*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 3785–3788, June 1982
Cell Biology

# Temperature-compensated oscillations in respiration and cellular protein content in synchronous cultures of *Acanthamoeba castellanii*

(respiratory control/cell cycle/mitotic oscillator/circadian rhythm)

DAVID LLOYD*, STEVEN W. EDWARDS*, AND JOHN C. FRY†

*Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, Wales; and †Department of Applied Biology, University of Wales Institute of Science and Technology, Cardiff, CF1 3NU, Wales

*Communicated by B. Chance, March 22, 1982*

ABSTRACT **Synchronous cultures of the soil amoeba *Acanthamoeba castellanii*, established by a selection procedure, show significant oscillations of respiration and total cell protein. There was little difference between the period of these oscillations, which averaged 76 min, although the five incubation temperatures used varied between 20°C and 30°C and the cell division time increased from 7.8 to 16 hr. The phase of these oscillations also corresponded approximately at all incubation temperatures. Similar observations made over the whole division cycle at three temperatures indicated that similar oscillations occurred, with a constant period of 65 min, although these data were too variable to show this unequivocally. Control (asynchronous) cultures show that the oscillations are not a consequence of metabolic perturbation produced by the centrifugal selection procedure. It is suggested that these temperature-compensated epigenetic oscillations serve a dual role in cell cycle and circadian timekeeping and that cell cycle time is quantized.**

We have shown, in minimally perturbed synchronous cultures established by a simple and rapid selection procedure (1), that the respiration of the soil amoeba *Acanthamoeba castellanii* ocsillates during the cell division cycle with a period of ≈1 hr and a mean amplitude (peak–trough) of ≈30% (2). Thus, at 30°C, seven respiratory subcycles occur in a cell cycle time of 7.8 hr. Measurements of adenine nucleotide pools (2), redox state of mitochondrial flavoproteins (3), and effects of inhibitors of electron transport and an uncoupler of energy conservation (2) indicate that the respiratory oscillation involves cycles of energization and deenergization of mitochondria. The phase relationships between oscillatory components suggest that the concentration of ADP is the key factor controlling this process of *in vivo* respiratory control (2). Furthermore, total cellular protein content oscillates approximately in phase with respiration, so that tight coupling between energy demand and its supply occurs via the adenine nucleotide system (4).

Processes that occur in living organisms can be classified on the basis of their characteristic relaxation times (5, 6). Those that involve metabolic intermediates have half-times of the order of minutes ("metabolic time domain") whereas those particularly associated with cellular growth in eukaryotes often have half-times of the order of hours ("epigenetic time domain"). The upper limit of the epigenetic domain corresponds to the cell cycle time, and epigenetic processes include biosynthesis of macromolecules and their processing, transport, and interaction [i.e., the consequences of gene expression and altered activities of genes (6)]. We have suggested that the phase relationships and time scales of the oscillations observed here place them in the epigenetic (6), rather than the metabolic, time domain. We have argued, therefore, that the fundamental (driving) oscillator is probably located in a feedback loop involving transcription and translation (7, 8); oscillatory levels of cellular (i.e., mostly ribosomal) RNA support this suggestion. Here we show that cell division cycle-dependent oscillations of respiration and cellular protein content are unlike metabolic oscillations (9) but rather resemble circadian rhythms (10), in that they have a temperature-compensated periodicity.

