The Pool Sizes of Adenine Nucleotides in Exponentially Growing, Stationary Phase and 2'-Deoxyadenosine-synchronized Cultures of *Schizosaccharomyces pombe* 972 h-

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(Received 18 November 1977)

The intracellular concentrations of adenylates and the value of the adenylate energy charge in *Schizosaccharomyces pombe* have been determined. Dilution of extracts before enzymically converting AMP and ADP to ATP was necessary for the quantitative measurement of all three adenylates. Using trichloroacetic acid or perchloric acid extracts of exponentially growing cultures, the energy charge was calculated to be 0.8 to 0.9; the value rose to about 0.95 in the stationary phase of growth. Lower energy charge values (0.6 to 0.64) were obtained using chloroform extracts. During the last 2.5 h of a 4 h treatment of an exponentially growing culture with 2'-deoxyadenosine, and during the induced synchronous growth that followed subsequent removal of the inhibitor, the pool sizes of all three adenylates oscillated. Minimal ATP/ADP ratios (and energy charge values) occurred concurrently with maximal rates of respiration that were relatively insensitive to stimulation by carbonyl cyanide *m*-chlorophenylhydrazine. Cultures not treated with 2'-deoxyadenosine but centrifuged and resuspended in fresh medium, showed a transient extensive increase in the ATP/ADP ratio 1 to 2 h after resuspension. However, rates of O₂ uptake, in the absence or presence of carbonyl cyanide *m*-chlorophenylhydrazine, increased smoothly throughout this period. The results suggest that, in 2'-deoxyadenosine-induced synchronous growth, respiration rates may be controlled by the intracellular ATP/ADP ratio, and demonstrate that division synchrony is not induced by depletion of ATP pools.

INTRODUCTION

Respiratory oscillations occur in synchronous cultures of the fission yeast *Schizosaccharomyces pombe* prepared either by size selection of cells from a sucrose gradient (Poole, Lloyd & Kemp, 1973) or by an induction method that involves treating an exponentially growing culture for 4 h with 2 mM-2'-deoxyadenosine (dAdo) (Poole & Pickett, 1976; Poole, 1977*b*). However, the response of the oscillating rates of O₂ uptake to the uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) reveals that the mechanisms underlying the oscillations in these two types of synchronous culture are different. In dAdo-synchronized cultures, O₂ uptake is more susceptible to stimulation by CCCP at those times during growth when respiration rates are minimal; it has been suggested that during these periods, respiration is ADP-limited (State 4) and that CCCP relieves respiratory control (Poole, 1977*b*). One test of this hypothesis would be the measurement of intracellular adenine nucleotide pools during synchronous growth. The determination of adenine nucleotide pools would also be of interest in the light of the suggestion (Penman & Duffus, 1975) that, in the yeast *Kluyveromyces fragilis*, dAdo may act as a synchronizing agent by reducing intracellular ATP levels to below those required for the energy-demanding process of mitosis.

The intracellular pools of AMP, ADP and ATP and the derived value of adenylate energy charge have been determined in a wide range of prokaryotic and eukaryotic organisms (for recent reviews, see Knowles, 1977; Chapman & Atkinson, 1977). In synchronous cultures of *Crithidia fasciculata*, complex oscillations in the levels of all three adenylates and in the adenylate energy charge were correlated with changes in rates of O_2 uptake (Edwards, Statham & Lloyd, 1975). In synchronous cultures of *Escherichia coli*, the pool sizes of ATP and dATP were maximal later in the cycle, decreasing near the onset of cell division; AMP and ADP were not assayed (Huzyk & Clark, 1971).

In this paper, we describe investigations of the validity of methods used for the quantitative extraction and assay of ATP, ADP and AMP, and the application of three extraction procedures to cells in the exponential and stationary phases of growth. We describe the fluctuations in pool sizes of the three adenylates both during the period of treatment of *S. pombe* with dAdo and during the subsequent synchronous growth. The varying ATP/ADP ratio observed is consistent with the hypothesis that respiration rates are modulated by acceptor (respiratory) control in these cultures.

METHODS

Organism, growth and synchronization. *Schizosaccharomyces pombe* strain 972 h⁻ was grown and maintained in glucose-containing defined medium as described previously (Poole, 1977b). Cultures were grown in 200 ml medium, with vigorous magnetic-stirring, in a 250 ml Erlenmeyer flask fitted with a short side-arm that was closed by a no. 41 Suba-seal (Astell Laboratory Service Co., Catford, London). Synchronous growth and division was induced by 4 h treatment with dAdo (Mitchison & Creanor, 1971; Poole, 1977b).

