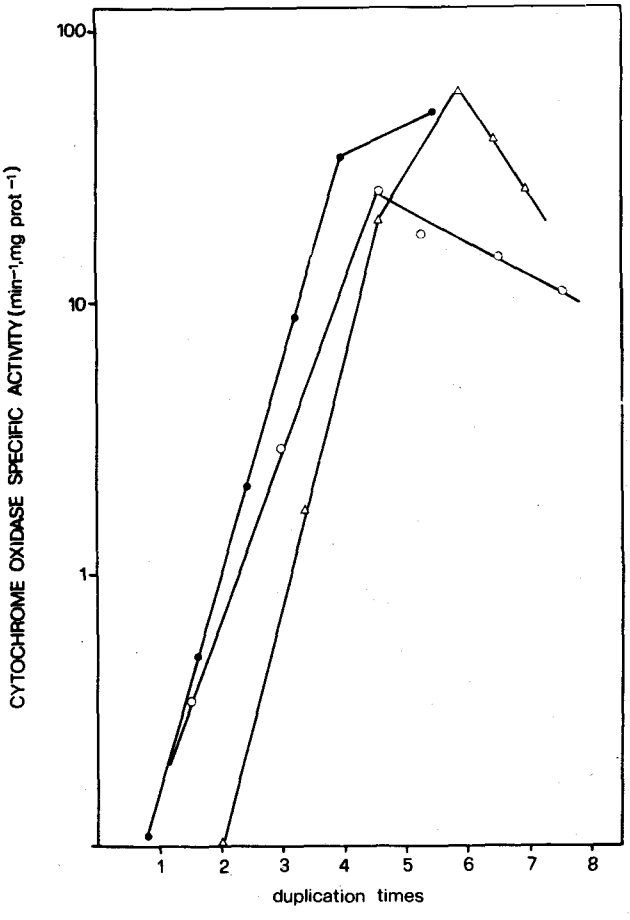
Change of the Cytochrome Oxidase Level During Exponential Growth in *Neurospora crassa*

During the exponential phase of growth in *Neurospora crassa* cultures, the syntheses of RNA, proteins and DNA are balanced, i.e. each of the rates of synthesis can be expressed by the following equation: $dM/dt = KM$, where K is the constant of the exponential rate of growth and M is the cellular content of each macromolecular component. Also the levels of RNA and protein, that is the amounts of these compounds referred to an amount of DNA equivalent to one haploid genome, remain constant[1,2]. But a question remains open: during the exponential phase in a defined medium when the cellular composition is constant, are the cells endowed with the same enzymatic activities? Or to put the same question in a more immediate manner: are *Neurospora* cells collected at different times during exponential phase of growth identical? In the experiment reported in the present paper, we determined the levels of a key respiratory enzyme, cytochrome oxidase, during the exponential phase of growth in different media and found that the level of cytochrome oxidase varies extensively during the exponential phase in all the nutritional conditions we have considered.

Materials and methods. The wild type strain 74A (St. Lawrence) of *Neurospora crassa* has been used. The growth conditions in shaken liquid media at 30 °C were as indicated in detail in a previous paper[3]. Conidia, which had been obtained on solid sucrose minimal medium in wide-mouth flasks incubated for 7 days in a lighted thermostat (25 °C) and stored in the cold room for 2–4 weeks, were used to inoculate the liquid cultures. The constants (K, h^{-1}) of the exponential rates of growth were determined[3] and from them the duplication times ($t_D = ln2/K$) referred in the Figure were calculated. Cell extracts were prepared as follows. Mycelia grown at 30 °C for the required period were quickly collected over a glass fibre filter with suction, washed in cold distilled water and frozen at −70 °C. The frozen pads were homogenized with sea sand in 2–3 volumes of 0.1 *M Tris*-HCl pH 7.8, 1 m*M* EDTA, 0.1% sodium deoxycholate. The supernatant of 1,500 × *g* per 15 min was saved and the pellet extracted again with the same buffer and centrifuged as described. The 2 supernatants were pooled and immediately used for the enzymatic determinations. Extraction of the pellets after the second centrifugation gave supernatants with no detectable enzymatic activities. Cytochrome oxidase activity was measured according to SMITH[4] using a recording spetrophotometer and was expressed as K (min^{-1}), mg protein^{-1}. The malate dehydrogenase reaction was assayed spectrophotometrically according to standard procedures[5]. Protein was determined on cell extracts as described previously[1,6]. Glucose was assayed in the culture medium by an enzymatic spectrophotometric test which employs the glucose oxidase reaction (reagents obtained from Böehringer Mannheim, Germany).

Results and discussion. The specific activity of cytochrome oxidase was measured in extracts of *Neurospora* cells after different periods of exponential growth in various liquid media. As indicated in the Figure, it changes very markedly during growth, being very low in early exponential growth, reaching a maximum after a growth period equivalent to 4–5 duplication times, then declining slowly. The maximal specific activity is quite high in glucose plus casamino acids (about 60 min^{-1}, mg $prot^{-1}$) and in minimal acetate (about 50 min^{-1}, mg $prot^{-1}$) and lower in minimal glucose (about 25 min^{-1}, mg $prot^{-1}$). To test whether the extracts of early exponential cells contained some inhibitor(s) of the cytochrome oxidase, mid-exponential and early exponential cells were extracted



The level of cytochrome oxidase in *Neurospora* cells in exponential growth in different media. The cells were grown at 30 °C after an overnight preincubation in minimal acetate (●), t_D = 2.464 h; minimal glucose (○), t_D = 1.971 h; glucose plus casamino acids (△), t_D = 1.533 h.

