

NUCLEOSIDE DIPHOSPHOKINASE, AN ENZYME
WITH STEP CHANGES IN ACTIVITY DURING THE
CELL CYCLE OF THE FISSION YEAST
SCHIZOSACCHAROMYCES POMBE

I. PERSISTENCE OF STEPS AFTER A BLOCK TO THE
DNA-DIVISION CYCLE

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SUMMARY

In confirmation of earlier results, nucleoside diphosphokinase is shown to be a 'step' enzyme in *Schizosaccharomyces pombe* with a sharp doubling in activity at the beginning of the cell cycle. These doubling steps occur at the same time in the cycle in the smaller cells of the mutant *wee1.6*. An important result is that the activity steps persist with normal cell cycle timing after a block to the DNA-division cycle imposed by the cell cycle mutants *cdc2.33* and *cdc2.33 wee1.6*. This is clear proof that oscillatory controls of some cell cycle events can persist after the main periodic events of the DNA-division cycle have been abolished.

INTRODUCTION

Enzymes that show stepwise increases in activity during the cell cycle were once believed to be relatively common (Mitchison, 1971). Although such patterns of activity undoubtedly exist, especially in enzymes concerned with DNA synthesis and mitosis, many of the earlier results may have been flawed by technical artefacts (Mitchison, 1977; Creanor *et al.* 1983).

Dickinson (1983) showed that nucleoside diphosphokinase (NDPK) has step increases in activity during the cell cycle of *Schizosaccharomyces pombe* and is the first genuine 'step' enzyme to be discovered in this yeast. He also showed that activity in asynchronous cultures increases, as does protein content per cell, after a block to the DNA-division cycle imposed by a *cdc* mutant.

We have confirmed most of Dickinson's results, though our estimates of the timings of steps is somewhat different. More important, we have extended his results by examining the NDPK activity in blocked synchronous cultures. This leads to the surprising conclusion that the step pattern persists for at least three cycles after a

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block. An oscillatory control must therefore continue to function after the main periodic events of the DNA-division cycle have been eliminated.

MATERIALS AND METHODS

Strains

The wild-type (WT) strain 972h⁻ was originally obtained from Professor U. Leupold, Bern. The mutants *cdc2.33* and *cdc10.129* are temperature-sensitive conditionals that form colonies at 25°C but not at 35°C (Nurse *et al.* 1976). The *cdc2*⁺ function is required both for mitosis and for the initiation of DNA synthesis (Nurse & Bisset, 1981) but in normal growing cultures only the mitotic block is apparent in *cdc2.33* since nearly all the cells are in S and G₂ and they arrest in G₂ after a shift-up in temperature. The *cdc10*⁺ function is required for the initiation of DNA synthesis. The mutant *wee1.6* divides at about half the size of the WT strain (Nurse *et al.* 1976). The double mutant *cdc2.33 wee1.6* was constructed by the appropriate crosses and tetrad analysis (Benitez *et al.* 1980).

Synchronous cultures and medium

Cultures were grown up overnight in a shaking water bath to a cell density of 4×10⁶ to 6×10⁶ cells ml⁻¹. Synchronous cultures were prepared from these overnight cultures by selecting the smallest cells in an elutriating rotor (Creanor & Mitchison, 1979). Control asynchronous cultures were prepared by pumping all the cells out of the rotor and diluting appropriately. A minimal medium EMM3 (Creanor & Mitchison, 1982) was used throughout.

Enzyme assays

We have had considerable difficulty in finding reliable assays for NDPK activity, most of which probably stems from having to use permeabilized cells rather than cell extracts, since it is impossible to get consistent cell breakage from the small samples that are available from synchronous cultures. We list below the assay methods used. The first method produces variability, primarily because of the difficulty of getting complete localization of the labelled ATP on the PEI (polyethyleneimine) plates. The second method is not consistently reliable, for unknown reasons. The third method, which we have only adopted recently, appears to be the best.

