Biochem. J. (1982) **202**, 453–458
Printed in Great Britain

The mitochondrial adenosine triphosphatase of *Acanthamoeba castellanii*

Oscillatory accumulation of enzyme activity, enzyme protein and F_1-inhibitor during the cell cycle

Steven W. EDWARDS,* J. Barbara EVANS, Judith L. WILLIAMS and David LLOYD
Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, Wales, U.K.

(*Received 17 September 1981/Accepted 2 November 1981*)

1. The mitochondrial ATPase of *Acanthamoeba castellanii* accumulated discontinuously in synchronous cultures prepared by a minimally perturbing size-selection technique. 2. Enzyme activity per ml of culture doubled overall during one cell cycle time of 8h, but oscillated to give seven maxima during this period. Similar oscillations were observed in the specific activities of ATPase and of the naturally occurring inhibitor protein. 3. These variations in enzyme activity reflected changes in amount of enzyme protein as assayed by an immunological technique. 4. Large variations in I_{50} values (μg of inhibitor/mg of protein necessary for 50% inhibition of inhibitor-sensitive activity) for inhibition of ATPase activity by seven different inhibitors of energy conservation were observed. Activity was more sensitive to inhibition by oligomycin, efrapeptin, citreoviridin and quercetin when values were highest. 5. The results are discussed in relation to the phased organization of biosynthesis and degradation of cellular components known to occur during the cell cycle of this organism.

Extensive studies on the mitochondrial ATPase (EC 3.6.1.3) of yeast have revealed structural and functional similarities to the enzyme from mammalian sources (Kagawa & Racker, 1966; Schatz *et al.*, 1967; Tzagoloff & Meagher, 1971). The enzyme comprises three functional components: F_1-ATPase, the oligomycin-sensitivity-conferring peptide, and the membrane factor (F_0). The synthesis and assembly of the entire enzyme complex is under the dual control of mitochondrial and non-mitochondrial protein-synthesizing systems, and is coded for in part by nuclear DNA and in part by mitochondrial DNA (see Tzagoloff *et al.*, 1979, for a review). A naturally occurring inhibitor protein of F_1-ATPase has been isolated from many sources, and the presence or absence of this inhibitor in *Schizosaccharomyces pombe* is correlated with marked changes in sensitivities of ATPase to inhibitors of energy conservation (Lloyd & Edwards, 1976); when the natural inhibitor is present, sensitivities to inhibitors acting on F_1-ATPase increase 2-fold, whereas sensitivities to inhibitors acting on the membrane factor show a greater increase. Removal of the inhibitor protein by gel filtration (Racker & Horstman, 1967) decreases the sensitivity of the mitochondrial ATPase of *Acanthamoeba castellanii* to inhibitors of energy conservation by between 1.5- and 14-fold (Edwards *et al.*, 1982).

There have been few reports on the expression of ATPase activity during the cell cycle (Lloyd *et al.*, 1982). In *Escherichia coli* ATPase activity reaches two maxima per cell cycle at 0.37 and 0.8 of the cycle, whereas sensitivity to Ruthenium Red is maximal at the middle of the cycle (Scott *et al.*, 1980). In *Alcaligenes eutrophus* ATPase activity is maximal at 0.4 and 0.9 of the cycle, corresponding to mid-step rises in respiratory activities (Edwards & Jones, 1977) and minimal at 0.1 and 0.6 of the cycle (Edwards *et al.*, 1978); inhibition by 4-chloro-7-nitrobenzofurazan was greatest at maximal ATPase activity, whereas maximal inhibition by *NN'*-dicyclohexylcarbodi-imide was when ATPase activity was lowest. Discontinuous accumulation of ATPase activity was also observed during the cell cycle of *S. pombe* (Edwards & Lloyd, 1977), and 11 inhibitors of energy conservation all showed cell-cycle-dependent variations in their I_{50} values (μg of inhibitor/μg of protein necessary for 50% inhibition of inhibitor-sensitive activity) (Lloyd & Edwards, 1977).

The cell cycle of *A. castellanii* is divided into phases of biosynthesis separated by degradative phases, whereby total RNA and total cellular protein accumulate discontinuously (Edwards & Lloyd, 1980). Thus, at 30°C the generation time is 8h (Edwards & Lloyd, 1978) and total protein oscil-

* Present address: Research Unit for Tropical Diseases, International Institute of Cellular and Molecular Pathology, Avenue Hippocrate 74, B1200 Brussels, Belgium.

