Respiratory Oscillations and Heat Evolution in Synchronous Cultures of Candida utilis

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Synchronous cultures of the budding yeast Candida utilis prepared by continuous-flow size selection showed respiratory oscillations when the energy source was either glucose, acetate or glycerol. The period of the oscillations was about one-third of the cell cycle time (i.e. about 0.5 h). No fluctuations in heat evolution could be detected. In organisms growing with acetate or glycerol, the effects of cyanide, N,N'-dicyclohexylcarbodi-imide and carbonyl cyanide m-chlorophenylhydrazone (maximum inhibition of respiration at respiratory maxima, maximum uncoupling of energy conservation at respiratory minima) suggest that the control mechanism responsible for the oscillations is mitochondrial respiratory control in vivo. The effects of cyanide and N,N'-dicyclohexylcarbodi-imide on the respiration of cultures growing synchronously with glucose were different from those for cultures growing with the non-fermentable substrates; this suggests that the mitochondrial respiratory system interacts with the early reactions of glucose utilization.

INTRODUCTION

Changes in oxygen consumption rates during the growth of synchronous cultures of eukaryotic micro-organisms provide insights into mechanisms of development and control of mitochondrial activities (see Lloyd, 1974). It is essential in such studies to prepare synchronously dividing populations by a procedure which does not disturb their energy metabolism; synchrony with minimal perturbation of respiratory rates and adenine nucleotide pools can be achieved using either the continuous-flow selection method (Lloyd et al., 1975) or, in the case of Acanthamoeba castellanii, by a low force-time centrifugal field in tubes (Chagla & Griffiths, 1978). In these synchronously dividing populations the rate of respiration was found to oscillate with a period of about 1 h in both Tetrahymena pyriformis (Lloyd et al., 1978) and A. castellanii (Edwards & Lloyd, 1978), when the protozoa were growing in complex media. In the present investigation we have studied the development of respiration in continuous-flow selected synchronous cultures of Candida utilis growing in chemically defined media with either glucose, glycerol or acetate as sole source of carbon and energy for growth. Although the periodicity of the respiratory oscillations is similar with different carbon sources, the effects of KCN and of N,N'-dicyclohexylcarbodi-imide (inhibitors of electron transport and energy transfer, respectively) are different on cells growing with glucose from those observed for organisms growing with the non-fermentable carbon sources.

METHODS

Abbreviations. CCCP, Carbonyl cyanide m-chlorophenylhydrazone; DCCD, N,N'-dicyclohexylcarbodi-imide.

Maintenance and growth of the organism. Candida utilis NCYC 193 was maintained on 10% (w/v) glucose/malt extract/agar slopes at 2 °C. Stock cultures were subcultured monthly and maintained at 2 °C.

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The chemically defined media contained (per litre): 10 g glucose or 12.2 g glycerol or 3.8 g sodium acetate (anhydrous), 5 g (NH₄)₂SO₄, 2 g NaH₂PO₄·2H₂O, 100 ml salts solution, 1-0 ml trace elements solution and 0.25 mg biotin. The salts solution contained (per litre): 10 g KCl, 10.8 g MgCl₂, 132 mg CaCl₂, 2H₂O and 24 mg CoCl₂·6H₂O. The trace elements solution contained (per litre): 50 mg H₃BO₃, 52.8 mg MnSO₄·4H₂O, 40 mg ZnSO₄·7H₂O, 20 mg FeCl₃, 16 mg H₃MoO₄·H₂O, 10 mg KI, 4 mg CuSO₄·5H₂O and 100 mg citric acid. NaH₂PO₄ and glucose solutions were autoclaved separately, and biotin was sterilized by filtration.

Starter cultures (250 ml in 1 l conical flasks) were inoculated from stock cultures and shaken at 75 rev. min⁻¹ on a rotary shaker at 30 °C. Batch cultures (8 l) were grown in a 14 l Microferm Fermentor (New Brunswick Scientific Co.) at 30 °C, with aeration at 1 l air min⁻¹ (l culture)⁻¹ and stirring at 400 rev. min⁻¹. Organisms were counted following the criteria of Williamson & Scopes (1960) in a Thoma haemocytometer slide (Hawksley, Lancing, Sussex) after suitable dilution. A particle with a single bud was scored as one cell, and a particle containing two or three buds was counted as two cells.