Assay 1. Radioactive assay measuring the transfer of labelled phosphate from ATP to TDP (after Pederson & Catterall, 1979). Samples of 1×10⁵ to 5×10⁵ cells were mixed with ≈2×10⁶ boiled cells as a carrier, centrifuged down and frozen-dried. These samples were further permeabilized by suspension in 15 μl Hepes buffer (0.1 M at pH 7.5) containing 2.5% isoamyl alcohol and kept for 15 min on ice. To each sample was then added 30 μl of the Hepes buffer containing 10 mM-ATP, 10 mM-TDP and 0.75–1.5 MBq ml⁻¹ adenosine 5'-[γ-³²P]triphosphate (normally 150 TBq mmol⁻¹). This reaction mixture was incubated for 15–30 min at 25°C and then spotted on PEI thin-layer chromatography plates (Randerath & Randerath, 1966). The plates were run with MeOH followed by 1.0–1.3 M-LiCl saturated with boric acid at pH 7.0 (Neuhard *et al.* 1965). The labelled TTP spots generated by the enzyme reaction were visualized under ultraviolet light, cut out and counted in a scintillation counter using Čerenkov radiation.

Assay 2. Spectrophotometric assay measuring the transfer of phosphate from TTP to dADP (after Mourad & Parks, 1966). Samples of 1×10⁶ to 2×10⁶ cells were prepared as for assay 1 but without boiled cells. To each sample was added 15 μl Tris buffer (0.1 M at pH 7.4) containing 5 mM-TTP, 2.5 mM-dADP, 2.5% isoamyl alcohol, 25 mM-MgCl₂ and 10 mM-KCl. The samples were incubated for 10–20 min at 20–25°C and the reaction was stopped by raising the temperature to 90–100°C for 2 min. The dATP generated was then measured by adding to each sample 100 μl of the Tris buffer containing 50 mM-glucose, 5 mM-NADP, with 8 units hexokinase and 1 unit glucose-6-dehydrogenase. The samples were incubated again at 35°C for 20 min and the reaction was stopped by raising the temperature to 70°C for 2 min. After centrifuging, the supernatant was measured at 340 nm.

Assay 3. Radioactive assay measuring the transfer of phosphate from ATP to labelled UDP (after Ingraham & Ginther, 1978). Samples of 0.5×10⁵ to 3×10⁵ cells were prepared as for

assay 1. To each sample was added 20 μ l Hepes buffer (0.1 M at pH 7.5) containing 25 mM-ATP, 10 mM-UDP, 0.9–1.9 MBq ml⁻¹ [³H]uridine diphosphate (370–925 GBq mmol⁻¹), 2.5% iso-amyl alcohol, 10 mM-MgCl₂ and 2 mM-mercaptoethanol. Samples were incubated for 10–12 min at 12°C and then spotted onto PEI plates as in assay 1. The plates were run with MeOH for 2 cm, then with 2 M-sodium formate (pH 3.4) for 3.5 cm and then in 4 M-sodium formate (pH 3.4) for a further 6.5 cm. The labelled UTP spots were visualized under ultraviolet light, cut out and extracted with 1 ml 4% (v/v) NH₄OH, neutralized with acetic acid and counted in a liquid scintillation counter.

Radioisotopes were obtained from Amersham International and other chemicals from Sigma Chemicals. In the figure legends, 1 enzyme unit (e.u.) = 1 fmol PO₄ transferred min⁻¹ cell⁻¹.

Cell collection and cell counting

Cell samples were collected by centrifugation rather than filtration. Cell numbers were determined with a Coulter Counter (Industrial model D) with a 100 μ m aperture.

RESULTS

Enzyme specificity

NDPK (ATP:nucleoside diphosphate phosphotransferase, EC 2.7.4.6), the last enzyme on the pathway to nucleoside triphosphate, is relatively non-specific and will catalyse transphosphorylation of a variety of ribonucleotides and deoxyribonucleotides in both higher eukaryotic cells and budding yeast (Parks & Agarwal, 1973; Ratcliff *et al.* 1964). In accord with this, Table 1 shows that the NDPK assay with TDP as substrate is inhibited in *S. pombe* by other nucleoside diphosphates.

