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Acquisition and Loss of Rotenone Sensitivity in *Torulopsis utilis*

Richard KATZ, *Laurie* KILPATRICK, *and Britton* CHANCE

Johnson Research Foundation, Philadelphia

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Torulopsis utilis yeast cells growing exponentially on synthetic medium with ethanol as carbon and energy source are insensitive to rotenone; the cells acquire the rotenone-sensitive component of the mitochondrial electron transport chain upon entering an ethanol-depleted stationary phase. The rotenone-sensitive site is lost when growth is restimulated with ethanol, but not when growth is restimulated with glycerol or acetic acid. Growth limitation by iron or by glucose or glycerol is also characterized by acquisition of the rotenone-sensitive site, upon entering stationary phase. The transition from rotenone-sensitive to -insensitive can also be studied by the use of semi-continuous culture in a chemostat. Studies with the protein synthesis inhibitors cycloheximide and chloramphenicol indicate that protein synthesis on cytoplasmic ribosomes is required both for the acquisition and the loss of the rotenone-sensitive site. The acquisition of rotenone sensitivity is apparently associated with an increased phosphorylation efficiency, as evidenced by measurements of P/O ratios.

In a previous paper [1], it was reported that *Torulopsis utilis* yeast cells harvested from an exponentially growing culture are insensitive to rotenone and Piericidin A, inhibitors of mitochondrial electron transport. However, respiration of cells harvested from a culture which has reached stationary phase due to depletion of ethanol (the carbon source used in the experiments reported) and has been in this starved state for approximately 15 min, is cut 60% to 80% by rotenone. It was also reported that if additional substrate (ethanol) is administered to the starved stationary culture, rotenone sensitivity is lost within 15 min. In this communication we present some further data on the property of rotenone sensitivity in *Torulopsis utilis*, seeking to explain the sort of transition that takes place when a culture of rotenone-insensitive yeast cells exhaust their carbon source and enter stationary phase, thus acquiring the rotenone-sensitive component of the electron transport chain.

MATERIALS AND METHODS

The techniques used for culturing *T. utilis* were as described previously [1]. Rotenone sensitivity was assayed as in [1]. Yeast mitochondria were prepared by the method reported by Light *et al.* [2]. P/O ratios in isolated mitochondria were determined by the method of Chance and Williams [3]. Cycloheximide and chloramphenicol were obtained from Sigma. Absorbance was measured using a Klett instrument; the units are those on the instrument.

RESULTS

Effect of the Protein Synthesis Inhibitors Cycloheximide and Chloramphenicol on the Acquisition and Loss of Rotenone Sensitivity

Cycloheximide inhibits *de novo* protein synthesis on cytoplasmic ribosomes in eukaryotic organisms, including yeast [4]. The effect of cycloheximide on the loss of rotenone sensitivity is shown in Fig. 1. 750 ml of culture medium containing 0.1% ethanol were inoculated from an exponentially growing preculture of *T. utilis*. Throughout exponential growth there was a nearly linear drop in pH and the rate of culture oxygen consumption increased exponentially. Oxygen consumption of samples of intact cells removed from the culture during this time (from zero time to 6 h) was not inhibited by rotenone even at concentrations of 5 mM. Shortly before growth ceased due to depletion of ethanol the respiratory rate of the culture fell, followed by a last burst of oxygen consumption, and acid secretion stopped. Midway through this final burst of respiration, the first cells appeared which were partially inhibited by rotenone. This sensitivity to rotenone increased to approximately 50% with subsequent aliquots. The culture was kept in this carbon-starved state for approximately 3 h. At this point, cycloheximide was added at a final concentration of 100 μM. 15 min later, ethanol was added (final concentration 0.1%). In contrast to the experiments reported in [1], where rotenone sensitivity was lost within 15 min after the addition of ethanol, rotenone sensitivity decreased only slightly for 2 h, and never reached zero.