0306-3283/82/020453-06$01.50/1

lates to give seven maxima per cell cycle (Edwards & Lloyd, 1980). The accumulation of catalase [a peroxisomal enzyme in this organism (Müller & Møller, 1969; Morgan *et al.*, 1973)] is also oscillatory during the cell cycle, as determined by assays of both enzyme activity and enzyme protein by an immunological method (Edwards *et al.*, 1981).

In the present study we have examined changes in ATPase activity during the cell cycle of *A. castellanii*, as well as changes in sensitivities to inhibitors of energy conservation, and show that the changes in ATPase activity are paralleled by changes in ATPase protein, as assayed by an immunological method.

Experimental

Maintenance and growth of the organism

Acanthamoeba castellanii was maintained and grown with shaking at 30°C exactly as described previously (Edwards *et al.*, 1977). Cells were counted in a Fuchs–Rosenthal haemocytometer (Baird and Tatlock, Chadwell Heath, Essex, U.K.) after a suitable dilution in 50mM-$MgCl_2$ (pH7.4).

Preparation of synchronous cultures

Synchronous cultures were prepared by centrifugation of exponentially growing cultures, in growth medium at 800rev./min (150*g*; $r_{av.}$ 21cm) for 2min in the 6 × 1 litre head of an MSE Mistral centrifuge. Approx. 80% of the supernatant, containing the slowest-sedimenting cells of the culture (approx. 10%), was decanted and grown as a synchronous culture. Assessment of synchrony was as described previously (Edwards & Lloyd, 1980), by using the synchrony index of Blumenthal & Zahler (1962).

Preparation of cell-free extracts

Samples removed from synchronous cultures (50–100ml) were centrifuged at 3000rev./min (750*g*; $r_{av.}$ 7.6cm) for 2min in the 6 × 250ml head of an MSE 18 centrifuge. The pellet was resuspended in 50mM-Tris/H_2SO_4, pH8.1, and a cell-free extract was produced by treatment with ultrasound (Branson Sonicator, 40W for 10s). This treatment resulted in 100% cell breakage and the suspension produced was used as a cell-free extract.

Preparation of submitochondrial particles and ATPase-inhibitor protein

Mitochondria were isolated from mid-exponential-phase cultures essentially as described previously (Edwards & Lloyd, 1978) in a disruption buffer containing 0.32M-sucrose, 0.2mM-EGTA and 10mM-Tris/H_2SO_4, pH8.1. The mitochondrial suspension in 50mM-Tris/H_2SO_4 (pH8.1) was treated with ultrasound for 10s to produce submitochondrial particles. This suspension was used to assay the inhibitor protein after gel filtration, as described below.

The ATPase-inhibitor protein was prepared as described previously (Edwards *et al.*, 1982) from cell-free extracts prepared from synchronous cultures.

Other methods

ATPase was assayed by the proton-release method (Edwards *et al.*, 1982) in a stirred reaction mixture at 30°C containing 4mM-ATP, 6mM-$MgSO_4$ and 10mM-Tris/H_2SO_4, pH8.1. Activity was measured within 6h of preparation of extracts.

The activity of the ATPase-inhibitor protein was measured as described previously (Edwards *et al.*, 1982); one unit of activity is defined as the volume of inhibitor preparation required to inhibit ATPase activity of a preparation of inhibitor-depleted submitochondrial particles by 50%; the inhibitor activity was then expressed as units per mg of protein. The inhibitor was removed from submitochondrial particles by gel filtration through a Sephadex G-50 column (0.9cm × 15cm). Elution was with 50mM-Tris/H_2SO_4, pH8.1, and the particles were eluted in the void volume (3.6ml).

Immunological determination of ATPase protein was by complement fixation by using serum prepared against F_1-ATPase as described previously (Edwards *et al.*, 1982).

Protein was measured by the method of Lowry *et al.* (1951).

Chemicals

The sources of chemicals and inhibitors have been described previously (Edwards *et al.*, 1982).

