OSCILLATIONS OF REDOX STATES IN SYNCHRONOUSLY DIVIDING CULTURES OF *ACANTHAMOEBA CASTELLANII* AND *SCHIZOSACCHAROMYCES POMBE*

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ABSTRACT The redox state of the mitochondria of *Acanthamoeba castellanii* and *Schizosaccharomyces pombe* was assessed with a flying-spot fluorometer (Chance et al. 1978. *Am. J. Physiol.* **235**:H 809) that provides excitation appropriate for oxidized flavoprotein or reduced pyridine nucleotide. Fluorescence signals could be resolved from thin films of cultures that were only one cell deep. In both organisms anoxia was associated with an increased pyridine nucleotide and decreased flavoprotein fluorescence. The addition of mitochondrial uncoupling agents increased the flavoprotein fluorescence and the fluorometer was able to resolve uncoupler-sensitive and uncoupler-insensitive fractions of *S. pombe* cultures. In both synchronous and asynchronous cultures of *A. castellanii* and *S. pombe* the mitochondrial redox state oscillates with a period of 4.5 ± 1.0 min. Oscillations with much longer period, of the order of an hour, are observed in synchronous cultures and these oscillations correlate with similar oscillations in respiratory rate, uncoupler sensitivity, and adenine nucleotide pool sizes. The results are consistent with the hypothesis that synchronous cultures of *A. castellanii* and *S. pombe* oscillate between the ADP-limited (state 4) and ADP-sufficient (state 3) respiratory states, i.e., exhibit in vivo respiratory control.

INTRODUCTION

Cultures of microorganisms synchronized with respect to cell growth and cell division have been used to follow temporally organized biochemical events during the cell cycle. Discontinuous increases in respiration rates and adenine nucleotide pool sizes have been observed in yeast (Poole et al., 1973; Lloyd, 1974) and in protozoa (Edwards et al., 1975; Lloyd et al., 1978). In particular, respiratory oscillations with periods of the order of hours have been reported for synchronously dividing cultures of *Acanthamoeba castellanii* (Edwards et al., 1978), obtained by a method that does not measurably perturb energy metabolism or growth, and for *Schizosaccharomyces pombe* after 2′-deoxyadenosine-induced synchrony (Poole, 1977; Poole and Salmon, 1978). However, in these studies, the rates of oxygen uptake were

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not correlated with the oxidation-reduction state of respiratory chain pigments, although in both organisms the concomitant changes in ATP/ADP ratios and the effects of uncouplers of energy conservation indicate that the respiratory oscillations reflect in vivo respiratory control. In preparations of isolated mitochondria, changes in the rate of respiration induced by ADP (Chance and Williams, 1956) and uncoupling agents (Chance et al., 1963) cause characteristic changes of redox state in the respiratory chain components; similar changes have also been demonstrated in vivo, for example, in suspensions of starved bakers' yeast (Chance, 1959).

Oscillations with periods of the order of an hour, which are observed in synchronous cultures of microorganisms, are classified as epigenetic and are distinguished from oscillations with periods of ~24 h (circadian rhythms) and those of much higher frequency, which have periods of the order of minutes ("metabolic" oscillations). The best characterized metabolic oscillations occur in glycolysis and in mitochondrial respiration. These oscillating systems and their possible relationships with oscillations of longer period, perhaps by frequency diminution mechanisms, have recently been reviewed by Hess and Chance (1978).

Recently a two-dimensional flying-spot scanner has been developed that reads out in real time a histogram display of the distribution of redox states as measured by the fluorescence of mitochondrial flavoprotein or NADH (Chance et al., 1978*a*, *b*). The ratio of the flavoprotein and NADH signals provides an estimate of mitochondrial redox state largely free of distribution and screening errors (Chance et al., 1978*a*). In the experiments reported here the flying-spot scanner has been employed to follow the flavoprotein and NADH fluorescence of cultures of *A. castellanii* and *S. pombe* during synchronous and exponential growth. Oscillations of mitochondrial redox state are found both on short (minutes) and longer (hours) time scales. The oscillations with the longer period seen in synchronous cultures are comparable with those of the respiration rates measured previously.