Cell numbers and volumes. These were measured using a Coulter counter model Z_{BI} as described by Poole (1977a) except that the probe had an aperture diameter of 50 μ m.

Cell plate index. This was determined on formaldehyde-fixed cells using a Nikon phase contrast microscope (total magnification 1000 to 1500). Between 200 and 500 randomly selected cells were scored in each sample.

Estimation of yeast dry weight. Duplicate samples of culture (50 ml) were filtered through preweighed, dried (18 h at 105 °C) membrane filters (0.2 μ m pore size). Filters were reweighed after 24 h drying at 105 °C and cooling in a desiccator.

Measurements of oxygen uptake. The technique using conventional polarographic apparatus was described by Poole (1977b).

Glucose assays. These were done with Sigma reagent kit 510 on culture supernatants obtained by centrifugation.

Sampling and preparation of extracts. Three methods were used. (i) Extraction with chloroform (Dhople & Hanks, 1973). Culture (1 ml) was removed via the sampling port using a 1 ml sterile disposable syringe and needle, and squirted into a boiling tube containing 300 μ l chloroform (20 °C). The sample was placed in a boiling water bath for 2 min and then vigorously mixed for 30 s.

(ii) Extraction with trichloroacetic acid (Bagnara & Finch, 1972; Lundin & Thore, 1975). Cold (0 to 4 °C), freshly prepared 0.56 M-trichloroacetic acid (500 μ l) was loaded into a 2 ml glass/stainless steel syringe held in a specially constructed spring-loaded device similar to that described by Niven, Collins & Knowles (1977) and designed to minimize sampling and mixing times. A sterile needle was attached and 0.76 ml of culture was withdrawn via the Suba-seal stopper into the trichloroacetic acid under the action of the spring-loaded plunger of the syringe. The needle and spring were removed and the sample was ejected into a small tube on ice. After 15 min, trichloroacetic acid was removed by three extractions (15 s each, while mixing vigorously) each with 2 ml of water-saturated diethyl ether. The first phase separation was facilitated by centrifugation for 5 min in a Microangle centrifuge, which also removed cell debris. After the last extraction, water-saturated air was bubbled through the sample (aqueous phase) for 3 to 5 min at room temperature to remove dissolved ether.

(iii) Extraction with perchloric acid (Niven *et al.*, 1977; Bagnara & Finch, 1972). Cold 0.25 M-HClO₄ (200 μ l) was loaded into the spring-loaded syringe and 0.76 ml of culture was withdrawn as described above. The sample was ejected into a small tube on ice and after 15 min the perchlorate was removed by a modification of the two-step method of Bagnara & Finch (1972). Most of the perchlorate was neutralized by adding 5 M-KOH (150 μ l); addition of 2.5 M-KHCO₃ (150 μ l) neutralized the remainder and left the mixture buffered at about pH 7.5. This method has the advantages that the volumes of KOH and KHCO₃ are not critical and that the use of a pH electrode to monitor the single-step neutralization is obviated. The extract was then centrifuged (3000 g; 15 min; 4 °C) and the clear supernatant was retained.

The extracts from all three procedures were immediately frozen, maintained at -20°C and assayed within 48 h. On thawing, extracts were diluted with 4 or 9 vol. of cold buffer that contained 20 mM-Tris/ H_2SO_4 and 2 mM-EDTA, pH 7.75.

Preparation of samples for the luciferase assay. For ATP determinations, diluted extract (200 μl) was added to 50 μl 100 mM-Tris/ H_2SO_4 , pH 7.75, containing 25 mM- MgSO_4 and 62.5 mM- K_2SO_4 (Lundin & Thore, 1975). In addition, the Tris buffer contained 0.5 mM-phosphoenolpyruvate and 2 μg pyruvate kinase (EC 2.7.1.40) for the assay of ATP plus ADP, and 0.5 mM-phosphoenolpyruvate, 2 μg pyruvate kinase and 10 to 25 μg adenylate kinase (EC 2.7.4.3), for the assay of total adenylates. These mixtures were incubated at 30°C for 30 min and then held on ice until assayed (< 30 min). Each batch of adenylate kinase was tested to determine the optimal amount required for quantitative conversion of adenylates to ATP.

