Temperature-controlled alternative respiration and outgrowth rate from conidia of *Neurospora crassa*[1]

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Summary. Incubation of conidia of wild type *Neurospora crassa* at temperatures ranging from 25 to 46°C modulates their respiratory type. Between 37 and 41°C, the transient activity of the cyanide-insensitive respiratory pathway parallels, with a maximal extrusion of protons into the medium, the optimal rate of germ tube outgrowth.
Key words. Conidia; *Neurospora crassa;* temperature; respiration; germination.

Heat treatment of conidia of *Neurospora crassa* at 46°C shifts their terminal respiration from the cytochromic, cyanide-sensitive to the cyanide-insensitive, salicylhydroxamic (SHAM)-sensitive pathway, while allowing only isometric conidial growth instead of normal polarized germination[2]. In such heat-treated 'overswollen' conidia, mitochondria show a negative cytochemical reaction for cytochrome oxidase activity[3]. Since the alternative, SHAM-sensitive respiratory pathway is a non-electrogenic, low ATP-producing pathway[4,5], and a general acidification of the cytoplasm has been measured in hyphae of *N. crassa* treated with cyanide[6], it could therefore be expected that the induction of cyanide-insensitive respiration should also be paralleled by an increase in the cytoplasmic proton concentration.

Polarized acidification has indeed been shown to be involved in fungal germ tube outgrowth normally occurring at 20°C and 25°C[7,8], and the protons vectorially expelled are ascribed to ATP hydrolyzed in mitochondria, as suggested from experiments in which activation of their latent ATPase by uncoupling agents stimulated outgrowth rate in parallel[9].

We were therefore led to assume that the full shift to the alternative respiratory pathway induced at 46°C would provoke an exaggerated, generalized acidification of the cytoplasm incompatible with its polarization. Reciprocally, however, it should be possible to define more moderate temperatures of incubation modulating the acidifying alternative pathway to levels not only compatible with, but beneficial to the polarization process. Outgrowth of germ tubes has therefore been assayed within a range of intermediate temperatures between 25 and 46°C. An optimum was found between 37 and 41°C, productive in parallel of a short and transient cyanide-insensitive respiratory pathway and a maximally increased, presumably vectorial, extrusion of protons into the incubation medium.

Materials and methods. Conidia of the wild type *N. crassa* strain STA_4 (FGSC 262A) were produced in Fernbach flasks containing solid nitrate minimal medium[10] and harvested with sterile distilled water as described previously[2]. Generally 3.5 ± 0.17 g of conidia (wet weight) was obtained from each Fernbach flask. The inoculum was prepared by the addition of 1 ml of sterile distilled water to each g of harvested conidia. The concentrated homogeneous conidial suspension containing about 1.5×10^9 conidia/ml was used as inoculum. 500-ml Erlenmeyer flasks containing 150 ml of Vogel's minimal medium supplemented with 2% (w/v) sucrose were inoculated with 0.5 ml of conidial suspension (7.5×10^8 conidia) and incubated in a reciprocal water-bath shaker at 110 strokes/min. Conidia were grown at temperatures varying from 25 to 46°C and all assays were carried out at regular intervals during heat treatments. Germination rates were determined every hour on cells fixed in 2.5% glutaraldehyde to stop development. Conidia were scored as germinated when emergence of germ tubes was first detectable.

From the early germ tube outgrowth period up to 8 h of germination, changes in the pH of the growth medium were determined with the glass electrode of a standard pH-meter (Radiometer, model PHM82).

The whole cell oxygen uptake and the percent age of cyanide-sensitivity were measured polarographically as previously described[2] with the Clark-type oxygen electrode (Gilson Oxygraph Model K-IC). for each point of measurement, aliquots of cultures were centrifuged for 5 min at 3000 × g at 20°C, and the cells resuspended in sterile Vogel's medium. The reaction chamber contained 1.6 ml of Vogel's medium and 100 µl of cell suspension corresponding to 0.5–1.0 mg of total cell protein. Because we had no possibility of centrifuging cultures at their corresponding growth temperatures, all of the respiratory measurements were determined at 25°C. KCN and SHAM were used as inhibitors of respiration. SHAM was dissolved in dimethyl-sulfoxide and KCN was freshly prepared in distilled water. The rate of the alternate respiratory pathway was determined by recording the O_2 uptake in the presence of KCN and SHAM after 2–3 min respiration. At the end of each respira-

