# GROWTH PHASE AND ROTENONE SENSITIVITY IN *TORULOPSIS UTILIS*: DIFFERENCE BETWEEN EXPONENTIAL AND STATIONARY PHASE

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## 1. Introduction

Rotenone, the principal insecticidal constituent of derris root, has been shown to inhibit respiration and electron transport in isolated mitochondria from rat liver [1], beef heart [2], pigeon heart [3], insect flight muscle [4], as well as from a number of other sources. It has recently been shown to inhibit the oxidation of low potential flavoproteins associated with the oxidation of NADH and of pyruvate and α-ketoglutarate dehydrogenases [5]. Rotenone has no detectable effect on the activity of the electron transport system in *Saccharomyces* yeats [6, 7]; rotenone insensitivity has been refered to as constitutive in these organisms [8].

Rotenone sensitivity in *Torulopsis utilis* yeast, (and some other yeasts), however, has been the subject of much recent investigation and controversy. Ohnishi et al. [7] reported in 1966 that *T. utilis* grown in batch culture was sensitive to rotenone. Light et al. [9] reported in 1968 that respiration of *T. utilis* cultured in a chemostat on a glycerol-limited medium was inhibited 50–70% by rotenone; however, if the chemostat was switched from glycerol-carbon-limited growth to iron-limited growth, cells were obtained in which respiration was completely insensitive to rotenone. Ohnishi [10] reported in 1970 that analogous results could be obtained with batch cultures of *T. utilis*. She found that if the initial iron concentration in the culture was 1.1 μM Fe or more the cells produced were sensitive to piericidin A (a compound identical to rotenone in its effect on electron transport [11, 12]), but if the initial iron concentration was less than 1.1 μM the cells were insensitive. Recently DeMaille et al. [8] reported data supporting this effect of iron-limited growth.

The present report presents rather different data, demonstrating that *T. utilis* is insensitive to rotenone during exponential growth in a batch culture (when iron concentration is obviously not limiting). When growth stops and the cells enter the stationary phase (in this case due to carbon limitation), rotenone sensitivity appears after a short lag.

## 2. Materials and methods

*T. utilis* was cultured at 30° on the synthetic medium of Galzy and Slonimski [13], the only modifications being increase of iron concentration to 50 μM $FeCl_3$, a tripling of copper concentration and omission of manganese. The carbon and energy source was ethanol. The culture vessel used was a 1.0 liter cylinder, approximately 5 × 50 cm, equipped with a sparger, stirrer, and Teflon-covered oxygen electrode (Yellow Springs Instr. Co.) immersed in the culture medium. The electrode output activated a solenoid valve when the culture oxygen concentration fell to approximately 15% saturation, giving a pulse of oxygen sufficient to raise the $O_2$ concentration to approximately 25% saturation. With this oxystat (designed by Dr. Dieter Mayer) the rate of oxygen consumption by the culture was monitored, by recording the rate at which the oxygen concentration fell from 25 to 15%. Cells were siphoned continuously from the culture throughout the experiment and collected in a fraction collector,