Results

Changes in cell numbers, ATPase activity and F_1-inhibitor activity during the cell cycle

A synchronous culture, containing 9% of the original exponential culture, was prepared as described in the Experimental section and followed for 9h of growth. Cell numbers remained constant for the initial 5h, then increased and doubled by just less than 8h of growth (Fig. 1*a*). Throughout this period, ATPase activity per ml of culture doubled overall, but the pattern of accumulation was discontinuous (Fig. 1*b*), in that activity rose to seven maxima during the cell cycle. These oscillations in activity were also observed in measurements of specific activities (Fig. 1*c*) and the mean amplitude of these (peak–trough, % of minimum value) was 37%. Similar oscillations were observed in the activities of the F_1-inhibitor protein in these extracts (Fig. 1*d*); the mean amplitude (peak–trough, % of minimum value) was 74%. The timings of maxima and minima of enzyme activity and F_1-inhibitor activity closely

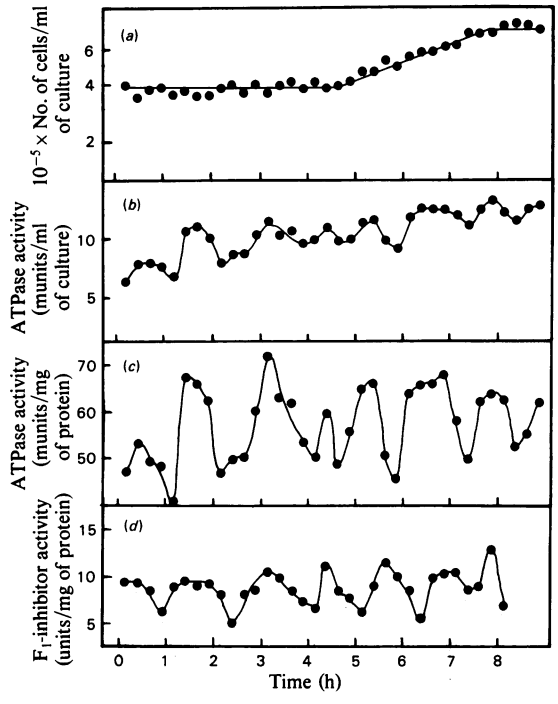

Fig. 1. *Changes in cell numbers, ATPase activity and F_1-inhibitor-protein activity during synchronous growth of A. castellanii*

An exponentially growing culture (3.5×10^6 cells/ml) was centrifuged at 800 rev./min for 2 min as described in the Experimental section. The supernatant (containing 9% of the exponential culture) was decanted and grown as a synchronous culture. At frequent time intervals, samples were removed for measurement of (*a*) cell numbers, (*b*) ATPase activity/ml of culture, (*c*) ATPase activity/mg of protein and (*d*) F_1-inhibitor activity/mg of protein. The synchrony index, *F* (Blumenthal & Zahler, 1962), was 0.68.

coincided. Similar changes in ATPase activity were observed in an experiment in which the disruption buffer contained the following proteinase inhibitors: 2 mM-phenylmethanesulphonyl fluoride; 5 mM-*p*-aminobenzamidine hydrochloride; 5 mM-ε-aminohexanoic acid (results not shown).

Effect of inhibitors on ATPase activity during the cell cycle

The effect of oligomycin on ATPase activity of extracts prepared at different stages of synchronous growth is shown in Fig. 2. ATPase activity over the initial 2.5 h varied by 46%, and sensitivity to oligomycin also showed large changes and varied over a 4-fold range. Maximum sensitivity to oligomycin was observed when ATPase activity was highest, whereas minimum sensitivity was observed when activity was lowest.

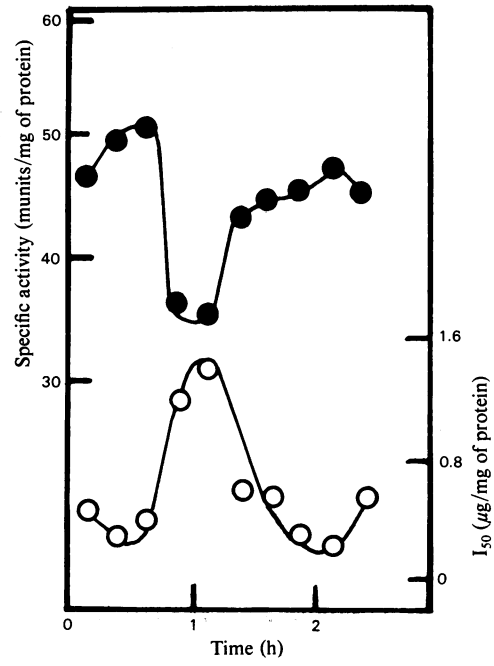

Fig. 2. *Changes in ATPase activity and sensitivity to oligomycin during a portion of the cell cycle of A. castellanii*

A synchronous culture (containing 15% of the exponential culture) was prepared as in the legend to Fig. 1. The initial cell population after centrifugation was 7×10^5 cells/ml, and growth was followed for 9 h. The doubling time was 8 h and the synchrony index (*F*) was 0.7. ●, ATPase activity/mg of protein, and ○, I_{50} values (μg of oligomycin/mg of protein required for 50% inhibition), over the initial 2.5 h of synchronous growth.