Preparation of firefly lantern extract. Commercial firefly lantern extract was reconstituted with cold water, gently mixed by inversion, and centrifuged at 27000 g for 15 min (4°C). The supernatant was diluted with up to 1 vol. 50 mM- Na_2HAsO_4 /20 mM- MgSO_4 , brought to pH 7.4 with H_2SO_4 and stored in the dark at 5°C . The preparation could be kept for several days without appreciable loss of activity, but was usually centrifuged once a day to remove precipitate. During the assays, the preparation was kept at room temperature.

Luciferase assays. Incubated samples (100 μl , or 50 μl mixed with 50 μl 20 mM-Tris/ H_2SO_4 , pH 7.75, containing 5 mM- MgSO_4 and 12.5 mM- K_2SO_4) were added to 9×55 mm fluorimeter tubes, followed by 200 μl 3 mM- MgSO_4 in 100 mM-glycylglycine brought to pH 7.75 with KOH. Light emission is enhanced at a pH approaching 8 relative to that obtained at the more widely used pH 7.4 (Strehler, 1968; Kimmich, Randles & Brand, 1975). The sample tube was placed in a fluorimeter (The Locarte Group, North End Road, London W14), modified to allow injection of 100 μl firefly lantern extract through a Suba-seal without removing the cap of the sample compartment. The maximum light intensity emitted was recorded on a potentiometric recorder with a response time for 100 % full scale deflection of 0.25 s.

The apparatus was routinely calibrated with stock solutions of ATP (dissolved in 20 mM-Tris/ H_2SO_4 plus 2 mM-EDTA, pH 7.75). ADP and AMP were determined by difference (Pradet, 1967). Nucleotide concentrations were corrected for dilutions, standards, blanks and inhibition by the extracts (see Results). The method of internal standardization was used (Strehler, 1968).

Chemicals. ATP (Sigma grade, disodium salt), ADP (grade III, sodium salt), phosphoenolpyruvate (tricyclohexylamine salt), glycylglycine (free base), firefly lantern extract (FLE-50 or FLE-250), adenylate kinase (myokinase, grade III), pyruvate kinase (type II), CCCP and dAdo were obtained from Sigma. AMP (disodium salt) was from BDH.

RESULTS

Evaluation of the methods for extraction and assay of adenine nucleotides

Solutions containing a mixture of 10 μM -ATP, 10 μM -ADP and 10 μM -AMP in 20 mM-Tris/ H_2SO_4 plus 2 mM-EDTA, pH 7.75, were subjected to the trichloroacetic or perchloric acid extraction procedures; the resulting extracts were assayed (undiluted) for each of the adenylates. In trichloroacetic acid extracts, the recoveries of ATP, ADP and AMP were, respectively, 100 to 110 %, 59 % and 6.5 %. Using perchloric acid, the corresponding recoveries were 44 to 51 %, 32 to 49 % and 3 to 5 %. These low and variable recoveries were shown in the following experiments to be due to inhibition by the 'extracts' of the enzymic conversion of ADP and AMP to ATP. To three portions of Tris/EDTA buffer (containing no adenylates) were added, respectively, chloroform, trichloroacetic acid or perchloric acid; these samples were subsequently treated as described in Methods. The effect of including increasing amounts of these 'extracts' on the assay of adenylates in a solution that contained 10 μM -ATP, 10 μM -ADP and 10 μM -AMP was investigated (Table 1). Up to 100 μl of the chloroform-treated buffer in a total enzyme incubation volume of 250 μl was without significant effect on the recovery of the adenylates. However, the inclusion of buffer treated with either trichloroacetic acid or perchloric acid resulted in a marked decrease in the assayed amounts of the adenylates, particularly AMP and to a lesser extent ADP (results not shown). To overcome these inhibitions, all extracts described subsequently were diluted 1:5 (chloroform and trichloroacetic acid preparations) or 1:10 (perchloric acid preparations) with Tris/EDTA buffer as described in Methods. After correction for this dilution factor and the small degree of inhibition ($< 10\%$) caused by the remaining extract (equivalent to 20 or 40 μl undiluted preparation), 85 to 100 % recoveries of all three adenylates were obtained when the three extraction methods were applied to solutions containing known

Table 1. *Inhibition of the assay of adenylates in a standard solution by buffer subjected to three extraction methods*

Three portions of Tris/EDTA buffer were subjected to the treatments (chloroform, trichloroacetic acid or perchloric acid) used for extraction of adenylates from cells. Various volumes of these treated buffers were incubated with a mixture of 10 μM -ATP, 10 μM -ADP, 10 μM -AMP and the reagents required for the conversion of all adenylates to ATP. Total adenylates (as ATP) were then assayed by the luciferase method. Detailed analytical procedures are given in Methods.