METHODS

Organisms and Growth Conditions

A. castellanii and *S. pombe* 972 h^- were maintained and growth with shaking at 30°C as previously described (Edwards et al., 1977; Poole et al., 1973; Poole, 1977).

Preparation and Harvesting of Synchronous Cultures

Synchronous cultures of *A. castellanii* were prepared by size selection using the procedures described by Edwards and Lloyd (1978). Synchronous cultures of *S. pombe* were prepared using 2′-deoxyadenosine induction as described by Mitchison and Creanor (1971) and Poole (1977).

Analytical Methods

Flavoprotein and NADH fluorescence was recorded from cell suspensions using a two-dimensional flying-spot scanner (Chance et al., 1978*a*, *b*). Excitation was provided by a Lexel argon ion laser (model 96; Lexel Corp., Palo Alto, Calif.). The combined lines at 351.1 and 363.8 nm were used for NADH excitation and the 457.9-nm line was used to excite flavoprotein fluorescence. The lines were selected by employing appropriate interference filters in the laser beam. NADH fluorescence emission was detected at 460 nm using a suitable filter combination (Kodak Wratten, numbers 2c and 47b; Eastman Kodak Co., Rochester, N.Y., and a Corning 9863 infrared cut-off filter; Corning Glass Works, Corning, N.Y.); flavoprotein emission was monitored through a 20-nm band pass interference filter with peak transmission at 550 nm. The laser spot was focused to a diameter of ~50 μm on cultures that had been

either poured into a petri dish (~1.5 mm depth) or pipetted into the well of a hemocytometer slide (0.2 mm depth). Evaporation was minimized by enclosure in a humidified atmosphere, and in some experiments by covering cultures with a Teflon membrane (DuPont Instruments, Wilmington, Del.). The temperature of the culture in the illuminated area, measured *in situ* by means of a thermocouple, was 28°C. The growth rate of organisms in thin layers and under intermittent laser illumination was indistinguishable from that in flasks shaken in a rotary incubator as described previously (Poole, 1977; Edwards and Lloyd, 1978). Fluorescence emission spectra were recorded in a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) utilizing the front face optical arrangement.

Presentation of Results

Histogram displays present frequency distributions of the intensity of fluorescence amongst 2×10^4 data points, with frequency represented on the ordinate and with intensity, sorted into 64 channels, represented on the abscissa. For each set of experiments the area under the histogram (equivalent to the total number of fluorescence determinations per scan) remained constant. On the figure axes "histogram peak position" refers to the mode of the frequency distribution, and, as the distributions showed little or no skew, closely represents the mean intensity for the population. The position of the histogram mode was measured both from the intensity channel of highest frequency and from the midpoint of the intensity width at half maximal histogram height. The agreement between the measurements, and the reliability of the determination, was better than 1%. The parameter "percent histogram changing" represents the fraction of the histogram area that did not overlap with the control histogram and was calculated from the difference histograms (test-control, see Fig. 3), which were computed from stored information. The area of the control histogram (in arbitrary units) was determined according to the formula: $A = \frac{1}{2} \times$ width $\times$ height of the histogram. The area of the histogram that changes was determined from the difference histograms according to the formula $\Delta A = \frac{1}{2} \times$ width $\times$ height of area above (or below) the baseline; "percent histogram changing" is given by $100 \times \Delta A/A$. For histograms of constant cumulative frequency, as is the case for all the experiments reported here, the areas above and below the baseline of difference histograms are always equal, regardless of changes in dispersion. The error of the triangulation method, ~3%, was assessed independently by cutting and weighing tracings of the histograms.