Effect of cycloheximide

Dickinson (1983) found that NDPK activity in a growing asynchronous culture continued to rise after the addition of cycloheximide and suggested that this rise, at any rate transiently, was due to activation rather than *de novo* synthesis of the enzyme protein. We have repeated this experiment but have been unable to confirm it. Fig. 1 shows that cycloheximide stops the rise in activity. Thereafter, the activity only drops slightly over a period of 3 h, indicating that the enzyme is stable under these conditions.

Enzyme activity in synchronous cultures of WT and weel.6

NDPK activity follows a step pattern in synchronous cultures of WT cells (Fig. 2). The absence of these steps in a control asynchronous culture that has been through the elutriating rotor shows that the steps are genuine cell cycle effects and not an artefact of the method of making the synchronous cultures. The doubling in activity takes only a little more time than the doubling in cell numbers, so the step must be sharp at the level of the single cell.

The results from 12 similar experiments are shown in the cell cycle map in Fig. 3. There is no significant difference between the timing of the steps in the first and in the second cycle, and the mean timing is at 0.11 of the cycle, near to the *S/G*₂ boundary (Mitchison & Creanor, 1971).

Seven experiments were also done with the mutant *wee1.6* in which the cell size at division is about half that of WT and in which the *S* period is delayed by about 0.38

of the cycle (Dickinson, 1983; Nasmyth *et al.* 1979). There were the same steps in synchronous cultures, with the same timing as in WT (Fig. 3). Altering the position of the S period does not therefore alter the timing of the NDPK steps.

These results confirm the existence of the steps first shown by Dickinson (1983) in both WT and *wee1.6*. Our timings are somewhat different, but since they come from a larger number of experiments, we believe them to be more reliable.

Enzyme activity in blocked synchronous cultures

The procedure here was to make a synchronous culture by the same technique from an initial culture of a temperature-sensitive *cdc* mutant grown at the permissive temperature and then transfer it immediately after selection in the rotor to the higher restrictive temperature. Most of the experiments were done with the mutant *cdc2.33* in which mitosis and DNA synthesis are blocked at the restrictive temperature but growth continues in cell size and protein content. Since there were no stepwise increases in cell numbers, this was not a normal synchronous culture but we refer to it as 'synchronous' since the cells at the time of the shift-up in temperature were all at

Table 1. *Inhibition of NDPK assay by ribonucleoside and deoxyribonucleoside diphosphates*

	%		%
ADP	17	dADP	44
ADP (0.5 M)	0	dGDP	32
GDP	28	dUDP	81
UDP	60	dCDP	57

Assay 1 with 0.1 M-TDP as substrate. Other nucleotides added at 0.1 M (except where stated) and degree of inhibition shown as activity as a percentage of the control without any additions.

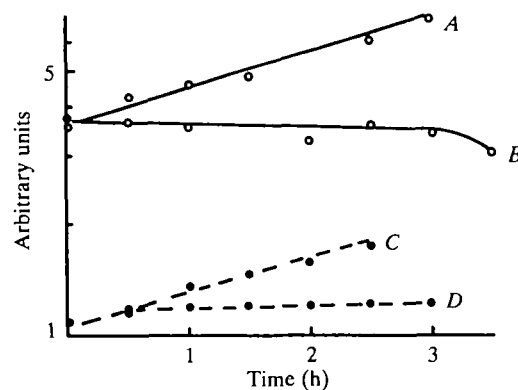


Fig. 1. Effect of cycloheximide on NDPK activity and cell number in an asynchronous culture of WT cells at 35°C. Cycloheximide ($100 \mu\text{g ml}^{-1}$) was added at time zero to a portion of the culture and the rest kept as a control. Curve A, NDPK activity in control; assay 1, 1 a.u. (arbitrary unit) = 0.38 e.u. (enzyme unit). Curve B, NDPK activity after addition of cycloheximide; 1 a.u. = 0.38 e.u. Curve C, cell numbers in control. Curve D, cell numbers after addition of cycloheximide; for both C and D, 1 a.u. = 3.1×10^6 cells ml^{-1} .

about the same point in the cell cycle. Cell numbers were also followed in a portion of the selected cells kept at the permissive temperature. The resulting step in numbers was a control for the degree of selection achieved in the rotor.