In a similar experiment where ATPase activity varied by 96% (trough–peak, % of minimum value), large variations in sensitivities to efrapeptin, *NN'*-dicyclohexylcarbodi-imide and 4-chloro-7-nitrobenzofurazan were observed (Fig. 3). I_{50} values for efrapeptin showed a reciprocal relationship to enzyme activity; thus sensitivity of ATPase to this inhibitor was maximal when activity was highest. Sensitivities to the other inhibitors tested in this experiment were not as clearly defined, however, except for the first minimum in activity, which was relatively insensitive to both *NN'*-dicyclohexylcarbodi-imide and 4-chloro-7-nitrobenzofurazan (Fig. 3*b*).

In another experiment, shown in Fig. 4, ATPase activity was measured and correlated with a totally independent assay for amount of ATPase protein by an immunological method. ATPase activity was high at the beginning of the experiment and again after 1 h and 2 h of synchronous growth (Fig. 4*a*); these changes in activity were closely paralleled by changes in amount of enzyme protein.

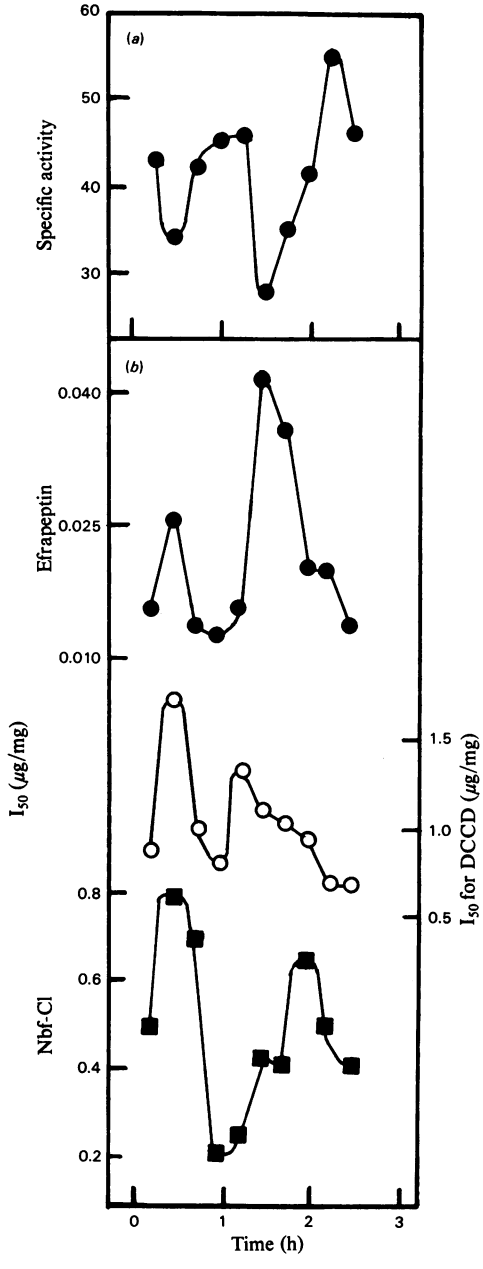

Fig. 3. *Changes in ATPase activity and sensitivity to efrapeptin, 4-chloro-7-nitrobenzofurazan (Nbf-Cl) and NN'-dicyclohexylcarbodi-imide (DCCD) during synchronous growth of A. castellanii*

A synchronous culture, initially containing 4.1×10^5 cells/ml (13% of the original exponential culture), was followed under conditions identical with those described in the legend to Fig. 2. The doubling time was 8.05 h and the synchrony index (*F*) was 0.71. (*a*) Specific activity; (*b*) I_{50} values (μg of inhibitor/mg of protein necessary for 50% inhibition) for efrapeptin (●), DCCD (○) and Nbf-Cl (■).