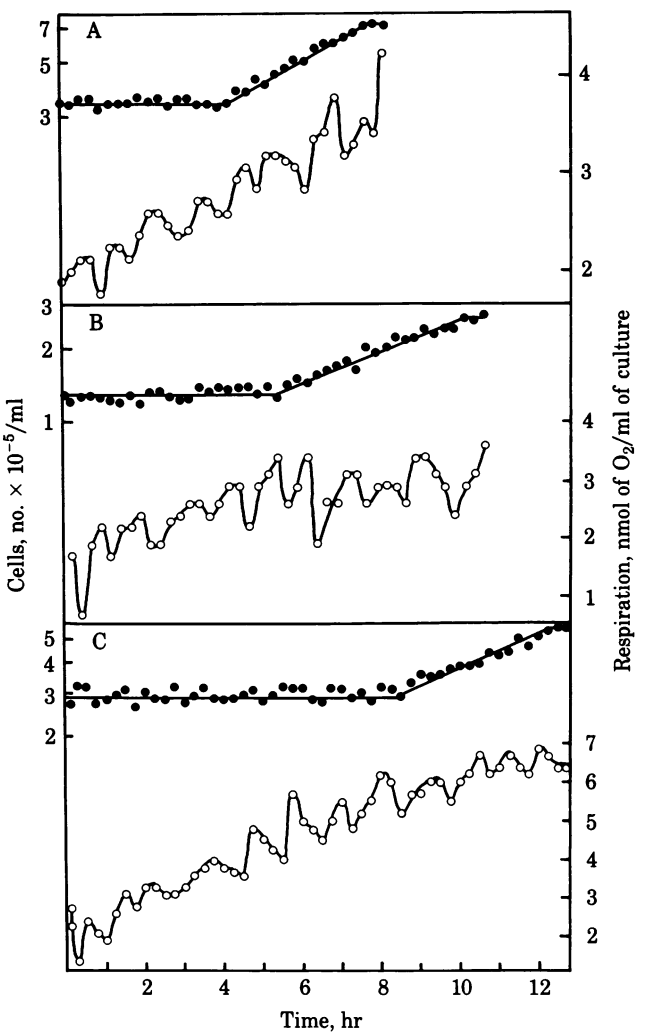


FIG. 1. Growth and respiration of synchronous cultures of *A. castellanii* at 30°C (*A*), 27°C (*B*), and 25°C (*C*). ●, Growth; ○, respiration rate.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

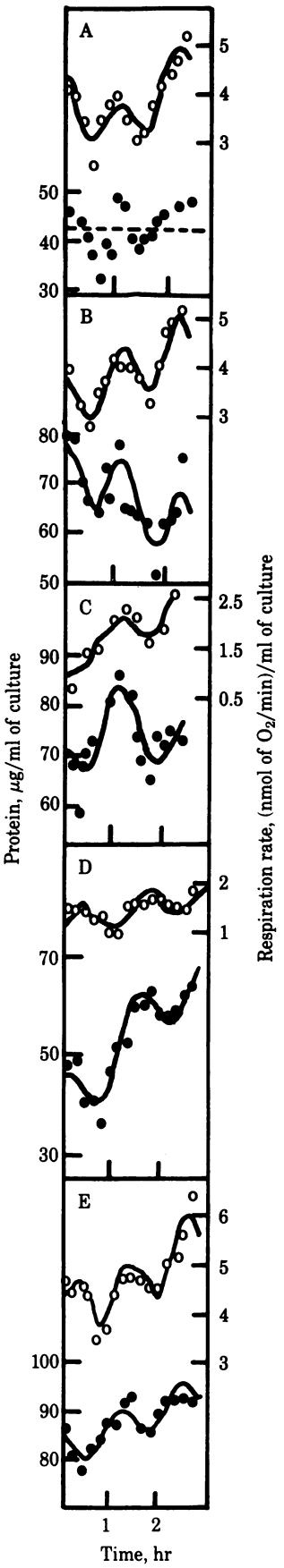


FIG. 2. Protein accumulation and respiration rate in synchronous cultures of *A. castellanii* at 30°C (*A*), 27°C (*B*), 25°C (*C*), 22.5°C (*D*), and 20°C (*E*). ●, Protein synthesis; ○, respiration rate.