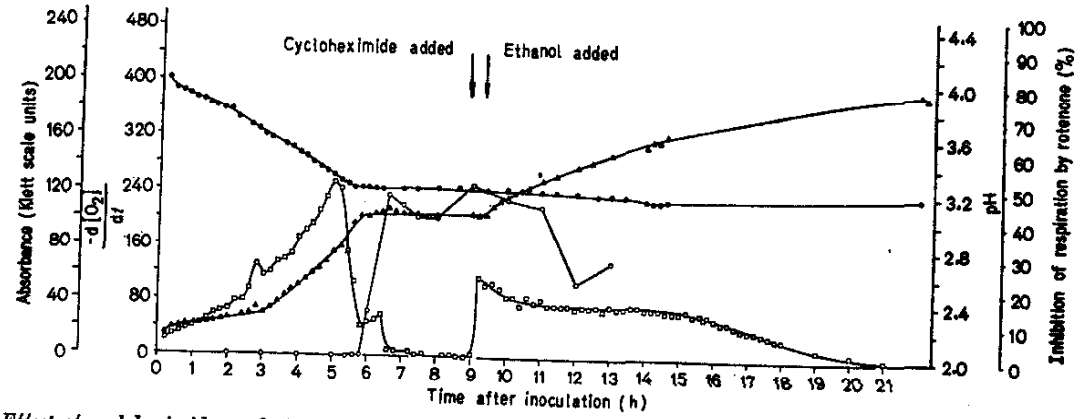

Fig. 1. *Effect of cycloheximide on the loss of rotenone sensitivity.* Measurements of turbidity by absorbance in Klett units, ▲, culture O_2 consumption ($d[O_2]/dt$) (□), and pH (●) were made as described under Methods. Culture O_2 consumption is given in arbitrary units per 5 min, one arbitrary unit being equivalent to a change of 3 μM oxygen. Rotenone sensitivity (○) is plotted as the percentage of inhibition of ethanol-stimulated respiration by rotenone in aliquots of washed and resuspended cells taken at the times indicated and measured polarographically

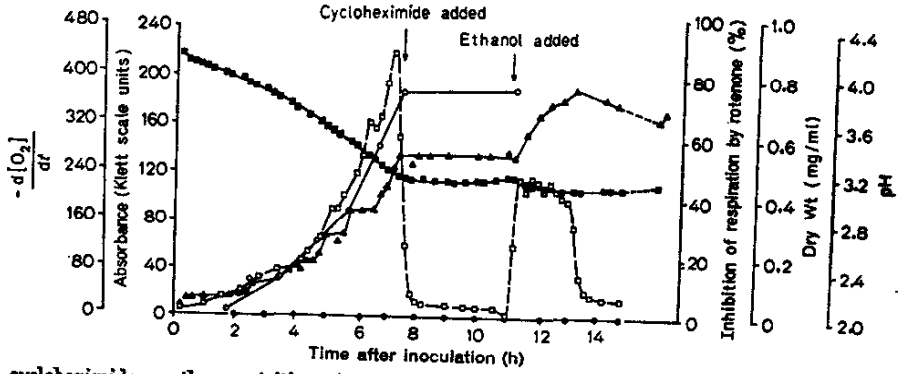

Fig. 2. *Effect of cycloheximide on the acquisition of rotenone sensitivity.* Measurements were made as described in the legend to Fig. 1. □, culture O_2 consumption; ▲, absorbance; ●, rotenone sensitivity; ○, dry weight; ■, pH

A second experiment was performed to determine the effect of cycloheximide on the acquisition of rotenone sensitivity. Taking advantage of our direct readout of culture oxygen consumption, cycloheximide was added immediately after the rate of culture oxygen consumption first decreased. As shown in Fig. 2, the expected final burst of respiration did not occur, and rotenone sensitivity did not appear. Addition of ethanol (final concentration 0.1 %) to this cycloheximide-inhibited culture produced some linear growth, a small drop in pH, and a period of anomalously low respiration similar to that in the experiment shown in Fig. 1. Rotenone sensitivity remained zero throughout.