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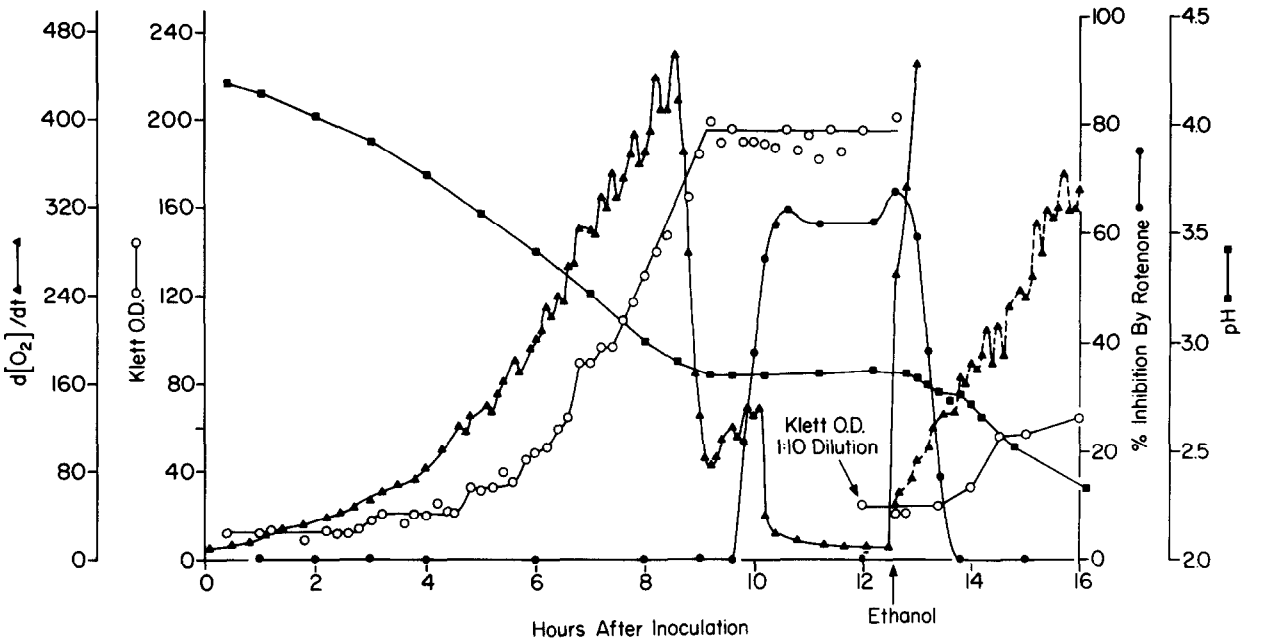


Fig. 1. Rotenone sensitivity, culture turbidity, culture pH, and culture oxygen consumption for batch of *Torulopsis utilis*. All measurements were made as described in the text. Symbols: ○—○: turbidity in Klett absorbance units; ●—●: rotenone sensitivity, percent inhibition of respiration (rotenone = 20 μM); ▲—▲: culture oxygen consumption in arbitrary units per 5 min, one arbitrary unit being equivalent to a change of 3 μM oxygen; ▲---▲: culture oxygen consumption in arbitrary units per one minute, one arbitrary unit being equivalent to a change of 3 μM oxygen; ■—■: pH of culture medium.

maintained at 30°, at a rate of 5 ml of cells in a 12 min interval. The turbidity of the fractions was measured in a Klett colorimeter. The fractions were then centrifuged at 1500 rpm and the pH of the supernatant culture medium determined. The cells were washed once in phosphate buffer, 50 mM, pH 6.8, and resuspended in a small volume of the same buffer. Rotenone sensitivity was assayed by measuring polarographically the percentage inhibition of ethanol-stimulated respiration by rotenone in the washed cells at room temperature.

## 3. Results

The results of a typical experiment are shown in fig. 1. The culture was inoculated from an exponentially growing preculture of *T. utilis*. Throughout the period of rapid growth, all samples obtained failed to be inhibited by concentrations of rotenone up to 5 mM. Approximately 25 min after growth stopped, due to ethanol limitation, the first partial sensitivity to rotenone became evident. This sensitivity increased steadily with subsequent aliquots, attaining a maximum of between 60 and 70 percent inhibition of respiration. The same maximum inhibition could be obtained with Piericidin A at a concentration of 4.0 μM. The half-time for acquisition of rotenone sensitivity was approximately 17 min (cf. fig. 1).

During the period of rapid growth, the pH of the culture medium declined. This decline in pH, presumably resulting from excretion of acid by the cells, ceased abruptly when cell growth (as indicated) by turbidity stopped. When cell growth stopped, there was also an abrupt fall in total culture respiratory rate, followed by one last gasp of $O_2$ consumption. It is approximately midway through this final burst of respiration that rotenone sensitivity is first observed.