## METHODS

**Growth of Cells.** *A. castellanii* was grown axenically on a complex medium in shaken cultures as described (1). Cells were counted in a Fuchs–Rosenthal hemocytometer. Synchronous cultures were prepared by centrifugal selection of the most slowly sedimenting organisms. Cultures in midexponential growth (1–2 × $10^6$ organisms/ml) were centrifuged at room temperature at 300 rpm for 2 min in an MSE bench centrifuge or at 750 rpm for 2.5 min in a 6 × 1 liter rotor of an MSE Mistral centrifuge. Approximately 80% of the supernatant, containing ≈10% of the original population, was carefully decanted into a sterile shaker flask and grown as a synchronous culture. Con-

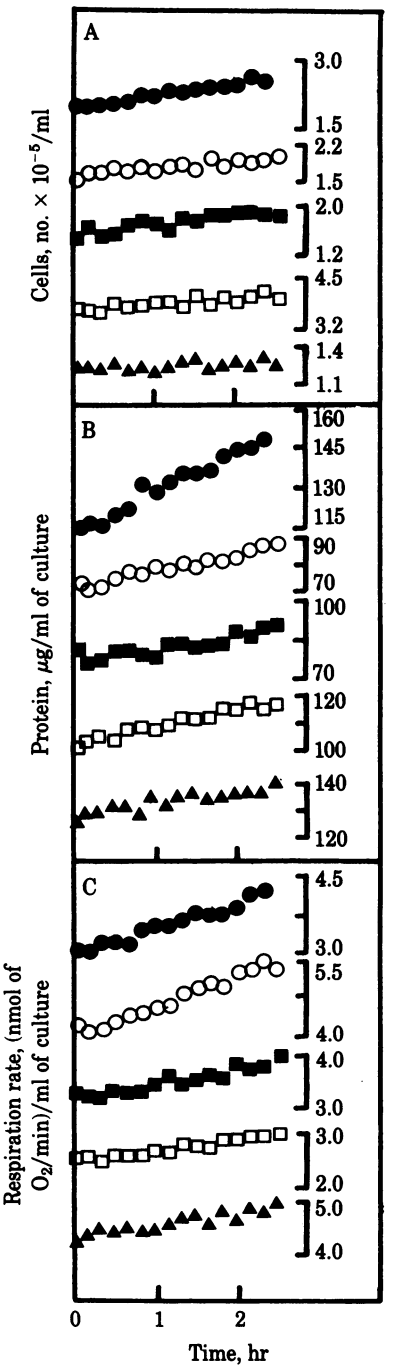


FIG. 3. Growth (*A*), protein accumulation (*B*), and respiration rate (*C*) of control cultures of *A. castellanii* at various temperatures. ●, 30°C; ○, 27°C; ■, 25°C; □, 22.5°C; ▲, 20°C.

Table 1. Periods of significant cycles in respiration and cellular protein over 2.5 hr in synchronous cultures of *A. castellanii* incubated at various temperatures

| | Period of significant cycle, min | | | | |
|---|---|---|---|---|---|
| | 20°C | 22.5°C | 25°C | 27°C | 30°C |
| Cellular protein | 73 (74) | 90 (93) | 86* (70) | 71 (61) | NSC |
| Respiration | 69 (81) | 80 (73) | 81 (85) | 66* (89) | 65; 23† (88) |

Cycles were deemed significant if they removed all the significant serial autocorrelation from the residuals or, in data sets having <15 values, when the Durbin–Watson statistic was >1.8. Values in parentheses are percentage of the variation in the data explained by the time series model. NSC, no significant cycles present in the data.

* Data set having <15 values.

† Two periods are given because two cyclic components were necessary to remove all significant serial autocorrelation from the residuals.

trol (asynchronous) cultures were obtained after identical centrifugal treatment by remixing sedimented organisms with supernatant and diluting (1:10) with conditioned medium.

**Analytical Techniques.** Samples withdrawn from cultures were used directly for polarographic measurement of respiration (11) and, after sedimentation of organisms, total cellular protein content (12).

**Analysis of Data.** Data were analyzed by time-series analysis (13). The multiple regression approach for irregular data was used in short experiments and the spectral analysis approach for regular data was used for longer experiments. ASPECT, a University of Warwick Computer Unit program, was used for the spectral analysis and the Statistical Package for the Social Sciences (14) and Minitab (15) programs were used for all other computations. Mean values of periods are given ±95% confidence intervals and were compared by one-way analysis of variance.