Table I. Recovery of cytochrome oxidase activity in different cell extracts

Type of extract	Specific Activity (min^{-1}, mg $prot^{-1}$)	Total activity (min^{-1})
Early exponential cells	3.54	30
Mid-exponential cells	20.30	120
Mixture of extracts from early exponential and mid-exponential cells	12.00	152

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Table II. Effects of glucose concentration of enzyme levels and glucose utilization in *Neurospora*

	Initial growth conditions					
	2% Glucose			0.5% Glucose		
Time (h)	Cytochrome oxidase (a)	MDH (b)	Glucose level (c)	Cytochrome oxidase (a)	MDH (b)	Glucose level (c)
3	1.5	0,835	100	3.1	2,473	30.0
5	6.0	1,860	94	8.5	4,420	29.2
7	25.0	2,360	86	31.3	6,942	28.5
9	22.0	4,900	80	24.7	9,050	28.0
10	19.0	6,520	78	21.7	9,270	27.5

Experimental conditions: the enzymatic activities were determined as indicated in the Methods and they are expressed as: a) K, mg prot^{-1}; b) units, mg prot^{-1}. The level of glucose in the culture media is given as c) μmoles of glucose/ml of culture.
After 10 h the yield of cells in the 2 conditions were: 0.28 mg dry weight/ml of culture in 2% glucose and 0.31 mg dry weight/ml of culture in 0.5% glucose. The constant of exponential growth, determined as dry weight[3] was 0.37 h^{-1} for both cultures.

together. The results reported in Table II exclude the occurence of an inhibitor because the recovered activity was the sum of the two activities measured independently. The increase of specific activity of cytochrome oxidase occurs in cells growing in glucose as well in those growing in acetate. Besides, both types of cells have been shown to have fairly similar mitochondrial structures[7]. Therefore it is unlikely that a release from catabolite repression plays a major role in enhancing the level of cytochrome oxidase. To gain more information on this point, the level of cytochrome oxidase was measured in cells growing in 2 different initial concentrations of glucose: 2% and 0.5%. As shown in Table II, it was found only slightly higher in cells growing with a lower initial availability of glucose. Also the level of malate dehydrogenase has been found to increase during the early exponential growth, although not as dramatically as cytochrome oxidase (Table II). The lower availability of glucose modifies the way the cells utilize glucose. In fact, although the yield at the 10th h of growth is the same for the culture started in 2% glucose or in 0,5% glucose (see legend of Table II), in the 3 to 10 h period the cells with more glucose available utilize 22 μmoles of glucose/ml, while the cells with less glucose available utilize 2.5 μmoles/ml.

The results presented here indicate that the level of a key respiratory enzyme may vary extensively during exponential growth in *Neurospora*, when conidia germinate into coenocytic hyphae, initiating a defined progression of events whose order cannot easily be changed, thus differing from the exponential phase of growth in bacteria. Indirect support of our results is offered by a recent study on respiratory and energy metabolism during exponential growth in *Neurospora*[8]. The oxygen uptake per unit of cell mass has been found to increase at the beginning of the exponential growth; then it slowly declines. The AMP and ADP levels increase as soon as the germination of conidia starts, reach a maximum at mid-exponential phase, then decline. The ATP level remains at the conidia level during early exponential growth, then it raises reaching a maximum during late exponential growth[8]. The pattern of change of the cytochrome oxidase level is very similar both for cells grown in glucose or in acetate, confirming the indication given by previous findings[7] that in *Neurospora* catabolite repression plays only a marginal role in controlling the synthesis of mitochondrial enzymes and structures. As for the question which this paper aimed to answer, it is clear that *Neurospora* cells collected during a given exponential phase of growth may differ widely in their enzymatic set-up and metabolic activities, although they appear identical when gross parameters such as RNA and proteins levels are considered. Several patterns of cytodifferentiation may be evoked in a temporal sequence by a given nutritional condition and all of them seem compatible with the same rate of exponential growth. This suggests the possibility that nutrients control the rate of cellular growth independently of the regulation of the synthesis and activity of metabolic enzymes.

Summary. In *Neurospora* cells growing in various media, the specific activity of cytochrome oxidase increases very markedly during early exponential growth, reaching a maximum after 4–5 duplication times, then it slowly declines.

Riassunto. L'attività specifica della citocromo ossidasi è stata determinata in miceli di *Neurospora crassa* (ceppo selvatico) in crescita esponenziale bilanciata in diversi mezzi liquidi di cultura. Indipendentemente dalla qualità della fonte di carbonio usata l'attività specifica è molto bassa durante la fase esponenziale precoce, raggiunge un massimo dopo 4–5 tempi di duplicazione, quindi decresce. Il valore massimo dell'attività specifica dipende dalle condizioni nutrizionali, basso in glucosio minimo più elevato in acetato minimo e in glucosio più casaminoacidi.

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Surface Features and Histochemistry of the Pollinial Wall of *Calotropis gigantea*

Angiosperm microspores normally develop to form granular pollen, but occasionally they adhere to form dyads or tetrads (Ericaceae) or even polyads of 8–16 spores (Mimosoideae). A greater degree of pollen aggregation occurs in some members of Asclepiadaceae and Orchidaceae, where the pollen of the entire sac are agglutinated to form a body of definite shape called pollinium. The ontogeny of the pollinium and the structure of its wall have not received much attention so far. Some aspects of the morphology and chemistry of the pollinial wall of *Calotropis gigantea* R & Br. (Asclepiadaceae) are examined in this report.

Pollinia from two varieties of *Calotropis gigantea* were used in this study. Freshly collected mature pollinia were