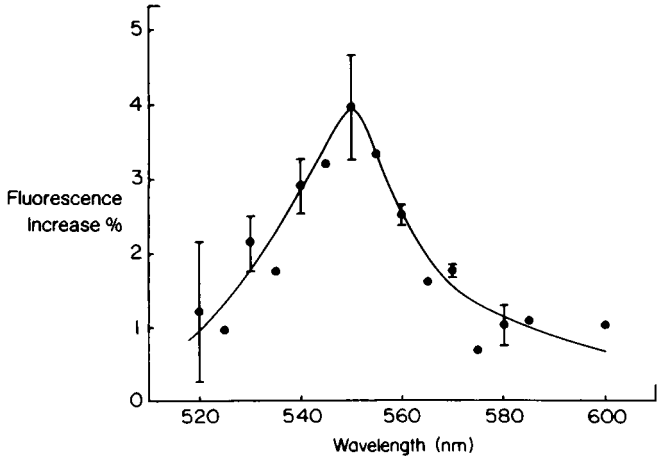

FIGURE 1 The oxidized-reduced fluorescence emission difference spectrum of *A. castellanii*. The change in fluorescence of *A. castellanii* (~5×10^9 cells/ml) suspended in 50 mM $MgCl_2$ during an anaerobic-aerobic transition was recorded as a function of emission wavelength with 458 nm excitation.