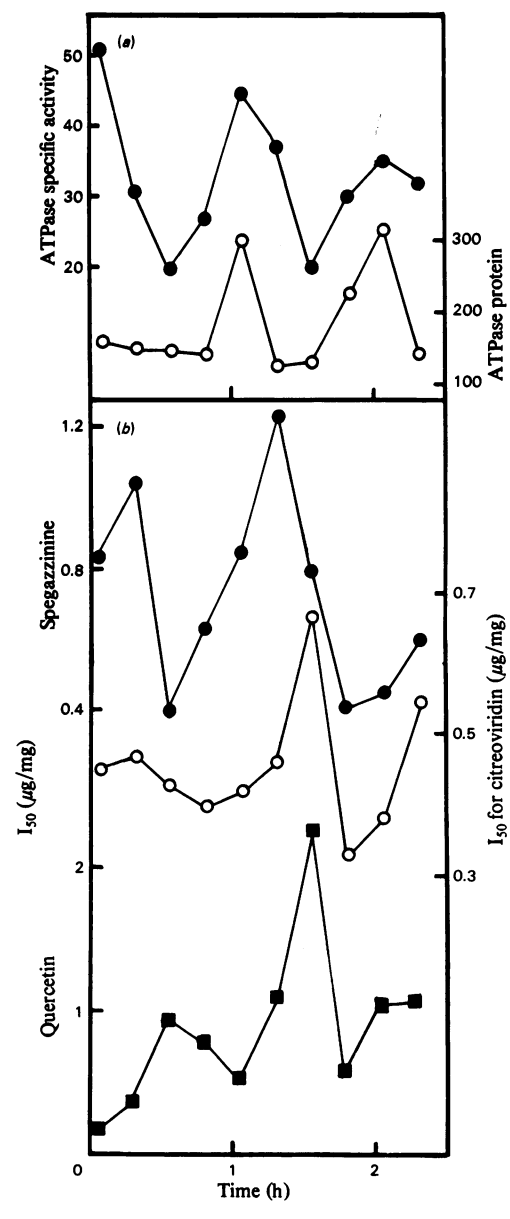

Fig. 4. *Changes in ATPase protein, ATPase activity and sensitivity to spegazzinine, citreoviridin and quercetin during synchronous growth of A. castellanii*

Experimental conditions were identical with those described in the legend to Fig. 2. The synchronous culture initially containing 5×10^5 cells/ml (16% of the original exponential culture) doubled in cell numbers by 8.1 h; the synchrony index (*F*) was 0.68. (*a*) ATPase activity (munits/mg of protein) (●) and ATPase protein (immunological units/mg of protein) (○). (*b*) I_{50} values (μg of inhibitor/mg of protein necessary for 50% inhibition) for spegazzinine (●), citreoviridin (○) and quercetin (■).

Sensitivity of ATPase activity to citreoviridin and quercetin were similar in that sensitivity was maximal at highest enzyme activity and was lowest at low activity (Fig. 4*b*). The sensitivity profile to spegazzinine was again less well defined, but lowest sensitivity to the inhibitor appeared to occur just after maximum activity.

Table 1 summarizes the effects of inhibitors on ATPase activity during the initial 2.5 h of synchronous growth.

Changes in ATPase activity, ATPase protein and F_1-inhibitor during growth of control, asynchronous cultures

To establish whether the variations in ATPase activity observed in Figs. 1–4 were genuinely associated with the cell cycle, or whether they resulted from artefacts arising from the method used to produce the synchronous cultures, the experiment in Fig. 5 was performed. An exponentially growing culture was centrifuged under conditions identical with those used to produce the synchronous cultures, but after centrifugation the pellet and supernatant were remixed and the entire contents were returned to the growth vessel. Cell numbers increased exponentially (doubling time 8 h), as did total cell protein and ATPase activity per ml of culture (Fig. 5*a*). The specific activities of ATPase did not vary significantly and averaged 20.3 munits/mg of protein. The coefficient of variation (v) for the data points shown in Fig. 5(*b*) (v = 100 × standard deviation/mean) was 3.8%. A similar coefficient of variation for the F_1-inhibitor-protein activity was 7%. ATPase protein as determined by an immunological method also showed no fluctuations during this period.