Volume of treated buffer (μl)	Inhibition (%) of the recovery of total adenylates in the presence of buffer treated by various extraction methods		
	Chloroform	Tri-chloroacetic acid	Perchloric acid
10	0	2.3	3.5
20	0	3.8	18.3
100	3.3	9.4	27.8

amounts of adenylates. A similar technique has been used by Lundin & Thore (1975) in the preparation and assay of bacterial extracts. The lower limit of sensitivity for each of the adenylates using our system was about 1 pmol in a sample volume of 50 μl ; the sensitivity was limited by the degree of luminescence of the firefly extract in the absence of added ATP and by the sensitivity of the fluorimeter. The assay was linear, for all three adenylates, up to at least 5 nmol adenylate in a 50 μl sample. The presence of 2 mM-dAdo in either a mixture of known amounts of adenylates or in a culture was without effect on the extraction, assay and recovery of adenylates. The presence of EDTA in the extraction reagents at a final concentration of 20 $\mu\text{mol ml}^{-1}$ inactivates nucleotide-converting enzymes in extracts of bacteria (Lundin & Thore, 1975). There was no significant difference in the concentrations of adenylates in trichloroacetic acid extracts of *S. pombe* prepared in the absence or presence of EDTA, and so it was omitted from our extraction procedures.

Adenine nucleotide pools in exponentially growing and stationary phase cultures

Extraction of the yeast by either trichloroacetic acid or perchloric acid gave very similar results for the concentrations of ATP and total adenylates and for the values of energy charge and ATP/ADP ratio (Table 2). ATP concentrations and, to a lesser extent, the total adenylate pools were higher in stationary phase cells than in exponentially growing cells. Similarly, energy charge values increased from 0.86 to 0.88 (in exponentially growing cultures) to about 0.95 (in stationary phase cultures). This increase was due mainly to a three- to four-fold increase in the ATP/ADP ratio; AMP concentrations were usually below the sensitivity limits of our assay. Table 2 also confirms that insufficient dilution of the perchloric acid extracts results in anomalously low concentrations of ADP (and consequently an increased ATP/ADP ratio and energy charge value), due to the inhibition of the enzymic conversion of ADP and AMP to ATP. Extraction of cells with chloroform resulted in a value of ATP (mg dry wt cells) $^{-1}$ for exponentially growing cells similar to that obtained after extraction with trichloroacetic acid and perchloric acid, but a significantly lower value for the ATP pool in stationary phase cells. The ATP/ADP ratio and the energy charge value were both higher in stationary phase cells than in exponentially growing cells but were significantly lower than the values obtained in trichloroacetic acid or perchloric acid extracts. This may be due to insufficiently rapid and irreversible quenching of the activity of enzymes in adenylate metabolism (Chapman & Atkinson, 1977). In view of the greater inhibition of the assay of ADP and AMP by perchloric acid extracts (and the consequent need for greater dilution of the extracts), all subsequent measurements of adenylates were performed using trichloroacetic acid extracts.

Table 2. Intracellular pools of adenine nucleotides, ATP/ADP ratios and energy charge values in exponentially growing and stationary phase cultures of *S. pombe*

Extracts of cultures growing exponentially (2.35×10^7 organisms ml^{-1} ; glucose concn in medium, 8.22 mM) and 24 h after entering the stationary phase (4.22×10^7 organisms ml^{-1} ; glucose concn in medium, 0.01 mM) were prepared using chloroform, trichloroacetic acid or perchloric acid. Extracts were diluted 1:5 (chloroform and trichloroacetic acid) or 1:10 (perchloric acid), except where indicated, and assayed for ATP, ADP and AMP using the firefly luciferase system. Detailed analytical procedures are given in Methods. The adenylate charge is given by $([\text{ATP}] + \frac{1}{2}[\text{ADP}])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$ (Atkinson, 1968). All values based on adenylate determinations are the means of four to six experiments $\pm$ S.D.

