously suggested hypothesis that magnesium concentration is the transducer for size, and consequently time, related control of the cell cycle. The fundamental proposition is that a fall in magnesium concentration, accompanying an increase in cell size, eventually reaches a level which permits tubulin polymerization and spindle formation. Once chromosome separation is complete, a rapid influx of magnesium causes breakdown of the spindle, and nuclear and cell division ensue. The results quoted here show a much greater magnesium influx than had previously been envisaged, and elucidation of the uptake mechanism involved must be a high priority for future research. Further work is also needed on the tubulins known to occur in yeast (Water & Kleinsmith, 1976; Baum, Thorner & Honig, 1978), since the hypothesis depends at present on the assumption that yeast tubulin has similar properties to tubulin from sea urchins and mammals. Finally, there is the postulated correlation of tubulin with the 'division proteins' suggested by Zeuthen and his coworkers (Zeuthen & Ramussen, 1972). If such a correlation can be demonstrated, it should be possible to integrate our hypothesis into the currently accepted cell cycle control models without too much difficulty.

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