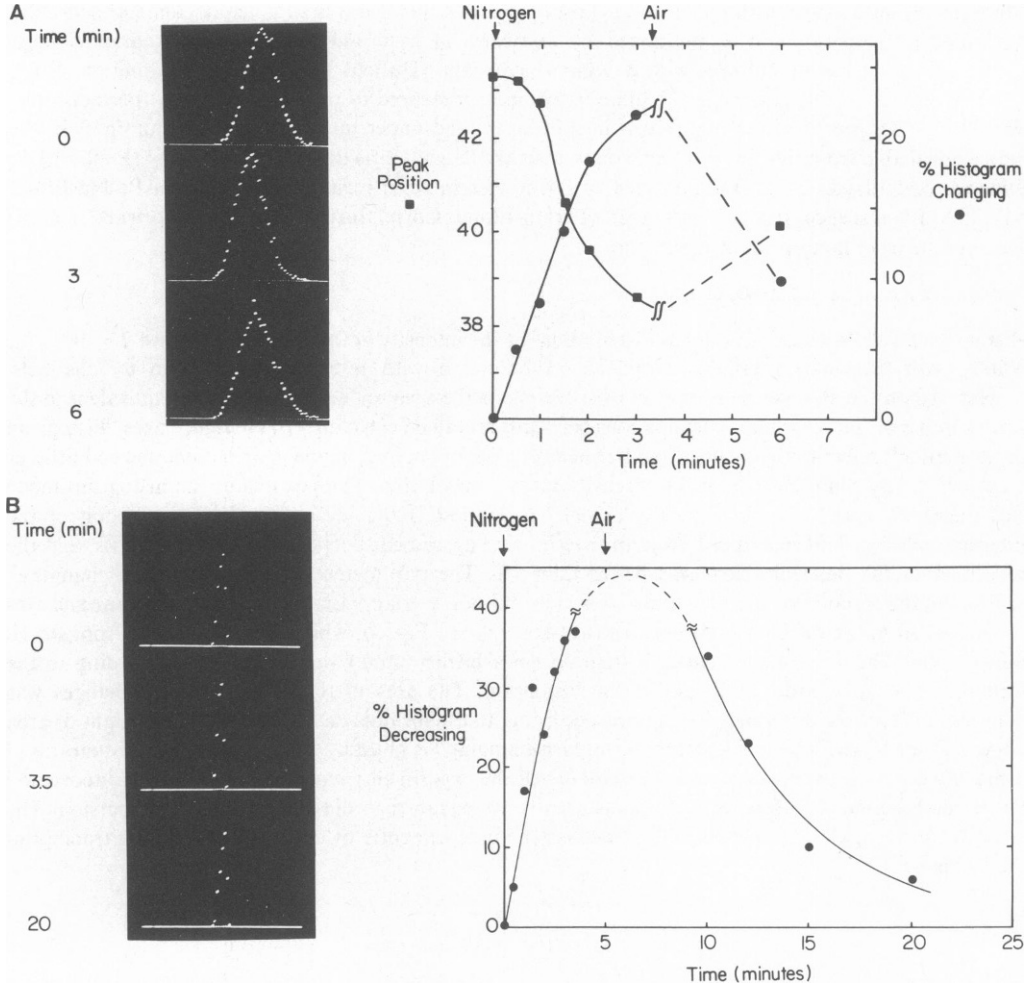

FIGURE 2 The effect of anaerobiosis on the flavoprotein fluorescence emission of *A. castellanii* and *S. pombe*. The emission at 550 nm was recorded with the flying-spot scanner with 457.9 nm excitation. At the start of each experiment the culture was perfused with 100% nitrogen. Air was reintroduced to the culture as indicated. (*A*) *A. castellanii* (6×10^6 cells/ml) from a late-exponential phase culture were pipetted into the well of a hemocytometer slide to give a 0.2-mm film. The area illuminated by the flying spot contained 1.5×10^4 cells. (*B*) *S. pombe* (5×10^6 cells/ml) from an exponentially growing culture were poured into a petri dish to a depth of 3 mm. The illuminated area contained 10^5 cells. The mode of the fluorescence distribution shifted to 4% lower intensity during anoxia.

Chemicals

Carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP) and α, α'-bis (hexafluoroacetonyl)-acetone (1799) uncouplers of mitochondrial energy conservation were the kind gift of Dr. W. D. Bonner, Jr., University of Pennsylvania School of Medicine. All other chemicals were of reagent grade.

RESULTS

Fig. 1 illustrates the fluorescence emission spectrum of *A. castellanii* during an anaerobic-aerobic transition. With 458 nm excitation the changes in emission reached a maximum at

550 nm, showing a 4% increase during aerobiosis. The spectrum closely resembles that of the flavoproteins of isolated avian mitochondria (Chance et al., 1978*a*). Typical records of the anaerobic-aerobic transition monitored by the flying-spot scanner are shown in Fig. 2. When the cultures are exposed to a nitrogen atmosphere the flavoprotein fluorescence frequency distribution shifts to the left in response to the decreased fluorescence of reduced flavoprotein. Taking the normoxic control histograms as 100%, the decrease in flavoprotein fluorescence during the anoxia was 9 and 4%, judged by the mode of the distributions, and 22 and 38%, judged by the area change, for *A. castellanii* and *S. pombe*, respectively. The fraction of the histogram changing during the normoxic to anoxic transition proceeded exponentially with a halftime of ~1 min. However, particularly in shallow cultures, the change in the mode of the distribution was not exponential, there being <1% decrease during the first minute of anoxia.

The effect of uncouplers of mitochondrial energy conservation on the flavoprotein fluorescence of both *A. castellanii* and *S. pombe* cultures is illustrated in Fig. 3. The addition of uncoupler at the edge of the illuminated area leads to an immediate shift of the flavoprotein fluorescence distribution to higher intensities. The shift of the distribution is exemplified by the difference histograms that represent the distribution in the presence of uncoupler minus