	Exponential phase culture	Late-stationary phase culture
Chloroform extracts		
Yeast adenylate concn [nmol (mg dry wt) $^{-1}$]		
ATP	3.70 ± 0.58	4.68 ± 1.44
Total adenylates	8.02 ± 1.28	9.30 ± 0.60
ATP/ADP ratio	1.66 ± 0.45	1.88 ± 0.82
Energy charge	0.61 ± 0.12	0.64 ± 0.11
Trichloroacetic acid extracts		
Yeast adenylate concn [nmol (mg dry wt) $^{-1}$]		
ATP	3.85 ± 0.33	7.57 ± 0.45
Total adenylates	5.11 ± 0.31	8.32 ± 0.31
ATP/ADP ratio	3.09 ± 0.41	10.27 ± 3.01
Energy charge	0.88 ± 0.01	0.95 ± 0.12
Perchloric acid extracts		
Yeast adenylate concn [nmol (mg dry wt) $^{-1}$]		
ATP	3.85 ± 0.71	6.44 ± 0.14
Total adenylates	5.02 ± 0.93	7.08 ± 0.31
ATP/ADP ratio	3.25 ± 0.15	12.88 ± 8.2
		$*32.78 \pm 11.5$
Energy charge	0.86 ± 0.04	0.95 ± 0.22
		$*0.98 \pm 0.005$

* Perchloric acid extracts were diluted 1:5 before assaying.

The presence of adenylates in the growth medium was investigated by rapidly filtering samples of culture and adding trichloroacetic acid to the filtrates. Adenylates were undetectable in the medium from either exponentially growing or stationary phase cultures. The adenylates measured in extracts of cultures therefore represent intracellular pools.

Freshly prepared trichloroacetic acid was used for all extractions. In one experiment, a trichloroacetic acid solution which had been stored for several weeks extracted only 1.3 nmol total adenylates (mg dry wt) $^{-1}$ from stationary phase cells (cf. Table 2), yet extracted 3.6 nmol total adenylates (mg dry wt) $^{-1}$ from exponentially growing cells. This indicates that stationary phase cells are more resistant to extraction than exponentially growing cells.

Adenine nucleotide pools in dAdo-synchronized cultures

The changes in cell numbers and in the cell plate index (Fig. 1a) were similar to those reported previously (Mitchison & Creanor, 1971; Sissons, Mitchison & Creanor, 1973). The number of organisms ml^{-1} approximately doubled during the 4 h treatment with dAdo, increasing only slightly during the last hour of the dAdo pulse and the first hour after the end of the pulse. Total cell volume (Fig. 1a) increased smoothly throughout this period, except during the transition into stationary phase when the mean cell volume increased from $32 \mu\text{m}^3$ (8 h) to $50.5 \mu\text{m}^3$ (10 h). The timings of the subsequent synchronous cell divisions were hard to determine from the steep rise in cell numbers which exhibits no clear plateaux. However, the cell plate index (the proportion of cells exhibiting visible cell plates)

Fig. 1. Changes in individual adenine nucleotide pools, total adenylates, energy charge and the ATP/ADP ratio in dAdo-synchronized cultures of *S. pombe*. Culture samples were rapidly mixed and extracted with trichloroacetic acid; adenylates were assayed by the luciferase method. (a) Cell numbers (●), total cell volume (○) and cell plate index (□). (b) Total adenylates (●) and the individual pool sizes of ATP (□), ADP (■) and AMP (○); all expressed as nmol (ml culture)⁻¹. (c) Energy charge (○) and ATP/ADP ratio (●). dAdo was added at the first arrow (and solid vertical line) and removed by centrifugation and resuspension in fresh medium at the second arrow (and solid vertical line). The vertical broken line indicates the mid-point of the first synchronous doubling in yeast numbers.