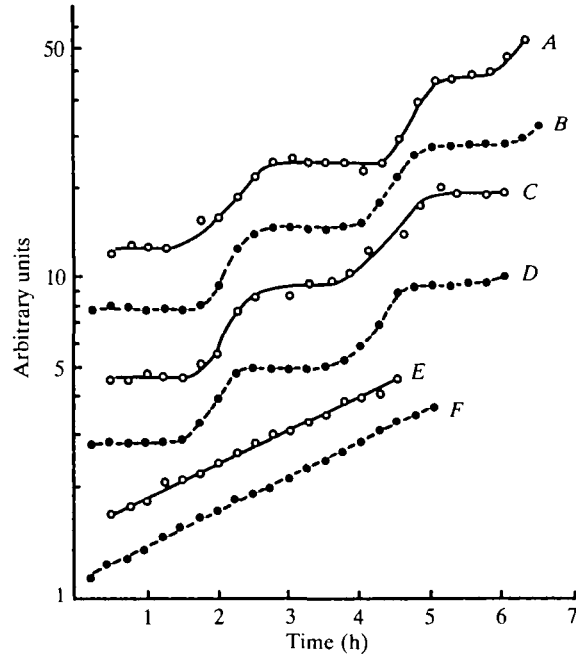


Fig. 2. NDPK activity in synchronous cultures of WT cells at 35°C. Curve A, NDPK activity in synchronous culture; assay 2, 10 a.u. (arbitrary unit) = 3.4 e.u. (enzyme unit). Curve B, cell numbers in culture above; 10 a.u. = 1.3×10^6 cells ml⁻¹. Curve C, NDPK activity in synchronous culture; assay 3, 1 a.u. = 1.0 e.u. Curve D, cell numbers in culture above; 1 a.u. = 0.48×10^6 cells ml⁻¹. Curve E, NDPK activity in control asynchronous culture; assay 1, 1 a.u. = 0.42 e.u. Curve F, cell numbers in culture above; 1 a.u. = 0.96×10^6 cells ml⁻¹.

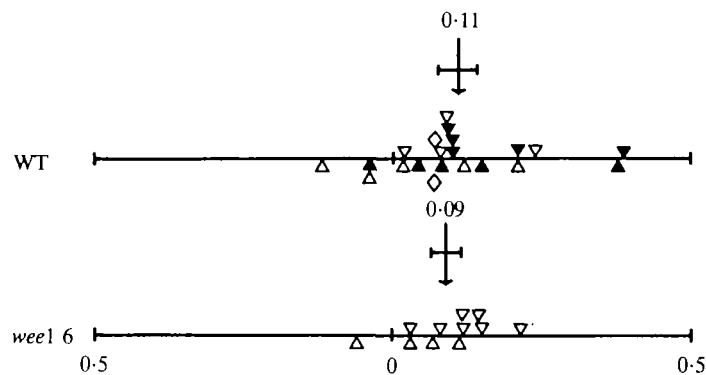


Fig. 3. Cell cycle maps of the activity steps of NDPK in WT and in *wee1.6*. Each triangle (and diamond) marks the mid-point (half-rise point) of a step in one experiment. Those above the line are in the first cycle and those below the line in the second cycle. The mean step position and its standard error are marked by an arrow and cross-bar. Values are in fractions of the cycle from 0 to 1. (▲) assay 1; (△) assay 2; (◇) assay 3.

Fig. 4 shows the NDPK activity in one asynchronous and two synchronous blocked cultures of *cdc2.33*. It is clear that the activity steps that occurred in normal synchronous cultures also persisted in these blocked cultures for up to three cycles (8 h) after the shift-up. There was a difference in the timing of the steps that was not due to differences in the degree of selection in the rotor, since the steps in cell number in the control cultures kept at the permissive temperature (25°C) were identical. This illustrates the fact that there was about three times more variability in the step timing in these blocked cultures (standard error of first step = 10.0 min) than in WT synchronous cultures (standard error of first step = 3.7).

The results from 10 similar experiments are shown in the cell cycle maps in Fig. 5; though, since there were no normal cell cycles, the steps are plotted with respect to time after shift-up rather than to stage in the cycle as in Fig. 3. The mean time to the first step (138 min), and between the first and second step (138 min), is close to the doubling time for protein/cell (143 min) in this mutant under these conditions (Novak & Mitchison, 1986).