## RESULTS

Growth curves and respiration of synchronous cultures of *A. castellanii* at 30°C, 27°C, and 25°C are shown in Fig. 1. Cell numbers showed a perfect doubling in each case and the cell cycle times were 7.8, 10, and 12 hr, respectively. These cycle times were similar to the doubling times of asynchronous cultures. Synchrony indices, calculated by the method of Blumenthal and Zahler (16), were 0.65, 0.63, and 0.72. It has previously been shown that estimates of cell numbers during the time when cells are dividing are subjective and that a more realistic assessment provided by DNA assays (2, 4) indicates that cell counting leads to underestimation of the degree of synchrony. Respiration doubled overall during the cell cycle time in each culture. Visual inspection of the results indicated that respiration oscillated with a period of ≈1 hr at all three temperatures.

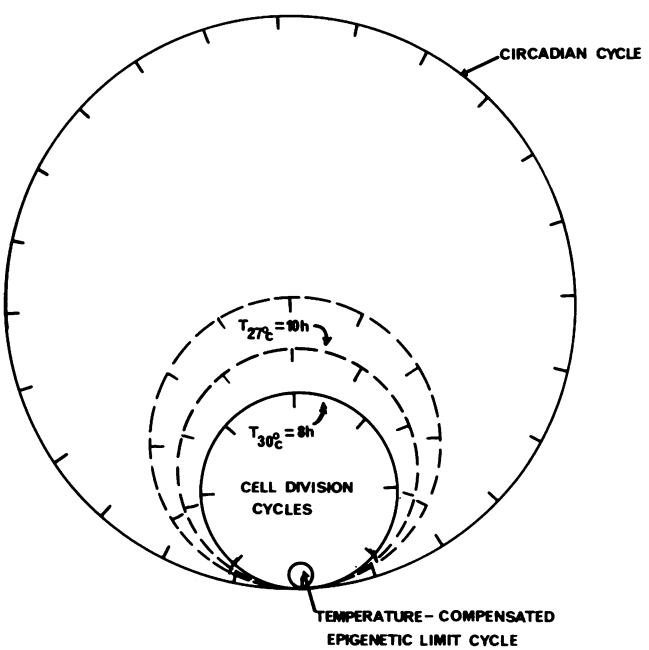


FIG. 4. Model for epigenetic oscillations that serve the dual role of cell cycle and circadian timekeeping. The outer circle represents the circadian cycle (period, ≈24 hr), the small inner circle represents the temperature-compensated epigenetic limit cycle (period, ≈1 hr), and the intermediate circles represent cell division cycles.

Spectral analysis of respiration data after the appropriate parabolic or linear corrections for trend effects showed up to about four peaks in the power spectrum. Only one frequency showed a peak at all three temperatures; this was clearly the major peak at 25°C and 30°C and the second largest peak, by only 15%, at 27°C. These peaks were consistent with a cycle having a period of 65 ± 3 min but were not significant ($P > 0.05$).

Time-series analysis of data from experiments in which sampling was more frequent during the first 2.5 hr of growth of synchronous cultures (Fig. 2) showed the presence of significant ($P < 0.05$) cycles in all but one set of data for both respiration and cellular protein (Table 1). This analysis also showed that the time series models used accounted for a large proportion of the variability in the data. There was little difference between the periods of the major cycles in these models (72 ± 10 min for respiration and 80 ± 15 min for cellular protein) and the mean values were not significantly different ($P > 0.1$).

Phase correspondence occurred with a subcycle in which cellular protein accumulated and then decreased: tight coupling between the two processes was observed over the temperature range 30–20°C and temperature compensation was evident. During the cell cycle, in each case, the increase in total protein approximated a doubling. That the oscillations observed in synchronously dividing cultures are genuinely associated with the undisturbed cell cycle has already been established (2, 4) and is confirmed in Fig. 3. In this series of experiments, exponentially growing cultures were treated by identical procedures but, after centrifugation, the pellet and supernatant were remixed and diluted (1:10) with conditioned medium (i.e., growth medium removed from an exponentially growing culture at the same stage of growth).