Chloramphenicol is an inhibitor of mitochondrial protein synthesis in yeast [4]. As shown in Fig. 3, chloramphenicol (3 mg per ml) was administered to a culture which had been in the starved stationary state for approximately 3 h and was approximately 50 % sensitive to rotenone. During the 2.5 h incubation with the drug, rotenone sensitivity increased to approximately 70 %. Following this incubation period, ethanol (0.1 %) was added. Growth, rapid oxygen consumption, and acid secretion resumed normally. Rotenone sensitivity fell to zero. Following the depletion of ethanol, rotenone sensitivity reappeared.

Effect of Growth Limitation by Substrates Other than Ethanol

Growth on Glucose. Fig. 4 shows the effect of growth on glucose rather than ethanol. The growth curve, as measured by turbidity, indicates that some diauxic growth took place. The second and lesser increase in turbidity was accompanied by a decreased but stable rate of culture oxygen consumption. All samples taken from the actively growing culture were insensitive to rotenone. Following the cessation of growth and the final drop in respiratory rate, there

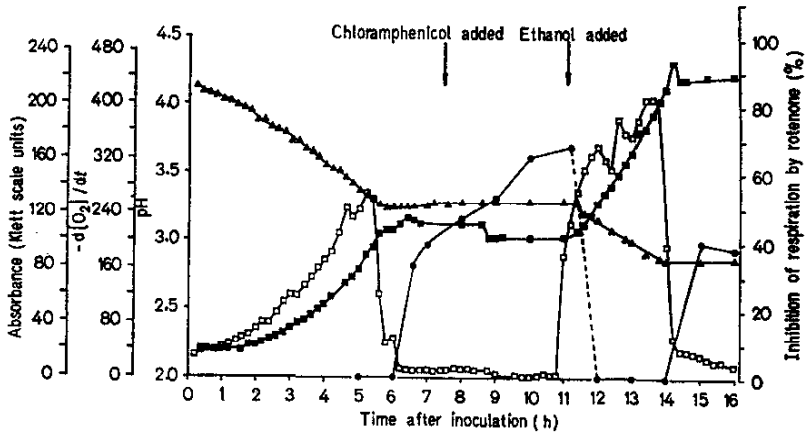

Fig. 3. Effect of chloramphenicol on acquisition and loss of rotenone sensitivity. Measurements made as described in the legend to Fig. 1. ■, absorbance; □, culture O_2 consumption; ▲, pH; ●, rotenone sensitivity