We have also done three similar experiments with the double mutant *cdc2.33 wee1.6*. The results were substantially similar to those with the single mutant *cdc2.33* and the step timings showed no significant difference (Fig. 5).

DISCUSSION

It is not surprising to find a cell cycle periodicity in NDPK activity since all the components of growth in *S. pombe* that have been measured over the last 30 years show such periodicities. These occur not only in extensive properties like total protein synthesis but also in the activities and the potential of individual enzymes (summarized by Novak & Mitchison, 1986). But these are all subtle changes in rate or even the rate of the rate, as in CO₂ production. What is unusual is to find stepwise doublings of enzyme activity. There is no other similar case in *S. pombe* and few, if any, authentic examples in other yeasts, such as *Saccharomyces cerevisiae*. It will obviously be of interest to try and discover whether these activity steps are paralleled by steps in enzyme protein and in gene transcript levels.

The most striking aspect of these results is the persistence of the periodic cell cycle pattern after the main periodic events of the DNA-division cycle have been blocked by the use of *cdc* mutants. Such persistence is now well known in eggs and early embryos (surface contraction: Yoneda *et al.* 1978, 1982; Hara *et al.* 1980: and maturation promoting factor (MPF); Gerhart *et al.* 1984) but there are only a few accounts of it in growing systems (summarized by Novak & Mitchison, 1986). Most of the current thinking about cell cycle controls, especially in yeast, assumes a dependence on the periodic events of the DNA-division cycle. Here, however, is an oscillatory control that may well be entrained by the events of the DNA-division cycle, but which can also function independently of them. Similar independence is shown by the controls of CO₂ production (Novak & Mitchison, 1986), of enzyme potential (Benitez *et al.* 1980) and perhaps of cell-length growth (Mitchison & Nurse, 1985). It is not, however, shown in the case of the rate of total protein

synthesis (Creanor & Mitchison, 1984) or of ribosomal RNA synthesis (Elliott, 1983).

It is as well to finish by pointing out some interesting but puzzling features of the present position. The steps in NDPK activity do not appear to be related to the *S* period since their timing remains unchanged in the *wee* mutant where the *S* period is substantially delayed. Given the likely importance of the enzyme in RNA synthesis and the fact that the DNA/RNA ratio in *S. pombe* is about 1/100, it would not be

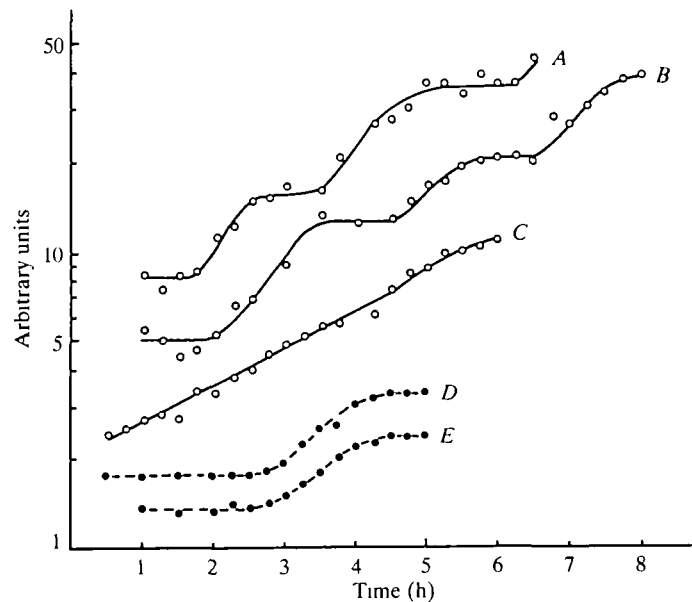


Fig. 4. NDPK activity in synchronous cultures of *cdc2.33* shifted up at time zero from 25°C to 36.5°C. Curve A, NDPK activity in synchronous culture; assay 2, 10 a.u. (arbitrary unit) = 14.1 e.u. (enzyme unit). Curve B, NDPK activity in synchronous culture; assay 2, 10 a.u. = 8.5 e.u. Curve C, NDPK activity in control asynchronous culture; assay 3, 1 a.u. = 2.5 e.u. Curve D, cell numbers in portion of culture for A but kept at 25°C; 1 a.u. = 0.65×10^6 cells ml⁻¹. Curve E, cell numbers in portion of culture for B but kept at 25°C; 1 a.u. = 0.73×10^6 cells ml⁻¹. Cell numbers in the cultures at 36.5°C remained constant after the first 40 min in C and from time zero in A and B (not shown).