Discussion

The results presented demonstrate that, in common with the majority of protein in this organism (Edwards & Lloyd, 1980), the accumulation of ATPase protein during the cell cycle is oscillatory. Since changes in ATPase activity closely parallel changes in enzyme protein, the possibility that these periodic fluctuations in activity of this enzyme are regulated by binding and release of the naturally occurring inhibitor protein can be eliminated. However, it cannot be discounted that the cell-cycle-dependent variations in sensitivities of inhibitors of energy conservation arise, at least in part, by alterations in the amount of bound inhibitor protein, since sensitivity to inhibitors can vary up to 14-fold, depending on the presence or absence of this natural inhibitor (Edwards *et al.*, 1982).

Immunological determination of amount of enzyme protein during the cell cycle has very rarely been reported (Klevecz, 1969; Martin *et al.*, 1969). This approach provides confirmation that enzyme activity represents amount of enzyme protein, and has confirmed that the accumulation of catalase protein in this organism is also discontinuous (Edwards *et al.*, 1981). That similar changes were also observed when extracts were prepared in the presence of proteinase inhibitors eliminates the possibility that restricted proteolysis occurs in cell-free extracts. Thus the turnover of ATPase protein during the cell cycle of this organism is rapid under ideal growth conditions. The periodicity of these oscillations is about 1 h, and it should be emphasized that, as discussed previously (Edwards & Lloyd, 1980), the degree of synchrony in these cultures is underestimated, since DNA replication is completed in about 1 h (Edwards & Lloyd, 1978, 1980). As clearly shown in Fig. 5, these oscillations are genuinely associated with the cell cycle, since no oscillatory increase was observed in control (asynchronous) cultures that were subjected to experimental procedures identical with those employed to produce the synchronous cultures. Oscillatory enzyme formation, and the control processes that

Table 1. *Variations in inhibitor-sensitivity of ATPase activity during the cell cycle*

Synchronous cultures were prepared as described in the Experimental section, and growth was followed for 2.5 h. During this period samples were removed from the culture and ATPase activity was measured in cell-free extracts, by the proton-release method. Sequential small additions of inhibitors (as concentrated solutions in ethanol) were added to the reaction mixture and progressive inhibition was monitored. A titration curve for each sample for each inhibitor was then obtained and I_{50} values (μg of inhibitor/mg of protein necessary for 50% inhibition of the total portion of inhibitor-sensitive activity) were obtained.

Inhibitor	Maximum inhibition (%)	Minimum inhibition (%)	I_{50} range
Oligomycin	71	43	1.4–0.3
Spegazzinine	79	32	1.2–0.5
Citreoviridin	94	52	0.7–0.3
NN'-Dicyclohexylcarbodi-imide	84	39	1.75–0.6
4-Chloro-7-nitrobenzofurazan	68	30	0.8–0.2
Efrapeptin	68	21	0.04–0.01
Quercetin	80	26	2.3–0.1

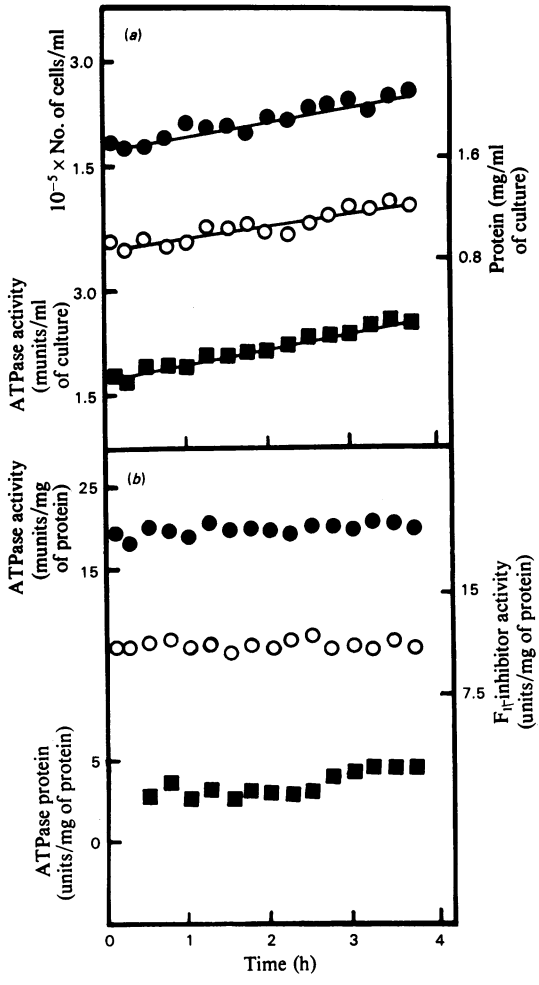

Fig. 5. *Changes in cell numbers, cell protein, ATPase activity, ATPase protein and F_1-inhibitor activity during growth of control asynchronous cultures*