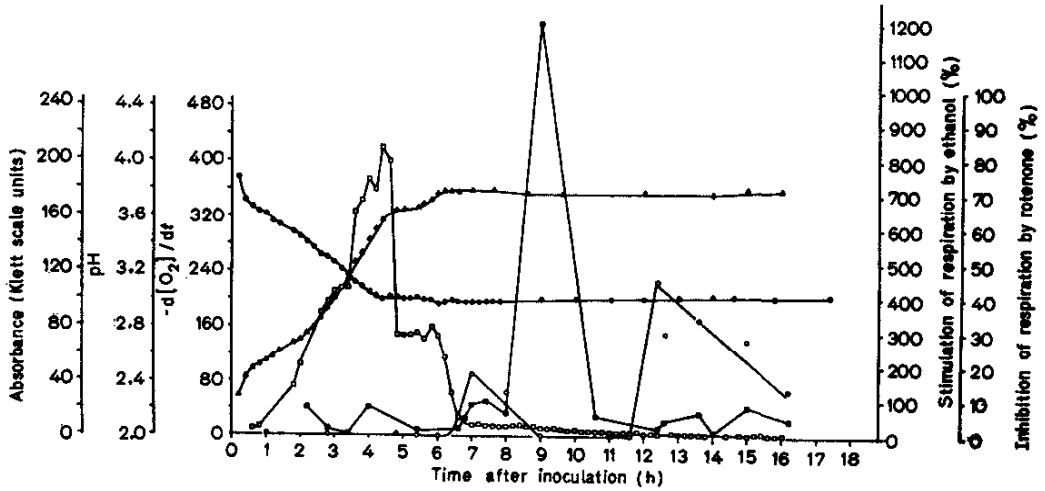

Fig. 4. *Growth of* T. utilis *on glucose.* Measurements made as described in the legend to Fig. 1. ▲, absorbance; ●, pH; □, culture oxygen consumption; ■, stimulation of respiration by ethanol; O, inhibition of ethanol-stimulated respiration by rotenone; *i.e.* rotenone sensitivity

was an episode of slight rotenone sensitivity (20% or less), followed by a period of approximately 3 h of rotenone insensitivity. Following this was a second episode of rotenone sensitivity (30—40% inhibition) which persisted for 4 h, whereupon the experiment was terminated.

Growth on Glycerol (not illustrated). Samples removed from a culture of *T. utilis* with glycerol as carbon and energy source during exponential growth were insensitive to rotenone. Samples taken during stationary phase were sensitive to rotenone, although the percentage of inhibition of respiration was always quite small, between 20% and 30%.

Growth on Ethanol; Restimulation with Glycerol and Acetic Acid. As shown in Fig. 5, an ethanol-limited culture of *T. utilis* was allowed to grow into a rotenone-sensitive stationary phase. Growth was restimulated with first glycerol, and then (when the glycerol was exhausted) acetic acid. The addition of glycerol (final concentration 0.1%) resulted in increased rotenone sensitivity in the samples taken. To the glycerol-depleted culture, an addition of glacial acetic acid (final concentration 0.1%) was made, allowing growth to resume for approximately 1.5 h. While there was some decrease in rotenone sensitivity, at no time did rotenone sensitivity fall to zero (as is the case with control cultures going through such feeding-starving cycles with ethanol).

Growth on Ethanol Limited by Iron. In this series of experiments, 300 ml shake-flasks containing 50 ml

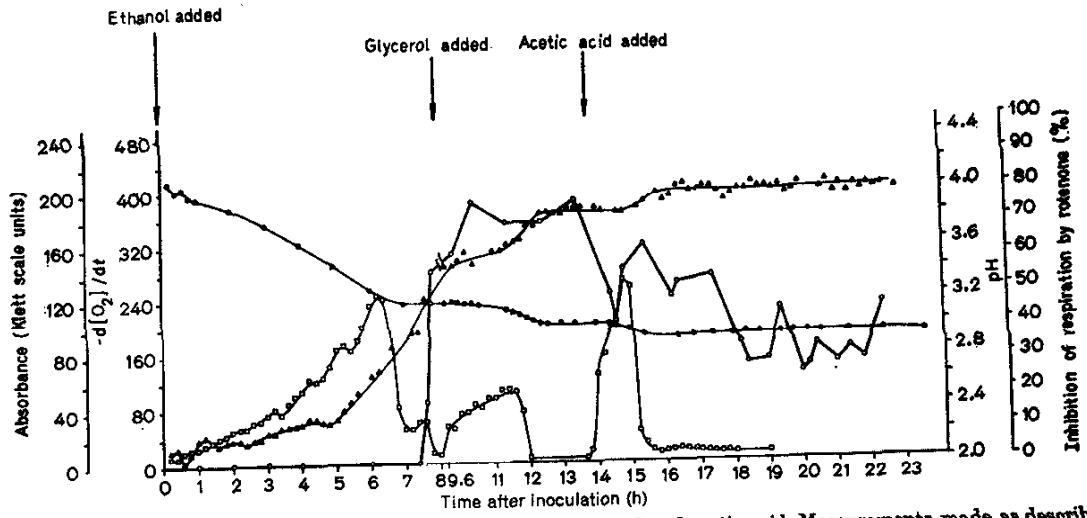

Fig. 5. *Growth of* T. utilis *on ethanol; restimulation of growth with glycerol and acetic acid.* Measurements made as described in the legend to Fig. 1. ▲, absorbance; □, culture oxygen consumption; ●, pH; ○, rotenone sensitivity