Preparation of synchronous cultures. Synchronous cultures were prepared by the continuous-flow selection method of Lloyd et al. (1975), using a flow rate of 420 ml min⁻¹ and a rotor speed of about 3500 rev. min⁻¹. Small adjustments were made to the rotor speed until exactly 10% of the original population escaped harvesting in the rotor before collection of the selected population was begun.

Assessment of synchrony. The degree of synchrony was assessed by the synchrony index (F) of Elumenthal & Zahler (1962), calculated from the equation:

\[ F = \frac{(N/N₀)}{2^{1/2}} \]

where \( F \) has a maximum value of 1·0 in a culture exhibiting theoretically perfect synchrony, \( N \) is the number of organisms at time \( t \), \( N₀ \) is the number of organisms at zero time and \( g \) is the mean generation time. In the presentation of results, vertical lines indicate the mid-points of doublings in cell numbers, and \( F₁ \) and \( F₂ \) denote the synchrony indices of the first and second doublings in yeast numbers, respectively.

Measurement of oxygen uptake. Samples were removed at 10 min intervals during growth and oxygen uptake was measured polarographically (Lloyd & Brookman, 1967). KCN was used as an aqueous solution (0.01 M). DCCD (10 mg ml⁻¹) was dissolved in 10% (v/v) dimethylformamide, as was CCCP (0.2 mg ml⁻¹).

Measurements of heat evolution. Measurements were made as described by Poole et al. (1973). The culture was pumped from the culture vessel at a constant rate of 54 ml h⁻¹ to a T-piece, where it was mixed with a constant flow of air (54 ml h⁻¹). The temperature of the thermostatic air bath was 30 °C; water at 15 °C was used to assist dissipation of heat from the instrument. The instrument was calibrated at the end of the experiment, with aerated medium flowing through the cell, by applying an internal current of 1·0 or 1·5 mA for 800 or 1000 s to achieve a steady state. The calibration was recorded in μJ s⁻¹.

Chemicals. KCN and DCCD were from BDH; CCCP was from Calbiochem.

RESULTS

Respiration during growth of asynchronous cultures

In asynchronous cultures growing on glucose, acetate or glycerol as sole carbon sources, cell numbers and respiration rates increased exponentially, with no detectable sign of perturbation even over the first 30 min after resuspension (Fig. 1). The maximum deviation from the best-fit line on the respiration plot was 3.3%. Mean generation times for cultures containing glucose, acetate or glycerol were 96, 122 and 105 min, respectively; corresponding times for doubling of oxygen consumption rates were 90, 96 and 105 min. The respiration of cultures growing exponentially with glucose or glycerol was 50% inhibited by 50 μM-KCN, whereas only 30 μM-KCN was required for a similar degree of inhibition in acetate-containing cultures. Complete inhibition of respiration of cultures growing on glucose, glycerol or acetate was produced by 150 μM-KCN. Concentrations of DCCD giving 50% inhibition were 75, 80 and 30 μM for cultures containing glucose, glycerol or acetate, respectively. Maximum uncoupling of respiration of all three types of culture was observed with 3 μM-CCCP.

Respiration of synchronous cultures and the effects of inhibitors and an uncoupler

The pattern of increase in respiration in a culture growing synchronously with glucose is shown in Fig. 2. The first mid-point of increase in cell numbers occurred after 94 min, and an exact doubling was observed after 130 min. Synchrony indices for the first and
second cycles were identical. The rate of respiration of the culture increased exponentially overall, but an oscillation superimposed upon this baseline showed a peak to trough amplitude of between 30 and 60% of the minimum respiration rates. The average periodicity was 26 min. Addition of 10 μM-KCN to samples withdrawn from the culture at respiratory minima gave almost 50% inhibition of oxygen uptake rates, whereas this concentration of KCN gave only about 30% inhibition of respiration in samples respiring maximally.