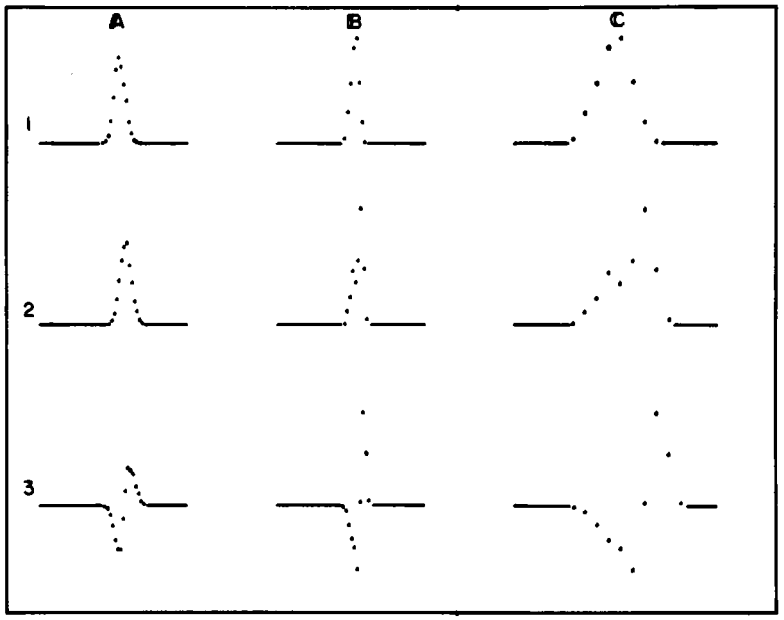

FIGURE 3 The effect of mitochondrial uncouplers on the flavoprotein fluorescence emission of *A. castellanii* and *S. pombe*. Fluorescence emission was recorded as indicated in the legend to Fig. 2. (*A*) *A. castellanii* (6×10^6 cells/ml) from a late-exponential phase culture were poured into a petri dish to a depth of 1.5 mm. The uncoupler FCCP was added to give a final concentration of 1.25 μM. (*B*) *S. pombe* (6×10^6 cells/ml) from an exponential culture were poured into a petri dish to a depth of 2 mm. The uncoupler 1799 was added to give a final concentration of 20 μM. (*C*) Histograms presented in (*B*) are redrawn with the abscissa expanded threefold. (1) Control histograms. (2) Histograms recorded 1 min after the addition of uncoupler to the edge of the illuminated area. (3) Difference histograms representing the distribution in the presence of uncoupler minus the distribution in the absence of uncoupler. In the presence of FCCP the percent histogram increasing for *A. castellanii* is 30; for *S. pombe* in the presence of 1799 the value is 44. The proportion of the *S. pombe* population not affected by 1799 was approximated from the peak heights of the two populations (height 1)/(height 1 + height 2), giving a value of 0.30.

the control distribution. The fraction of the distribution that changes in the presence of uncoupler is given by the area of the distribution above (or below) the baseline in the difference histogram divided by the total histogram area. The areas were approximated by triangulation (see above) and values of 0.30 and 0.44 were obtained for the fraction of the histograms that shifted to higher fluorescence for *A. castellanii* and *S. pombe*, respectively. In the presence of FCCP the *A. castellanii* culture behaved as a single population; the distribution shifted to higher intensities with no significant change of shape. On the other