If a second addition of ethanol is made to the stationary culture, there is a rapid decrease in rotenone sensitivity. The return to insensitivity has a half-time of approximately 14 min, roughly comparable to the half-time of the original induction. This second ad-

Table 1

Piericidin A sensitivity of intact cells and mitochondria isolated from them, harvested at different stages of growth. The reaction medium contained 0.6 M 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.

| Type of Mitochondria | $QO_2$ of Endogenous respiration (State 2) (natoms O/min/mg) | Substrate used (State 4) | $QO_2$ in State 4 | Maximum % inhibition of respiration by piericidin A[a] | | |
|---|---|---|---|---|---|---|
| | | | | Isolated mitochondria | | Intact cells |
| | | | | State 3 (excess of ADP) | State 4 (limiting ADP) | |
| 1) Isolated from exponential phase cells | 0.0 | Pyruvate and malate | 82.0[b] | – | 0 | |
| | 0.0 | NADH | 31.3[b] | 0 | 0 | 0 |
| | 0.0 | Ethanol | 167.5[b] | – | 73[c] | |
| 2) Isolated from ethanol-depleted stationary phase cells | 39.0 | Pyruvate and malate | 39.0[d] | 100 | – | |
| | 35.1 | NADH | 56.9[d] | 85 | 61 | 63 |
| | 37.4 | Ethanol | 64.4[d] | 100 | – | |

[a] Maximal piericidin A inhibition was obtained with 1.2 μM piericidin A in all assays except as noted (see c).
[b] State 4 respiration not stimulated by ADP.
[c] Piericidin A concentration = 60 μM; stimulated by pyruvate and malate (see text)
[d] State 4 respiration stimulated approximately 200% by ADP.

dition of ethanol also causes an immediate 25-fold increase in rate of culture oxygen consumption, a further lowering of pH, and after some lag a further increase in culture turbidity. Further cycles of feeding and starving (not shown) produce similar cycles in rotenone sensitivity, acid secretion, $O_2$ consumption and culture growth.

To test the possibility that the observed changes in rotenone sensitivity in the samples of intact cells were not due to an altered permeability to rotenone or piericidin A, mitochondria were isolated from cells harvested during both exponential and ethanol-depleted stationary phases of growth, according to the method reported by Light et al. [9]. As shown in table 1, the piericidin A sensitivity of the isolated mitochondria paralleled that of the cells from which they were isolated, even when substrate levels of NADH were employed. Results with rotenone were entirely comparable (not shown).

The one notable exception was for mitochondria respiring on ethanol, in which case piericidin A and rotenone (both at high concentrations) invariably inhibited respiration. This latter result is in agreement with Balcavage and Mattoon [14], who showed even in the case of *Saccharomyces cerevisiae* (in which the intact cells are invariably insensitive to rotenone), there is an 80% inhibition of respiration by rotenone in the isolated mitochondria. These investigators localized the source of this anomaly as yeast alcohol dehydrogenase (EC 1.1.1.1.), showing that rotenone inhibits the activity of the purified enzyme by 60% or more. The most conclusive proof that rotenone does not inhibit the rotenone sensitive site on the electron transport chain of the exponential phase *Torulopsis utilis* is that even after 73% of the ethanol-stimulated respiration has been inhibited by rotenone, the respiratory rate can be restored to 100% of control by the addition of pyruvate + malate. This result conclusively demonstrates that altered cell permeability is not a tenable explanation for the differential rotenone sensitivity

found in cells harvested from the two growth phases.

## 4. Discussion

These results suggest that in the "normal" physiological state of *T. utilis,* where "the best standard of normality is probably the cycle of the average single cell growing exponentially in constant conditions..." [14], electrons flow through a rotenone insensitive pathway, involving an NADH dehydrogenase flavoprotein similar to the one in yeast mitochondria which oxidizes externally added NADH ($fp_{Dex}$) [7–15]. When the cells cease to grow, they switch to a different physiological state, in this case characterized by a drop in $O_2$ consumption and cessation of acid production. The transition to this new physiological state apparently also involves a fundamental change in the nature of the electron flow pattern in the mitochondrial membranes, a change that can occur with a half-time of 14 min and can be repetitively stimulated. The acquisition of rotenone sensitivity may involve: a) diversion of electron flow from the external, rotenone/insensitive NADH dehydrogenase to a preexisting, rotenone/sensitive, internal dehydrogenase; b) a change in the location or conformation of the preexisting rotenone insensitive dehydrogenase, rendering it rotenone sensitive; or c) derepression of the biosynthesis of the rotenone sensitive, internal dehydrogenase by some intermediate of the stationary phase maintenance metabolism.

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