DCCD (75 μM), an inhibitor of energy transfer at the level of the ATP synthetase reaction (Kováč et al., 1968), also showed a differential effect depending on whether samples were tested at respiratory maxima or minima (Fig. 3). As with cyanide, sensitivity to inhibition was most marked when respiratory rates were at their minimum values.

Maximum stimulation of the respiration of cultures growing synchronously with glucose by the uncoupling agent CCCP (3 μM) was observed when this compound was added at respiratory minima. At these times over 60% acceleration of oxygen consumption rates was observed, in contrast to the small extent of uncoupling (20 to 30% stimulation of respiration) observed when CCCP was added at respiratory maxima. A similar result was
Fig. 3. Effect of DCCD on respiratory oscillations in a synchronously dividing culture of *C. utilis* with glucose as carbon source. The synchronous culture initially contained 10% of the original population of the exponentially growing culture. (a) Cell numbers; (b) respiration rate in the absence of DCCD; (c) respiration rate in the presence of 75 μM-DCCD; (d) inhibition of respiration (%) resulting from addition of DCCD.

Fig. 4. Effect of DCCD on respiratory oscillations in a synchronously dividing culture of *C. utilis* with acetate as carbon source. The synchronous culture initially contained 10% of the original population of the exponentially growing culture. (a) Cell numbers; (b) respiration rate in the absence of DCCD; (c) respiration rate in the presence of 30 μM-DCCD; (d) inhibition of respiration (%) resulting from addition of DCCD.

Fig. 5. Effect of cyanide on respiratory oscillations in a synchronously dividing culture of *C. utilis* with glycerol as carbon source. The synchronous culture initially contained 10% of the original population of the exponentially growing culture. (a) Cell numbers; (b) respiration rate in the absence of KCN; (c) respiration rate in the presence of 50 μM-KCN; (d) inhibition of respiration (%) resulting from the addition of KCN.
obtained previously with organisms synchronized by selection on a sucrose gradient (R. K. Poole & D. Lloyd, cited by Lloyd, 1974).

The changes in respiration rate in a synchronous culture growing with acetate are shown in Fig. 4; the time between mid-points in cell doublings in this experiment was 120 min. The respiration rate increased exponentially overall but, again, superimposed on this pattern was an oscillation with a periodicity of about one-third of the cell cycle. Addition of 30 μM-DCCD to culture samples gave about 90% inhibition of respiration at a respiratory maximum, but only 25 to 40% inhibition at a respiratory minimum. Addition of 30 μM-KCN similarly produced a greater degree of inhibition at a respiratory maximum. These inhibitor effects were therefore quite different from those observed with synchronous cultures growing with glucose. Uncoupling of the reactions of energy conservation in acetate-grown cultures with 30 μM-CCCP gave between 30 and 50% stimulation of respiration at respiratory minima but produced no detectable acceleration of respiration at respiratory maxima (not shown).

In synchronous cultures growing with glycerol, the effects on respiration of inhibition of electron transport, energy transfer and uncoupling of energy conservation by KCN, DCCD and CCCP, respectively, were qualitatively similar to those observed for acetate-containing cultures. For the culture in Fig. 5, the mean period of the respiratory oscillation was 33 min and the peak–trough amplitude of the oscillation was between 22 and 100% of the minimum value. The overall increase was exponential and the respiration rate approximately doubled over each cell cycle (95 min). Maximum inhibition (70%) by 50 μM-KCN observed at respiratory maxima contrasted with the low inhibition at respiratory minima (10 to 30%). DCCD (80 μM) also inhibited respiration preferentially at respiratory maxima. As with cells growing synchronously with glucose or acetate, CCCP (3 μM) gave most marked uncoupling of respiration at respiratory minima.

**Heat evolution in synchronous cultures**

The rate of heat evolution by a culture growing synchronously with glycerol increased smoothly (and almost linearly) through three cycles of growth and division (Fig. 6). Respiration rates showed the usual oscillation with a period of about one-third of the cell cycle. Similar results were obtained with cultures growing synchronously with glucose or acetate; we could not detect any fluctuations in the rates of heat dissipation irrespective of the carbon source utilized.