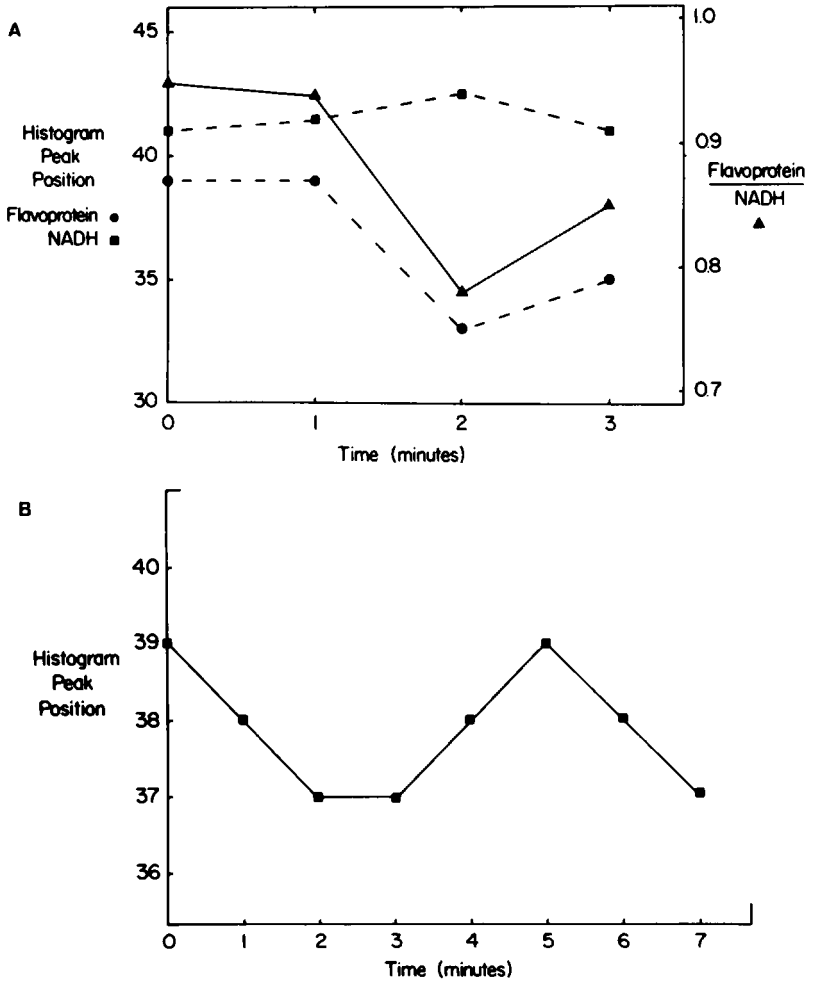

FIGURE 4 Oscillations of the flavoprotein and pyridine nucleotide fluorescence emissions of a synchronous culture of *S. pombe*. (*A*) 1.1 × 10^7 cells/ml were poured into a petri dish to a depth of 1.5 mm, 75 min after the addition of 2′-deoxyadenosine. Flavoprotein fluorescence was recorded as indicated in the legend for Fig. 2; NADH fluorescence was recorded by using 351.1 plus 363.8 nm excitation and by monitoring the emission at 460 nm. The position of the mode of the fluorescence frequency distribution (histogram peak position) was measured in arbitruary units. The flavoprotein/NADH ratio was determined from the ratio of the positions of the modes of the fluorescence distributions. (*B*) 1.55 × 10^7 cells/ml were poured into a petri dish to a depth of 1.5 mm 2.5 h after the addition of 2′-deoxyadenosine. The mode of the NADH fluorescence emission distribution was determined as above.

hand, the uncoupler 1799 caused the *S. pombe* population to split into two distinct classes, one of which retained a fluorescence distribution similar to that of the control, while the other, ~70% as judged by the relative frequency of the modes of the two populations, shifted to higher intensities (Fig. 3 *C*).

Fig. 4 illustrates the variations of flavoprotein and NADH fluorescence in cultures of *S. pombe* synchronized by treatment with 2′-deoxyadenosine. On a time scale of minutes changes in NADH fluorescence were accompanied by reciprocal changes of flavoprotein fluorescence and the ratio of the modal values of fluorescence (flavoprotein/NADH) exhibited a minimum during the experiment. The decrease of the flavoprotein/NADH ratio reflects an increase in the reduction state of the respiratory chain components (Chance et al., 1978*a*). In a series of eight experiments with both synchronous and asynchronous cultures of both *A. castellanii* and *S. pombe* the fluctuations in mitochondrial redox state had a period of 4.5 ± 1.0 (SD) min. No variations in the modes of the flavoprotein and NADH fluorescence distributions were detected over short time intervals (seconds), although with *S. pombe* there were suggestions that the dispersions of the frequency distributions might vary on this time scale.

Slower fluctuations of the flavoprotein fluorescence of a synchronized culture of *A. castellanii* are illustrated in Fig. 5. Histograms were recorded at a frequency close to that of the more rapid variations in an attempt to identify a longer term periodicity. The culture shows an oscillation with a period of 45 min which is absent in an exponentially growing culture. A similar periodicity can be seen in 2′-deoxyadenosine-synchronized cultures of *S. pombe* (Fig. 5), although in this case there were also significant, but not obviously periodic, fluctuations in the exponentially growing control culture. In the *S. pombe* population which was growing exponentially, the variations in flavoprotein/NADH ratio arose primarily from NAD(P)H without a significant contribution from flavoprotein (data not shown), suggesting a nonmitochondrial origin for the observed fluctuations.

DISCUSSION

The fluorescence emission spectrum of *A. castellanii* illustrated in Fig. 1 closely resembles that found for preparations of isolated mitochondria under similar conditions (Chance et al., 1978*a*). This observation identifies the mitochondria of the cells as the prime locus for flavoprotein fluorescence and suggests that, as in animal tissues, the highly fluorescent flavoprotein is localized in the low potential pyruvate and α-ketoglutarate dehydrogenases. The reduced pyridine nucleotide signal contains contributions from NADH in the mitochondrial and cytosolic spaces and also from NADPH. In perfused rat liver approximately half the signal originates from mitochondrial NADH (Chance et al., 1978*b*), but the relative contributions to the signals observed in this report have not been determined. However, in suspensions of bakers' yeast (Chance, 1959), the reduced pyridine nucleotide signal responds to changes in ADP levels in the same fashion as isolated mitochondria, suggesting that in these organisms the mitochondrial contribution to the fluorescence predominates.