The respiration of an asynchronous control culture incubated at 30°C and followed over an entire cell cycle that showed no obvious visual oscillations did not show either a peak in its power spectrum corresponding to a cycle with a period close to 65 min or any other significant peaks. Thus, the observed changes in respiration rates and total protein confirm that the oscillations observed in synchronous cultures were not a consequence of

metabolic perturbation due to the procedure used for establishing cell division synchrony.

## DISCUSSION

An extensive literature describes biological oscillations having periods of the order of seconds or minutes (metabolic oscillations; ref. 9) or of about a day (circadian rhythms; ref. 10). The former represent the inevitable consequence of feedback control in open systems; the latter are of paramount importance in all eukaryotes for accurate timekeeping. It is widely believed that long-period circadian oscillations must be derived from a higher frequency rhythm (10), but none of the metabolic oscillations that have been tested fulfils the criterion of temperature compensation.

Circadian rhythms have been observed in many lower eukaryotes under conditions in which mean generation times are longer than 1 day (17, 18). Under these conditions, the control of cell division is overridden by circadian control to give a circadian cell division time. A model has been proposed to suggest that a mitotic oscillator and the circadian oscillator are closely related (or perhaps even identical) limit cycles operating in two different modes (19). The present results suggest another class of oscillators in *A. castellanii*. These have a period of slightly >1 hr (Table 1) and are thus in the epigenetic time domain. Time-series analysis of the 2.5-hr experiments show unequivocally that similar significant cyclic components exist for both cellular protein synthesis and respiration. The subcycles observed in experiments over complete cell cycles were not significant, because the samples were too infrequent to detect the cycles against the high background variability. However, as the periods of the significant cycles from the 2.5-hr experiments were very similar to the 65-min cycles indicated in the longer experiments, it is likely that the cycles in these longer experiments are real.

Respiration and adenine nucleotide pools show these oscillations, but the fundamental oscillator appears to be in a transcriptional or translational feedback loop. Thus, total cellular protein and RNA contents oscillate and these systems slave the system of mitochondrial energy supply. The coupled oscillators are revealed in synchronous cultures established by a method that causes no measurable perturbation. It is suggested that these epigenetic oscillations may serve the dual role of cell cycle and circadian timekeeping (Fig. 4) as they fulfill the criterion of temperature compensation. The model proposed leads to the prediction of a quantized cell cycle time as has previously been suggested for mammalian cells in culture (20).

Between 30°C and 20°C, the cell cycle time was increased from 7.8 to 16 hr, whereas the period of the oscillations remained approximately constant (Table 1). The oscillations are not obscured by the apparent dispersion in cell cycle times. Alternative possible explanations for this include the following: (*i*) the degree of synchrony is underestimated by cell counts, (*ii*) dispersion arises late in the cell cycle, or (*iii*) an interactive phenomenon reminiscent of that observed in high-frequency glycolytic oscillations (21) operates to mutually entrain respiration and protein synthesis in cells imperfectly synchronized with respect to division.

Other examples of oscillatory accumulation of cellular protein with periods in the 0.3- to 4-hr range have been observed in several systems (18–20, 22–25).

It is remarkable that the process of turnover of cellular proteins should be a temporally discontinuous process, consisting of alternating synthetic and degradative phases, and that switching should be timed by a temperature-compensated mechanism.

Separate temporal compartments (e.g., for protein synthesis and degradation) may be necessary where mutually incompatible biochemical reactions occur in an identical spatial (subcellular) compartment (26). Temporal compartmentation then provides a function for the observed oscillations. These results also suggest that timing takes precedence over considerations of product conservation in the economy of the organism and that the interval between successive cell divisions is an integral multiple of timed subcycles.