An exponentially growing culture was centrifuged at 800 rev./min for 2 min in the 6 × 1 litre head of an MSE Mistral centrifuge. After centrifugation the pellet and supernatant were remixed and returned to the growth vessel. At frequent time intervals samples were removed for measurement of (*a*) cell numbers (●), cell protein (○) and ATPase activity/ml of culture (■), and (*b*) ATPase activity/mg of protein (●), F_1-ATPase-inhibitor activity/mg of protein (○) and ATPase protein by an immunological method (immunological units/mg of protein) (■).

underlie this phenomenon, are discussed further in several reviews (Thurston, 1972; Halvorson, 1977; Lloyd *et al.*, 1982).

A previous study of inhibitor sensitivity of ATPase activity during the cell cycle of *S. pombe* (Lloyd & Edwards, 1977) identified at least seven distinct inhibitor-binding sites on this enzyme. Clearly, in *A. castellanii*, no such model can be proposed. However, of those inhibitors tested, profiles for I_{50} values for oligomycin, efrapeptin, citreoviridin and quercetin were similar to each other, in that maximum sensitivity was observed when activity was highest.

Thanks are due to Dr. John Edwards for preparation of rabbit anti-(F_1-ATPase) serum. S. W. E. held a University of Wales Postdoctoral Fellowship.

References

Blumenthal, A. K. & Zahler, S. A. (1962) *Science* **135**, 724

Edwards, C. & Jones, C. W. (1977) *J. Gen. Microbiol.* **99**, 383–388

Edwards, C., Spode, J. A. & Jones, C. W. (1978) *Biochem. J.* **172**, 253–260

Edwards, S. W. & Lloyd, D. (1977) *Biochem. J.* **162**, 39–46

Edwards, S. W. & Lloyd, D. (1978) *J. Gen. Microbiol.* **108**, 197–204

Edwards, S. W. & Lloyd, D. (1980) *FEBS Lett.* **109**, 21–26

Edwards, S. W., Chagla, A. H., Griffiths, A. J. & Lloyd, D. (1977) *Biochem. J.* **168**, 113–121

Edwards, S. W., Evans, J. B. & Lloyd, D. (1981) *J. Gen. Microbiol.* **125**, 459–462

Edwards, S. W., Evans, J. B. & Lloyd, D. (1982) *Comp. Biochem. Physiol.* in the press

Halvorson, H. O. (1977) in *Cell Differentiation in Microorganisms, Plants and Animals* (Nover, L. & Mothes, K., eds.), pp. 361–376, Gustav Fischer, Jena

Kagawa, Y. & Racker, E. (1966) *J. Biol. Chem.* **242**, 1788–1792

Klevecz, R. R. (1969) *J. Cell Biol.* **43**, 207–219

Lloyd, D. & Edwards, S. W. (1976) *Biochem. J.* **160**, 335–342

Lloyd, D. & Edwards, S. W. (1977) *Biochem. J.* **162**, 581–590

Lloyd, D., Poole, R. K. & Edwards, S. W. (1982) *The Cell Division Cycle: Temporal Organization and Control of Cellular Growth and Reproduction*, Academic Press, London, in the press

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Martin, D., Tomkins, G. M. & Granner, D. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 248–255

Morgan, N. A., Howells, L., Cartledge, T. G. & Lloyd, D. (1973) *Methodol. Dev. Biochem.* **13**, 219–232

Müller, M. & Møller, K. M. (1969) *Eur. J. Biochem.* **9**, 422–430

Racker, E. & Horstman, L. L. (1967) *J. Biol. Chem.* **242**, 2547–2551

Schatz, G., Penefsky, H. S. & Racker, E. (1967) *J. Biol. Chem.* **242**, 2552–2560

Scott, R. I., Gibson, J. F. & Poole, R. K. (1980) *J. Gen. Microbiol.* **120**, 183–198

Thurston, C. F. (1972) *Process Biochem.* **8**, 18–20

Tzagoloff, A. L. & Meagher, P. (1971) *J. Biol. Chem.* **246**, 7328–7336

Tzagoloff, A., Macino, G. & Sebald, W. (1979) *Annu. Rev. Biochem.* **48**, 419–441