At low cell densities and with a well-focused spot it is possible for the flying-spot scanner to record fluorescence from single cells. In the experiments illustrated in Fig. 2 the scanner accumulated 20,000 data points for each histogram. In Fig. 2 *B* there were approximately 10^5 cells in the scanned area or about five cells per data point; under these conditions there was a

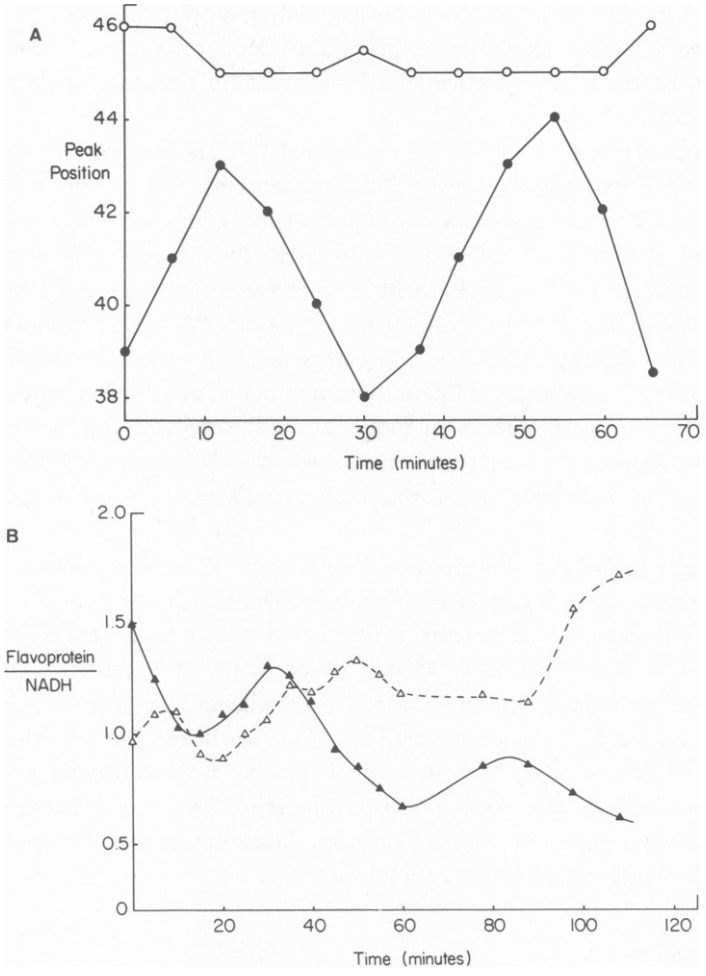

FIGURE 5 Oscillations of redox state in cultures of *A. castellanii* and *S. pombe*. Experimental conditions were as indicated in the legend for Fig. 4. (*A*) The modal position in arbitrary units of the flavoprotein fluorescence for both synchronous (•) and asynchronous (○) cultures of *A. castellanii*. The exponential culture contained 4.6×10^6 cells/ml at time zero. The synchronous culture was obtained by size selection of 10% of the original population and then concentrated tenfold. The selection procedure was completed 10 min before scanning was commenced. (*B*) *S. pombe* samples were removed from either an exponential (△) or a culture treated with 2′-deoxyadenosine (▲) and poured into a petri dish to a depth of 1.5 mm. The former experiment was started when the cell count in the exponential culture was 5.5×10^6 ml^{-1}. 2′-deoxyadenosine was added 3 h before the start of the experiment shown by the closed symbols; cell numbers during the period shown remained at 1.1×10^7 ml^{-1}.

very narrow distribution of redox states. In Fig. 2 *A* the number of cells in the area scanned was reduced to 1.5×10^4 and a much broader frequency distribution was recorded. In the latter case the heterogeneity can arise in at least two ways: not all cells will have the same fluorescence and not all data points will be collected from cells illuminated in the center of the spot. The heterogeneity imposed by the sampling procedure can be overcome both by increasing the size of the spot and by slowing down the scan rate. It is clear, however, that the

techniques can be used to record signals from cell suspensions that are only one cell deep (Fig. 2 *A*) and that the fluorescence distribution contains data recorded from single cells.