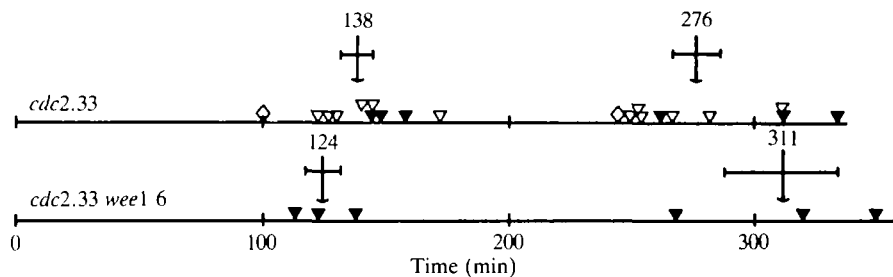


Fig. 5. Cell cycle map of the NDPK activity steps in blocked synchronous cultures of *cdc2.33* and *cdc2.33 wee1.6* at 36°C. Symbols as in Fig. 3 but the scale is in time (min) rather than in fractions of the cycle. Means are shown in minutes as well as by arrows.

unreasonable to associate the activity doublings with the sharp increase in the rate of ribosomal RNA synthesis that occurs a little earlier at 0.75 of the cycle (Elliott, 1983). However, these periodicities in RNA rate vanish after a DNA-division block (Elliott, 1983) while the NDPK activity steps persist. Perhaps the activity steps, which may have a metabolic function in the normal cycle, continue when their function is superfluous. A comparison with the steps in the rate of CO₂ production (Novak & Mitchison, 1986) brings out two points. The first is that the rate of increase in the rate of CO₂ production in blocked cultures (in common with other rates in *S. pombe*) reaches a constant value in due course. This limitation does not seem to apply to the NDPK activity within the time scale of the experiments. It is therefore possible to observe NDPK steps for longer than rate changes. The second is that the NDPK steps continue with a timing that is about the same as the normal cell cycle, whereas the rate changes in CO₂ production and in enzyme potential have timings that are appreciably shorter than the normal cell cycle. The significance of these differences may become apparent when we know more about these persistent oscillatory controls.

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REFERENCES

- BENITEZ, T., NURSE, P. & MITCHISON, J. M. (1980). Arginase and sucrase potential in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **46**, 399–431.
- CREANOR, J., ELLIOTT, S. G., BISSET, Y. C. & MITCHISON, J. M. (1983). Absence of step changes in activity of certain enzymes during the cell cycle of budding and fission yeasts in synchronous cultures. *J. Cell Sci.* **61**, 339–349.
- CREANOR, J. & MITCHISON, J. M. (1979). Reduction of perturbations in leucine incorporation in synchronous cultures of *Schizosaccharomyces pombe*. *J. gen. Microbiol.* **112**, 385–388.
- CREANOR, J. & MITCHISON, J. M. (1982). Patterns of protein synthesis during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **58**, 263–285.
- CREANOR, J. & MITCHISON, J. M. (1984). Protein synthesis and its relation to the DNA-division cycle in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **69**, 199–210.
- DICKINSON, J. R. (1983). Nucleoside diphosphokinase and cell cycle control in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **60**, 355–365.
- ELLIOTT, S. G. (1983). Coordination of growth with cell division: regulation of synthesis of RNA during the cell cycle of the fission yeast *Schizosaccharomyces pombe*. *Molec. gen. Genet.* **192**, 204–211.
- GERHART, J., WU, M. & KIRSCHNER, M. (1984). Cell cycle dynamics of an M-phase-specific factor in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* **98**, 1247–1255.
- HARA, K., TYDEMAN, P. & KIRSCHNER, M. (1980). A cytoplasmic clock with the same period as the division cycle in *Xenopus* eggs. *Proc. natn. Acad. Sci. U.S.A.* **77**, 462–466.
- INGRAHAM, J. L. & GINTHER, C. L. (1978). Nucleoside diphosphokinase from *Salmonella*. In *Methods in Enzymology*, vol. 51 (ed. P. A. Hoffee & M. E. Jones), pp. 371–386. New York, London: Academic Press.
- MITCHISON, J. M. (1971). *The Biology of the Cell Cycle*. Cambridge University Press.
- MITCHISON, J. M. (1977). Enzyme synthesis during the cell cycle. In *Cell Differentiation in Microorganisms, Plants and Animals* (ed. L. Nover & K. Mothes), pp. 377–401. Jena: VEB Gustav Fischer Verlag.