of medium replaced the usual elaborate culture apparatus, to facilitate completion of an entire iron titration simultaneously. As shown in Fig. 6, at all the iron concentrations tested (including those where final cell density, as indicated by absorbance, was certainly limited by iron), all samples taken during growth were insensitive to rotenone. Samples taken from stationary phase cultures (presumably depleted of iron, except for the 50 μM Fe culture) were sensitive to rotenone, albeit to varying extents and in different patterns. It should be pointed out that in these experiments shake-flask cultures, although more convenient and readily available, yield somewhat less reproducible results than the elaborate culture apparatus used in the rest of the studies reported here and in [1].

Chemostat Experiments

The transition of *T. utilis* from rotenone-insensitive to -sensitive and *vice versa* can also be studied (and more conveniently) by the use of semicontinuous culture in a chemostat. In the initial stages of chemostat operation, when the culture was not being diluted but was allowed to grow from an inoculum as a batch culture, cells removed from the culture were insensitive to rotenone. After a suitable cell density was reached, the vessel was diluted at a rate of 0.2 h^{-1}, with ethanol as the limiting substrate (final concentration 0.1 %). When the steady-state cell density was reached, it was found that the cells grown under these steady-state chemostat conditions were approximately 60 % sensitive to rotenone. This sequence was repeated by increasing the dilution rate to 2.0 h^{-1} until approximately half the cells were washed out, whereupon the dilution rate was set back to 0.2 h^{-1}. In the transient period while the culture was returning to the steady state, the cells were insensitive to rotenone. When steady-state conditions prevailed once more (as indicated by a constant cell density) the cells were sensitive.

Mitochondria Studies

To test the possibility that the transition from rotenone-insensitive to -sensitive was merely a change in whole-cell permeability to rotenone, mitochondria were prepared from *T. utilis* cells harvested during exponential phase; cells harvested from a stationary phase culture; and also cells harvested from the chemostat described above, operating at a dilution rate of 0.2 h^{-1} with ethanol as limiting substrate. The Table shows the various types of mitochondria prepared, their respiratory control and P/O ratios, and sensitivity to rotenone and Piericidin A. The rotenone-sensitivity of the isolated mitochondria paralleled that of the cells from which they were isolated, even when substrate levels of NADH were employed. The one exception was for mitochondria respiring on ethanol, in which case rotenone or Piericidin A at high concentrations invariably inhibited respiration, whether the intact cells were sensitive or not. This discrepancy has been discussed previously [1]. These studies *in vitro* make it highly unlikely that the acquisition of rotenone sensitivity in intact cells is due to an altered permeability to rotenone.

With mitochondria already prepared, it was of interest to assay the phosphorylation efficiency