Fig. 2. Maps of timings of minimal ATP/ADP ratios and maximal rates of O_2 uptake during the period between the end of a 4 h pulse with dAdo (0) and the mid-point of the first synchronous doubling in yeast numbers (1.0). Timings of the peaks of O_2 uptake (∇) were measured with an open O_2 electrode system (Poole, 1977*b*). To show the extent of the troughs in the ATP/ADP ratio, that are sometimes broad (see Fig. 1*c*), the width of each bar indicates those times during which the ATP/ADP ratio did not exceed by more than 13 % its minimum value during the same trough. The coefficient of variation for the measurement of the ATP/ADP ratio in trichloroacetic acid extracts in exponentially growing cultures is 13 % (see Table 2). The three blocks shown in each row were determined in one experiment; those in the lower row are derived from the experiment shown in Fig. 1.

revealed two synchronous divisions, separated by about 1.5 h (Fig. 1*a*). The pool of total adenylates (ATP+ADP+AMP) increased fairly smoothly with an overall doubling time of about 2.5 h during both the dAdo pulse and the following 4 h (Fig. 1*b*). ATP concentrations (Fig. 1*b*) followed closely those of total adenylates, giving rise to a high energy charge (Fig. 1*c*). In the period before adding dAdo and in the subsequent 1.5 to 2 h, the ADP pool (Fig. 1*b*) remained constant (± 20 %). After this, and throughout the remainder of the experiment, ADP concentration fluctuated markedly, rising to as much as one-third of the total adenylate pool or falling to below the limits of sensitivity of the assay. In most samples the concentrations of AMP were also below the limits of sensitivity of the assay; concentrations fluctuated particularly during the last 2 h of the dAdo pulse and at regular 1.75 h intervals throughout the ensuing period of the experiment (Fig. 1*b*). Figure 1(*c*) shows the ATP/ADP ratio and the energy charge values; since AMP levels throughout the experiment were too low to contribute significantly to the energy charge these two variables show qualitatively similar time-dependent fluctuations. Significant oscillations in the concentration of ATP occurred during the onset of stationary phase; these were also observed in a control, unsynchronized culture (see below) and so are probably unrelated to dAdo-induced synchronous growth.

Figure 2 summarizes the fluctuations in the ATP/ADP ratio after removing dAdo in two similar experiments and shows the temporal relation of these oscillations to those in O_2 uptake rates (Poole, 1977*b*). Minimal values of the adenylate energy charge (not shown) occurred at similar times to those of the ATP/ADP ratio. The timings of maximal respiration rates shown in Fig. 2 are those determined in an experiment in which good resolution of the oscillations was achieved by constantly monitoring O_2 tension in the growth vessel. The values of the timings obtained in three experiments involving measurements on samples removed at intervals and analysed by a conventional O_2 electrode are similar to those shown (Poole, 1977*b*). There is good correlation between maximal respiration rates (that are insensitive to CCCP, relative to the sensitivity of the troughs) and the minimum values of the ATP/ADP ratio (or energy charge). The periodicity of both oscillations is about one-third of the period between removal of dAdo and the mid-point of the first synchronous division. These findings are consistent with the earlier proposal (Poole, 1977*b*) that the respiratory oscillations reflect the modulation of mitochondrial respiration by ADP concentrations during synchronous growth.

Adenine nucleotide pools and respiration following centrifugation of a culture and resuspension in fresh medium

To investigate whether the observed fluctuations in adenine nucleotide pools were a consequence of the centrifugation and resuspension of cells in fresh medium, an exponentially

Fig. 3. Changes in individual adenine nucleotide pools, total adenylates, energy charge and the ATP/ADP ratio in cultures before and after centrifugation and resuspension in fresh medium. Cultures samples were rapidly mixed and extracted with trichloroacetic acid; adenylates were assayed by the luciferase method and are expressed as nmol (ml culture) $^{-1}$. Symbols are the same as those in Fig. 1. At the first vertical line (and first arrow) the culture was harvested by centrifugation; at the second vertical line (and second arrow) the cells were resuspended in fresh medium.

growing culture (to which dAdo had not been added) was studied before and after centrifugation. The rate of increase in cell numbers (doubling time 2.7 h) and the cell plate index were not affected by these procedures (Fig. 3a). The rate of increase in cell numbers and the cell plate index decreased as the culture approached the stationary phase of growth (Fig. 3a). Total adenylates increased smoothly following resuspension, but marked oscillations in these concentrations and in those of ATP and ADP occurred on the transition into stationary phase (Fig. 3b). These fluctuations did not cause large variations in the energy charge or ATP/ADP ratio, however, except for a brief period 1 to 2 h after resuspension (Fig. 3c), when a significant drop in the concentration of ADP (Fig. 3b) resulted in an ATP/ADP ratio greater than 20 and an elevation of the energy charge to 0.98 (Fig. 3c). AMP concentrations remained low or undetectable throughout the experiment; significant amounts were detectable in the first 1 h after resuspension and at irregular intervals thereafter (Fig. 3b). Similar results were obtained in a replicate experiment.