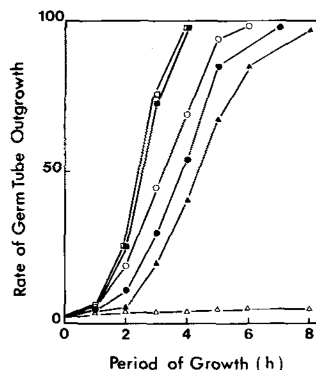

Figure 1. Germination rates of conidia from the wild type strain of *N. crassa* during 8 h of growth at temperatures varying from 25 to 46°C. At the intervals indicated, the percentage of the conidia that had germ tubes was determined with a hemocytometer. Conidia were incubated in Vogel's liquid shake cultures.
Symbols: —●—, 25–30°C; —○—, 33°C; —■—, 37°C; —□—, 41°C; —▲—, 43°C; and —△—, 46°C.

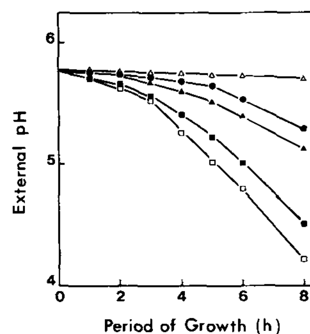

Figure 2. Time-course changes in the pH of the growth medium of conidia of wild type *N. crassa* incubated in Vogel's liquid shake cultures at temperatures varying from 25 to 46°C. At the intervals indicated, the pH of the medium was measured with a glass pH-electrode.
Symbols: —●—, 25°C; —■—, 37°C; —□—, 41°C; —▲—, 43°C; and —△—, 46°C.

tory measurement, the cells were recovered. Proteins were determined after hydrolysis at 37°C in 1 N NaOH by the method of Lowry et al.[11] using bovine serum albumin (BSA) as a standard. Oxygen uptake rates are expressed as natoms oxygen per min per mg of cell protein.

Results. Conidia of the wild type *N. crassa* strain STA_4 were incubated at 25, 30, 33, 37, 41, 43 and 46°C in Vogel's liquid shaken cultures. Since the rate of germination was found to be similar at 25 and 30°C, the same symbol has been used in figure 1, which illustrates the rates of conidial germination. No emergence was observed at any temperature during the first hour of incubation, but the burst of germ tube outgrowth within 60–90 min was most obvious at 37 and 41°C. After 2 h at 25–30°C, only 10% of conidia and by 5–6 h, 90% of conidia had germinated. A slight increase in the incubation temperature from 25 to 30°C to 33°C resulted in a stimulation of about 50% in the rate of germination. This heat-dependent acceleration in the process of germ tube emergence was observed up to 37°C.

A better synchronization of germ tube outgrowth from conidia was obtained at incubation temperatures of 37 and 41°C, with 70% of conidia germinated by 3 h of incubation (fig. 1); the same rate of germ tube outgrowth from conidia incubated at 25 or 30°C was obtained only after 4½ h of growth. When conidia were incubated at 43°C, there was a long period of latency (2 h) before their entry into the process of germ tube emergence: a sudden increase in the rate of germ tube outgrowth was observed between 2 and 3 h of incubation and 20% of the conidia had germinated by 3 h. However, all conidia were indeed able to produce germ tubes by 7–8 h of growth. Therefore, a significant decrease in the rate of germ tube emergence occurred when the temperature of incubation was raised from 41 to 43°C. At 46°C, the process of emergence was totally prevented unless conidia were transferred to the lower, normal temperature[12].

Figure 2 shows the time-course changes in the pH of extracellular medium of *N. crassa* incubated at temperatures varying from 25 to 46°C. At the time of inoculation, the pH of Vogel's medium was 5.8 and when conidia were incubated at 25 or 30°C, there was no change in the pH until 2 h of growth corresponding to the onset of germ tube outgrowth with a germination rate of 10% (fig. 1). As the number of conidia which entered into the process of germ tube emergence increased, the pH of the medium began to decrease slowly and after 5 h of incubation, the pH was 5.5, indicating a decrease of 0.3 unit as compared to the initial pH of the Vogel's medium. The process of acidification of the growth medium which resulted from proton extrusion out of the cells was accelerated when conidia were incubated at 37 and 41°C: after 2 h of incubation, a net decrease of 0.15 unit occurred in the extracellular pH, and at the end of the germination period (4 h), the respective pHs of the growth medium were 5.4 and 5.2. This observation shows that the decrease in the pH of the growth medium is related to the degree of germ tube emergence; when such outgrowth was prevented by incubation of conidia at 46°C, no significant change in the pH of the Vogel's medium could be measured (fig. 2).