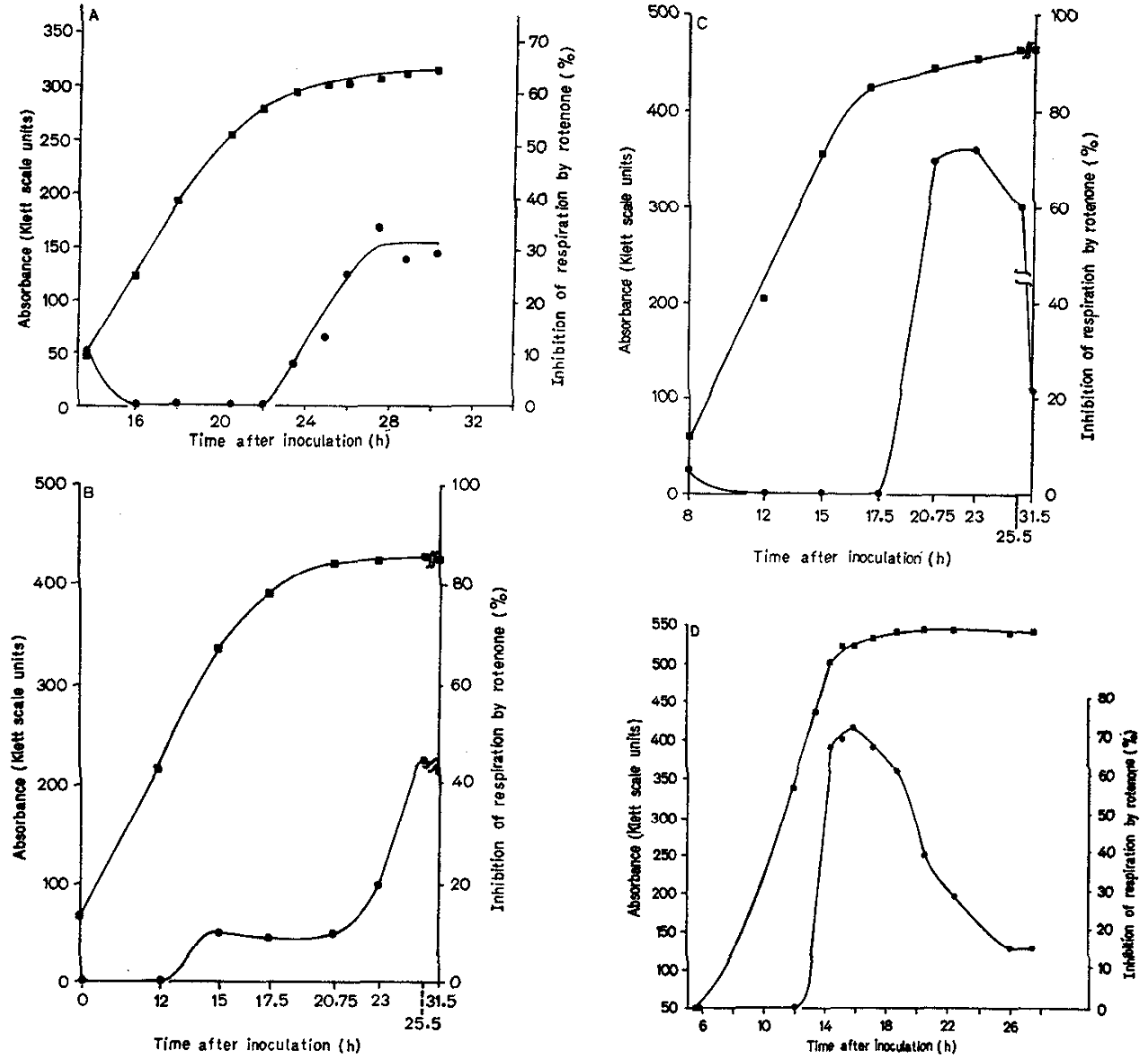

Fig. 6. *Growth of* T. utilis *on ethanol, limited by iron.* Four 300 ml sidearm flasks containing 50 ml of culture medium with 1% ethanol as carbon and energy source were inoculated from an exponentially growing preculture of *T. utilis*. A, no added iron; B, 1.5 μM $FeCl_3$ added; C, 2.5 μM $FeCl_3$ added; D, 50 μM $FeCl_3$ added. ■, Growth measured by absorbance; ●, rotenone sensitivity measured by inhibition of respiration

(P/O ratio) of the cells harvested from these three different culture conditions, *i. e.* exponential phase cells, ethanol-depleted stationary phase cells, and ethanol-limited chemostat cells. For both chemostat cells and stationary-phase cells, the isolated mitochondria were coupled (respiratory control ratio 2.0 or greater) and exhibited P/O ratios of approximately 3. However, mitochondria isolated by the same procedure in parallel experiments from exponential phase cells were nearly completely uncoupled, with a respiratory control ratio of approximately 1.0.