In view of the transient massive increase in ATP/ADP shown in Fig. 3(c), the experiment shown in Fig. 4 was performed. Oxygen uptake rates and their stimulation by CCCP were measured after centrifugation of an exponentially growing culture and resuspension of the cells in fresh medium. Before centrifugation, respiration rates (measured in the absence of CCCP) in this culture increased with a doubling time of 2.6 h (Fig. 4b), parallel with the increases in cell numbers and total cell volume (Fig. 4a). After resuspension, there was a period in which respiration rates (measured in the absence of CCCP) gradually increased to attain a doubling time of 1.8 h. Respiration rates measured in the presence of CCCP increased throughout with a doubling time of about 2.8 h (Fig. 4b), so that the percentage stimulation by uncoupler decreased (Fig. 4b). Respiration became insensitive to CCCP as the culture entered the stationary phase and during the 'overshoot' of respiration rates

Fig. 4. Oxygen uptake, and the effect of CCCP, in cell suspensions from a culture before and after centrifugation and resuspension of cells in fresh medium. (a) Cell numbers (●) and total cell volume (○). (b) O_2 uptake measurements made in the absence (□) or presence (▲) of $4.9 \mu\text{M}$ -CCCP and the calculated stimulation of respiration rate (■). At the first vertical line (and first arrow) the culture was harvested by centrifugation; at the second vertical line (and second arrow) the cells were resuspended in fresh medium.

(Fig. 4b). After prolonged incubation of the culture, respiration rates in the absence of uncoupler were unchanged and were stimulated about 10% by $4.9 \mu\text{M}$ -CCCP. Thus the fluctuations in the ATP/ADP ratio observed 1 to 2 h after resuspension of cells were not reflected in fluctuations in O_2 uptake although a slight variation in stimulation by CCCP occurred during this period.

DISCUSSION

The methods described and used here for extraction and assay of adenine nucleotides allow quantitative recovery of ATP, ADP and AMP at concentrations below which the sensitivity of assay is limited by the background luminescence of the firefly extract or instrument noise. Only a few workers (e.g. Lundin & Thore, 1975; Kimmich *et al.*, 1975) have established the suitability of their methods for the quantitative recovery of all three nucleotides. Since many of the widely used extraction methods were developed for extraction of ATP only, it seems imperative to assess their interference with the enzymic conversion of ADP and AMP to ATP. Low values for the energy charge, obtained here in chloroform extracts, were also found in extracts of various bacteria prepared with solvents (butanol and chloroform) by Lundin & Thore (1975). The use of trichloroacetic acid or perchloric acid presumably resulted in a complete and irreversible inactivation of nucleotide-converting enzymes and consequently a high energy charge. Caution must be exercised in the use of these reagents, however, since inadequate removal of the anion or insufficient dilution of the extract may also lead to an anomalously high energy charge (e.g. Table 2) due to incomplete conversion of AMP and ADP to ATP.

Schizosaccharomyces pombe, growing exponentially on glucose as carbon source, contained 3.7 to 3.9 nmol ATP (mg dry wt yeast) $^{-1}$. These values are in good agreement with those reported by Foury & Goffeau (1975) for a respiratory-deficient mutant of

S. pombe and with values obtained for a wide range of other eukaryotic micro-organisms (Knowles, 1977).

The elevated energy charge found in cultures 24 h after entering the stationary phase of growth (Table 2) and associated with the maintenance of a high respiration rate, was not observed during the early stages of the stationary phase (Figs 1 and 3). However, the early stages in the transition to a high steady state of the energy charge may be accompanied by oscillations in the levels of adenylates. To what extent these oscillations in adenylates, and perhaps the 'overshoot' in respiration rates, are connected with glucose derepression resulting from exhaustion of glucose from the medium (Poole & Lloyd, 1973) remains to be determined. A similar maintenance of the adenine nucleotide pools and the poising of the energy charge close to 0.9 has also been observed after aerobic growth on glucose and the ethanol-oxidizing phase of *Saccharomyces cerevisiae* (Ball & Atkinson, 1975).