- MITCHISON, J. M. & CREANOR, J. (1971). Further measurements of DNA synthesis and enzyme potential during the cell cycle of fission yeast *Schizosaccharomyces pombe*. *Expl Cell Res.* **69**, 244–247.
- MITCHISON, J. M. & NURSE, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **75**, 357–376.
- MOURAD, N. & PARKS, R. E. (1966). Erythrocytic nucleoside diphosphokinase isolation and kinetics. *J. biol. Chem.* **241**, 271–278.
- NASMYTH, K., NURSE, P. & FRASER, R. S. S. (1979). The effect of cell mass on the cell cycle timing and duration of S-phase in fission yeast. *J. Cell Sci.* **39**, 215–233.
- NEUHARD, J., RANDEATH, E. & RANDEATH, K. (1965). Ion-exchange thin-layer chromatography. XIII. Resolution of complex nucleoside triphosphate mixtures. *Analyt. Biochem.* **13**, 211–222.
- NOVAK, B. & MITCHISON, J. M. (1986). Change in the rate of CO₂ production in synchronous cultures of the fission yeast *Schizosaccharomyces pombe*: a periodic cell cycle event which persists after the DNA-division cycle has been blocked. *J. Cell Sci.* **86**, 191–206.
- NURSE, P. & BISSET, Y. (1981). Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature, Lond.* **292**, 558–560.
- NURSE, P., THURIAUX, P. & NASMYTH, K. (1976). Genetic control of the cell cycle in the fission yeast *Schizosaccharomyces pombe*. *Molec. gen. Genet.* **146**, 167–178.
- PARKS, R. E. & AGARWAL, R. P. (1973). Nucleoside diphosphokinases. In *The Enzymes*, vol. 8 (ed. P. Boyer), pp. 307–333. New York, London: Academic Press.
- PEDERSON, P. L. & CATTERALL, W. A. (1979). The use of thin-layer chromatography on poly(ethyleneimine) cellulose to facilitate assays of ATP–ADP exchange, ATP–P_i exchange, adenylate kinase and nucleoside diphosphokinase activity. In *Methods in Enzymology*, vol. 55 (ed. S. Fleischer & L. Packer), pp. 283–289. New York, London: Academic Press.
- RANDEATH, K. & RANDEATH, E. (1966). Ion-exchange thin-layer chromatography. XV. Preparation, properties and applications of paper-like PEI-cellulose sheets. *J. Chromat.* **22**, 110–117.
- RATLIFF, R. L., WEAVER, R. H., LARDY, H. A. & KUBY, S. A. (1964). Nucleoside triphosphate–nucleoside diphosphate transphosphorylase (nucleoside diphosphokinase). I. Isolation of the crystalline enzyme from brewers' yeast. *J. biol. Chem.* **239**, 301–309.
- YONEDA, M., IKEDA, M. & WASHITANI, S. (1978). Periodic change in the tension at the surface of activated non-nucleate fragments of sea-urchin eggs. *Dev. Growth Differ.* **20**, 329–336.
- YONEDA, M., KOBAYAKAWA, Y., KUBOTA, H. Y. & SAKAI, M. (1982). Surface contraction waves in amphibian eggs. *J. Cell Sci.* **54**, 35–46.

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