To assay phosphorylation efficiency in these cells, data were collected on total oxygen uptake and total increment in culture dry weight for several 1 h intervals, during the growth of a typical *T. utilis* culture with ethanol as carbon and energy source. Making use of the Elsden "constant" for Y_{ATP} [5], the

Table. *Piericidin A sensitivity of intact cells and mitochondria isolated from them, harvested at different stages of growth*
The reaction medium contained 0.6 mM mannitol, 10 mM potassium phosphate buffer (pH 6.5), 10 mM Tris-maleate buffer (pH 6.5), 10 mM KCl, and 0.1 mM EDTA. Substrates were added at a final concentration of 10 mM. P/O ratios were determined by the method of Chance and Williams [3]. Maximal Piericidin A inhibition was obtained with 1.2 μM Piericidin A in all assays except where indicated

Type of mitochondria	Qo_2 of endogenous respiration [state 2]	Substrate used [state 4]	Qo_2 in state 4	Piericidin A sensitivity of isolated mitochondria: Maximal inhibition of respiration by Piericidin A state 3 (excess of ADP)	Piericidin A sensitivity of isolated mitochondria: Maximal inhibition of respiration by Piericidin A state 4 (limiting ADP)	Respiratory control ratio $\frac{\text{state 3, } Qo_2}{\text{state 4, } Qo_2}$	P/O ratio	Piericidin A sensitivity in intact cells Maximal inhibition of respiration by Piericidin A
	natoms $O \times min^{-1} \times mg^{-1}$			%	%			%
Isolated from exponential phase cells[a]	0.0	pyruvate + malate	82.0	—	0	1.0	—	
	0.0	NADH	31.3	0	0	1.0	—	0
	0.0	ethanol	167.5	—	73[b]	1.14	—	
Isolated from ethanol-depleted stationary phase cells[c]	39.0	pyruvate + malate	39.0	100	—	3.5	2.8	
	35.1	NADH	56.9	85	61	2.5	2.5	63
	37.4	ethanol	64.4	100	—	—	—	
Isolated from chemostat cells, dilution rate = 0.2 h^{-1}, 0.1% ethanol substrate	44.8[d]	—	—	—	96	3.8	3.0	
	62.7	pyruvate + malate	74.5	—	100	2.7	2.9	50
	38.4	NADH	115.5	14	60	2.0	3.3	
	26.0	ethanol	74.8	—	100	3.1	3.6	

[a] State 4 respiration not stimulated by ADP.
[b] Piericidin A concentration = 60 μM; stimulated by pyruvate + malate (see text).
[c] State 4 respiration stimulated approximately 200% by ADP.
[d] Inhibited 100% by pyrazol; restimulated by pyruvate + malate.

following relationships were derived and used in evaluating a P/O ratio *in vivo*:

$$Y_{ATP} = \frac{\text{g dry weight produced/h}}{\text{moles ATP produced/h}} \simeq 10, \text{ the Elsden "constant" [5]} \quad (1)$$

$$\frac{\text{moles ATP produced/h}}{2 \times \text{moles } O_2 \text{ utilized/h}} = \text{P/O ratio}. \quad (2)$$

Rearranging Eqn (2),

$$\text{moles ATP produced/h} = (\text{P/O}) \times 2 \times \text{moles } O_2 \text{ utilized/h}. \quad (3)$$

Substituting Eqn (3) in Eqn (1),

$$\frac{\text{g dry weight produced}}{(\text{P/O}) \times 2 \times \text{moles } O_2 \text{ utilized}} \simeq 10. \quad (4)$$

Rearranging Eqn (4),

$$\frac{\text{g dry weight produced}}{20 \times \text{moles } O_2 \text{ utilized}} \simeq \text{P/O}. \quad (5)$$

Dry weight was measured by removing 25 ml samples from the culture, washing the cells in distilled water, resuspending in distilled water, and drying overnight at 105° C. Total O_2 consumption was measured by recording the amount of dissolved oxygen which had to be administered to maintain the level of dissolved oxygen between 15% and 25% of oxygen saturation (see [1]). In three 1 h intervals, the values of P/O ratios obtained by this method were 1.3, 1.6, and 1.4.