D.L. thanks Professor K. Brinkmann and Dr. W. Martin for expert advice. S.W.E. was a recipient of a University of Wales Postdoctoral Fellowship during this research.

1. Chagla, A. H. & Griffiths, A. J. (1978) *J. Gen. Microbiol.* **108**, 39–43.
2. Edwards, S. W. & Lloyd, D. (1978) *J. Gen. Microbiol.* **108**, 197–204.
3. Bashford, C. L., Chance, B., Lloyd, D. & Poole, R. K. (1980) *Biophys. J.* **29**, 1–12.
4. Edwards, S. W. & Lloyd, D. (1980) *FEBS Lett.* **109**, 21–26.
5. Goodwin, B. C. (1976) *Analytical Physiology of Cells and Developing Organisms* (Academic, London).
6. Lloyd, D., Poole, R. K. & Edwards, S. W. (1982) *The Cell Division Cycle: Temporal Organization and Control of Cellular Growth and Reproduction* (Academic, London).
7. Lloyd, D. & Edwards, S. W. (1979) in *Kinetics of Physiochemical Oscillations*, eds. Franck, U. K. & Wicke, E. (Mainz, Aachen, Federal Republic of Germany), Vol. 2, pp. 392–403.
8. Lloyd, D. & Edwards, S. W. (1980) *Ber. Bunsenges. Phys. Chem.* **84**, 417.
9. Chance, B., Estabrook, R. W. & Ghosh, A. (1964) *Proc. Natl. Acad. Sci. USA* **51**, 1244–1251.
10. Hastings, J. W. & Schweiger, A. G. (1975) *The Molecular Basis of Circadian Rhythms* (Dahlem Konferenzen, Berlin).
11. Lloyd, D. & Brookman, J. S. G. (1967) *Biotechnol. Bioeng.* **9**, 271–272.
12. Herbert, D., Phips, P. J. & Strange, R. E. (1971) *Methods Microbiol.* **5B**, 209–344.
13. Fry, J. C., Humphrey, N. C. B. & Iles, T. C. (1981) *J. Appl. Bacteriol.* **50**, 189–224.
14. Nie, H. H., Hull, C. H., Jenkins, J. G., Steinbrenner, K. & Bent, D. H. (1975) *Statistical Package for the Social Sciences (SPSS)* (McGraw-Hill, New York), 2nd Ed.
15. Ryan, T. A., Jointer, B. L. & Ryan, B. F. (1976) *Minitab Student Handbook* (Duxbury, North Scituate, MA).
16. Blumenthal, A. K. & Zahler, S. A. (1962) *Science* **135**, 724.
17. Szyszko, A. H., Prazak, B. L., Ehret, C. F., Eisler, W. J., Jr., & Wille, J. J., Jr. (1968) *J. Protozool.* **15**, 781–785.
18. Edmunds, L. N., Jr., & Funch, R. R. (1969) *Science* **165**, 500–503.
19. Wille, J. J., Jr. (1979) in *Biochemistry and Physiology of the Protozoa*, eds. Levandowsky, M. & Hutner, S. H. (Academic, New York), 2nd Ed., Vol. 2, pp. 67–149.
20. Klevecz, R. R. & Ruddle, F. H. (1969) *Science* **159**, 634–636.
21. Aldridge, J. (1976) in *Handbook of Engineering in Medicine and Biology*, eds. Fleming, D. G. & Feinberg, B. N. (CRC, Cleveland), pp. 57–147.
22. Brodsky, W. Y. (1975) *J. Theor. Biol.* **55**, 167–200.
23. Tsilimigras, C. W. A. & Gilbert, D. A. (1977) *S. Afr. J. Sci.* **73**, 123–125.
24. Mano, Y. (1970) *Dev. Biol.* **22**, 433–460.
25. Mano, Y. (1975) *Biosystems* **7**, 51–65.
26. Sel'kov, E. E. (1980) *Ber. Bunsenges. Phys. Chem.* **84**, 399–402.