At low cell densities the flavoprotein fluorescence distribution of *A. castellanii* becomes noticeably narrower under anaerobic conditions, indicating a decrease in heterogeneity of redox states. In the presence of oxygen the histogram recorded from an exponentially growing culture reflects the heterogeneity of the population that must contain cells at all stages of the cell cycle. During anoxia only a single state (i.e., complete reduction) is possible and the frequency distribution narrows. The onset of anoxia is associated first with the disappearance of small numbers of highly fluorescent cells from the right-hand limit of the distribution. This change is clearly evident in the difference histograms and hence the fraction of the histogram area that changed. However, the number of cells that become anaerobic quickly is not large enough to affect the mode of the frequency distribution. The time-course of the change in the mode consequently lags behind the fraction of the histogram area that is changing (Fig. 2 *A*). Similarly, during the recovery from anoxia, the first cells to recover are those at the right-hand (high intensity) limit of the distribution.

The respiratory chain becomes reduced during anoxia, leading to increased pyridine nucleotide and decreased flavoprotein fluorescence. On the other hand, uncouplers of mitochondrial energy conservation increase the rate of oxygen consumption by the respiratory chain and the components become more oxidized (Chance et al., 1963). Two different uncouplers increase the flavoprotein fluorescence of *A. castellanii* and *S. pombe* (Fig. 3), further confirming the mitochondrial origin of the signals. Significantly, the flying-spot scanner can resolve two distinct classes of cells in the *S. pombe* culture in the presence of an uncoupler, which represent ~30 and 70% of the total population, respectively (Fig. 3 *C*). The smaller fraction, which has a lower flavoprotein fluorescence than the larger fraction, appears to have a frequency distribution similar to that of the control population. In this experiment ~30% of the population was insensitive to uncoupler, presumably reflecting those cells that are at a stage of the cell cycle where uncouplers do not stimulate respiration (Poole et al., 1973). In contrast, the *A. castellanii* population responded to uncoupler in an apparently homogeneous fashion. This difference may arise because the sensitivity of *A. castellanii* respiration to uncouplers oscillates during the cell cycle with an amplitude (0–60% stimulation, Edwards and Lloyd, 1978) that is much smaller than that in the equivalent oscillations in the *S. pombe* cell cycle 0–300% stimulation, Poole et al., 1973). However, both oscillations have a similar period of ~1 h.

Oxygen consumption rates, measured in samples removed at ~10-min intervals from synchronous cultures of *A. castellanii* and *S. pombe*, oscillate with a periodicity of ~1 h (Poole, 1977; Edwards and Lloyd, 1978), although they have very different cell cycle times. Oscillations with a similar period also occur in the redox states of mitochondrial flavoprotein and pyridine nucleotide (Fig. 5) when the states are assessed at 5-min intervals. Oscillations with periods of ~1 h have been assigned to the epigenetic time domain (e.g., Poole, 1977; Edwards and Lloyd, 1978; Hess and Chance, 1978) and do not appear, in the experiments reported here, to be the result of aliasing (Gilbert, 1974), an effect caused by discontinuous sampling of a continuous periodic function, as similar periodicities are obtained at different sampling intervals in the range 5–20 min.

Oscillations in the epigenetic time domain were observed only in synchronous cultures of *A.*

castellanii and *S. pombe*, whereas more rapid, metabolic, oscillations with a period of ~5 min were observed in synchronous and asynchronous cultures of both microorganisms. Oscillations with a similar period have been observed in isolated mitochondria, and the possible relationship between metabolic and longer oscillations has been reviewed reeently by Hess and Chance (1978). At present, no experimental data is available from unperturbed synchronous cultures that might indicate a higher-frequency (5 min) oscillation in respiratory rate, adenine nucleotide pool, and uncoupler sensitivity. However, periodic respiratory rates are associated with periodic changes in the redox state of the respiratory chain, although further work is required to determine the involvement of higher frequency oscillations in redox states. Our results are consistent with the previous proposals that in synchronous *A. castellanii* cultures, which have not been significantly perturbed, and in 2′-deoxyadenosine-induced synchrony of *S. pombe*, organisms oscillate between the ADP-limited (state 4) and ADP-sufficient (state 3) respiratory states, i.e., exhibit the phenomenon of respiratory control (Chance and Williams, 1956).

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