DISCUSSION

As reported in [1], *Torulopsis utilis* yeast cells growing exponentially with ethanol as sole carbon and energy source are insensitive to rotenone; upon entering an ethanol-depleted stationary phase, the cells acquire the rotenone-sensitive component of the electron transport chain. The results of the series of experiments with the protein synthesis inhibitors cycloheximide and chloramphenicol imply that protein synthesis is required for the transition from rotenone-sensitive to -insensitive, and *vice versa*. This synthesis presumably takes place on cytoplasmic ribosomes, not on mitochondrial ribosomes.

The use of carbon sources other than ethanol, such as glucose and glycerol, shows that the effect of growth phase on rotenone sensitivity is not an effect peculiar to ethanol growth. When growth is

limited by a non-carbon substrate, *e. g.* iron, it is observed that the transition to stationary phase produces rotenone-sensitive cells, in a fashion quite similar to ethanol depletion. However, the effect of ethanol during active growth in producing rotenone insensitivity is apparently peculiar to ethanol among the substrates tested. When growth is restimulated with glycerol or acetic acid, for example, rotenone insensitivity does not reappear. Yet if the culture has not gone through a period of acquisition of rotenone sensitivity, then growth on glycerol and glucose, for example, still produces insensitive cells.

Recent results obtained in this laboratory [6] implicate another factor in control of the synthesis of the rotenone-sensitive site. Cells grown on ethanol under conditions of insufficient aeration, where the steady state concentration of dissolved oxygen in the culture is near zero, are sensitive to rotenone. This is in contrast to the experiments described previously, where the concentration of dissolved oxygen was maintained between 15% and 25% of saturation by an appropriate servomechanism (oxystat).

The studies performed to estimate the P/O ratio *in vivo*, or the P/O ratio with isolated mitochondria, from cells in different growth "phases", *i. e.* exponential phase, stationary phase, and from the chemostat, permit a good basis of comparison with the results of others working with the bioenergetics of *T. utilis*. Various workers [2,7] have reported data on possible correlations between rotenone or Piericidin A sensitivity and the presence or absence of energy coupling (phosphorylation) at site I of the respiratory chain. The loss of both rotenone sensitivity and site I phosphorylation was reported to result from iron limitation during growth. This does not appear too logical in the case of batch cultures [7], where iron limitation could only occur at the end of growth and at precisely the time when rotenone sensitivity appears. In the case of iron-limited continuous culture [2], rotenone insensitivity is only one of the many quirks produced by the chemostat with its highly unnatural growth "phase".

The results reported here show that the efficiency of energy conservation (as evidenced by P/O ratio measurements of two different kinds) is appreciably different depending on the growth phase of the culture, being much higher in the stationary phase than in exponential phase. This comparison may be specious since two very different methods were used for estimating P/O ratios in the different growth phases. This was made necessary by two factors: first, mitochondria isolated from rotenone-insensitive exponential phase cells were uncoupled, making the usual methods inapplicable; and second, the stoichiometric relationship of dry weight produced to O_2 utilized is inapplicable to non-growing cultures. On the basis of these results, however, it does appear that the acquisition of rotenone sensitivity is associated with an increased phosphorylation efficiency.

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L. Kilpatrick, and B. Chance
Johnson Research Foundation
University of Pennsylvania Medical School
Philadelphia, Pennsylvania 19104, U.S.A.

R. Katz' present address:
Department of Biochemistry, University of California
Berkeley, California 94720, U.S.A.