The pool sizes of ATP, ADP and AMP all oscillated dramatically in dAdo-synchronized cultures. In cultures not treated with dAdo, the ATP/ADP ratio showed a sharp peak 1 to 2 h after centrifugation and resuspension of cells, even though respiration rates did not oscillate during this period. These findings suggest that appropriate control experiments should be performed when adenylates are assayed after perturbation of cultural conditions (Chapman & Atkinson, 1977). Similar perturbations (e.g. centrifugation, filtration, anaerobiosis) often accompany procedures for the preparation of synchronous cultures by selection or induction methods. Unfortunately this observation makes it impossible to show an unequivocal correlation between the adenylate pools and respiration rates immediately following resuspension of cells. However, the similar periodicity of ATP/ADP ratios and respiratory oscillations in dAdo-synchronized cultures, together with previous observations on the uncoupler sensitivity of respiratory maxima and minima (Poole, 1977*b*), makes the control of respiration by adenylate pools appear likely. Furthermore, the ATP/ADP ratio remained relatively constant while the respiration rates increased smoothly during the beginning of the dAdo pulse; later, oscillations in respiration rate and the ATP/ADP ratio began almost coincidentally during the pulse. The correlation between adenylate pools and respiration rates in *Crithidia fasciculata* reported by Edwards *et al.* (1975) is quite different from the situation in *S. pombe*; in their study maximal rather than minimal respiration rates were more closely related to the maxima of adenylate energy charge. The timings of maximal ADP pool sizes were generally retarded by 0.1 of a cell cycle with respect to respiration rates, energy charge and pool sizes of ATP and AMP. The control of oscillating respiration rates in the cell cycle of *C. fasciculata* remains to be elucidated.

The adenine nucleotide translocase, which catalyses the exchange of ATP and ADP across the inner mitochondrial membrane of eukaryotic cells, is specific for these two nucleotides, so that the intra- and extra-mitochondrial pools of AMP remain separated (Klingenberg, 1970). Adenylate kinase catalyses the interconversions of extra-mitochondrial adenylates only, so that the concept of energy charge is applicable really only to these extra-mitochondrial pools (Knowles, 1977). We realize that the levels of ATP and ADP reported here represent both intra- and extra-mitochondrial pools. The validity of correlating total cellular pools of ATP and ADP with respiration rates is, however, supported by the recent observations of Holian, Owen & Wilson (1977). Respiration rates of mitochondrial preparations with high respiratory control ratios, and probably of mitochondria *in vivo*, are controlled by extra-mitochondrial [ATP]/[ADP] [P_i]; the translocase is probably not rate-limiting for mitochondrial respiration. We have not determined the contribution of substrate level phosphorylation to the adenylate levels, but it is likely to be considerable in this highly fermentative organism (R.Q. 4.3 to 10.5; Heslot, Goffeau & Louis, 1970; Hamburger *et al.*, 1977).

2'-Deoxyadenosine inhibits DNA synthesis in a variety of cells (Huez, Zampetti-Bosseler & Brachet, 1972; Wanka, 1974; Ingram & Fisher, 1972), probably by the formation of dATP (Munch-Peterson, 1960; Klenow, 1962) which subsequently inhibits the reduction

of purine and pyrimidine ribonucleotides (Larsson & Reichard, 1966*a, b*). Vornovitskaja, Shapot & Nicolskaja (1968) suggested that dATP may also inhibit the incorporation of deoxyribonucleotides into DNA. In addition, it has been reported that dAdo is incorporated largely into RNA (Plagemann & Erbe, 1974), is a precursor of deoxy- and 'native' nucleotides (Mah & Daly, 1975) and may inhibit adenylate cyclase activity (Blume & Foster, 1975). In contrast, Penman & Duffus (1975) suggested that, in *Kluyveromyces lactis*, dAdo and the ionophore A23187 both induce synchronous nuclear and cell division (but not DNA synthesis) by lowering intracellular ATP pools. This is clearly not the case in *S. pombe*, since ATP concentrations continue to increase, but oscillate, throughout the dAdo pulse, doubling overall in the same interval as the normal cell cycle time. dATP, and consequently dADP and dAMP in extracts, would be undetectable in our system using the firefly luciferase assay (Strehler, 1968).

We are grateful to Dr C. J. Knowles for helpful discussions. I.S. is the holder of an S.R.C. Research Studentship.

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