**DISCUSSION**

Synchronous cultures of *C. utilis* established by continuous-flow centrifugal size selection show respiration oscillations. Comparison with the smoothly exponential increases in respiration of asynchronously dividing control cultures exposed to a higher centrifugal force than that employed for selection during establishment of synchrony confirms that these oscillations do not arise as a consequence of metabolic perturbation by the selection procedure employed. It has been noted, however, that relatively short periods of anaerobiosis (15 min or more) can induce respiratory oscillations which differ in two respects from the cell cycle-dependent oscillations observed here (Lloyd & Ball, 1979): (i) their period is about 5 min, and (ii) they are highly damped and are not detectable after 50 min.

The cell-cycle dependent oscillations occur in cultures growing synchronously with either glucose, acetate or glycerol as sole carbon source. Thus *C. utilis* differs in this respect from *Schizosaccharomyces pombe*, which showed oscillations in respiration when grown with glucose (Poole et al., 1973) but not with glycerol (Poole & Lloyd, 1974), and also from *Saccharomyces cerevisiae*, which showed respiratory oscillations in glucose-grown but not in maltose-grown synchronous cultures (Dharmalingam & Jayaraman, 1973).

Table 1 summarizes the cell-cycle times and effects of KCN, DCCD and CCCP on *C. utilis* growing with the three different carbon sources. The response of acetate- and
Fig. 6. Oxygen uptake and heat evolution of a synchronously dividing culture of C. utilis with glycerol as carbon source. The synchronous culture initially contained 10% of the original population of the exponentially growing culture. (a) Cell numbers; (b) respiration rate; (c) heat evolution measured continuously using a flow microcalorimeter.

Table 1. Cell cycle times and the effects of KCN, DCCD and CCCP on the respiration of synchronous cultures of C. utilis

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>First cycle</th>
<th>Second cycle</th>
<th>Effect of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>94.6 ± 17.4 (10)</td>
<td>102.8 ± 12.1 (9)</td>
<td>− − −</td>
</tr>
<tr>
<td>Acetate</td>
<td>110.8 ± 16.4 (12)</td>
<td>118.1 ± 12.1 (11)</td>
<td>+ + −</td>
</tr>
<tr>
<td>Glycerol</td>
<td>91.4 ± 11.3 (9)</td>
<td>98.2 ± 9.5 (7)</td>
<td>+ + −</td>
</tr>
</tbody>
</table>

glycerol-grown cultures to the cytochrome oxidase and ATP synthetase inhibitors and to the uncoupler of energy conservation can be explained on the basis that the respiratory oscillation reflects *in vivo* mitochondrial respiratory control (Chance & Williams, 1956), as was shown to be the case in *A. castellanii* (Edwards & Lloyd, 1978). Thus the rate of respiration is primarily determined by the energetic requirements of biosynthetic reactions. The effect of CCCP on glucose-grown cultures is also consistent with this explanation, but the effects of KCN and DCCD on such cultures are anomalous. Secondary interactions between the products of mitochondrial energy conservation and the initial reactions of glucose catabolism via feedback loops and allosteric control sites complicate the interpretation of the effects of mitochondrial inhibitors on the respiration of glucose in growing organisms (see also Poole *et al.*, 1973). No fluctuations in heat evolution could be detected by flow microcalorimetric monitoring of synchronous *C. utilis* cultures: oscillations of heat evolution have been detected in synchronous cultures of *T. pyriformis* (Lloyd *et al.*, 1978). That heat dissipation did not parallel either growth or respiration was remarkable,
Respiratory oscillations in *C. utilis*

but a similar observation was made with *S. pombe* (Poole et al., 1973) and further work is necessary to quantify these relationships.

The period of the respiratory oscillations was similar in cultures growing synchronously with any of the three carbon sources. Whereas the respiratory oscillations previously described in *Crithidia fasciculata* (Edwards et al., 1975), *T. pyriformis* (Lloyd et al., 1978) and *A. castellanii* (Edwards & Lloyd, 1978) all showed periods of about 1 h at 30 °C, those in *C. utilis* were of a frequency of about 2 h⁻¹ at the same temperature.

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REFERENCES