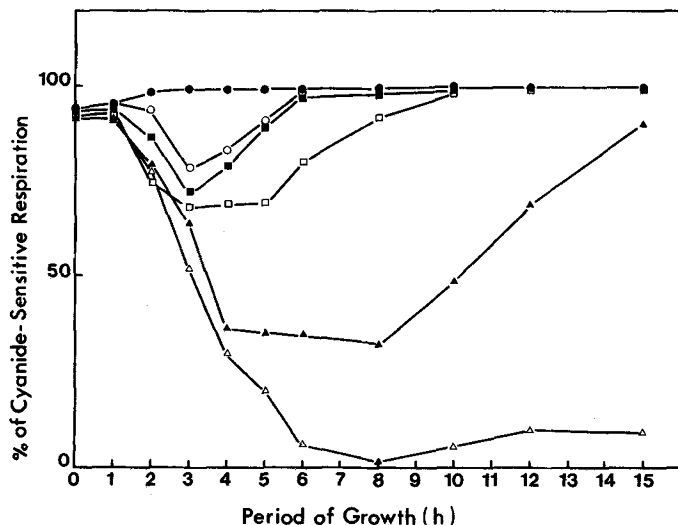

Figure 3. Heat and time-dependent changes in cyanide-sensitive respiration during the growth period of the wild type strain of *N. crassa*. Conidia were incubated in Vogel's liquid shake cultures at temperatures varying from 25 to 46°C. Whole cell respiration was measured and the percentage of inhibition by 1 mM KCN determined at regular intervals as indicated.
Symbols: —●—, 25°C; —○—, 33°C; —■—, 37°C; --□--, 41°C; —▲—, 43°C; and -△-, 46°C.

The whole cell respiration was also measured polarographically at regular intervals during 10–15 h of growth at various temperatures.

The oxygen uptake rate in freshly harvested conidia was about 150 natoms/min/mg protein and accelerated without any latency during the early stages of germination at temperatures up to 41°C, with an average maximum rate of 400 natoms O_2/min/mg protein. That peak of oxygen uptake rate corresponded to the end of the outgrowth stage, and reached an average of 425 natoms O_2/min/mg protein 1 h earlier at 37°C than at 25°C, at 4 h, in parallel with full outgrowth (fig. 1). The respiratory rate then sharply decreased when germ tubes started to elongate.

The pattern of oxygen uptake was markedly altered when conidia were incubated at 43°C. A maximum rate (250 natoms/min/mg protein) occurred after 3 h of incubation but was not followed immediately by the decrease found at lower temperatures. Conidia incubated at 46°C respired slowly and the maximum rate of oxygen uptake (180 natoms/min/mg protein) reached after 3–4 h remained unchanged up to 8 h of incubation before slowly decreasing (160 natoms O_2 at 10 h).

The percentage of inhibition of the whole cell respiration was measured with KCN at final concentration of 1 mM, in the absence of SHAM. Figure 3 shows time and heat-dependent changes in cyanide-sensitivity. The induction of the cyanide-insensitive respiratory pathways required a minimum time period of 1 h at all temperatures tested. Freshly harvested conidia respired via the cytochromic cyanide-sensitive pathway and 90–95% of O_2 uptake was inhibited by KCN. The remaining respiration was totally inhibited in the presence of 2 mM SHAM. This low SHAM-sensitivity usually disappeared when conidia were incubated in the growth medium at 25 or 30°C and the respiration remained totally cyanide-sensitive during the entire period of germ tube emergence up to 15 h of growth. However, when the incubation temperature was raised to 33°C, some brief, transient cyanide-insensitive respiration occurred during the very early stage of germination, but 80% of cell respiration was still inhibited with KCN by 3 h of growth. The recovery of the cytochromic pathway started thereafter and oxygen uptake was totally inhibited by KCN after 6 h of growth. In conidia incubated at 37 and 41°C, cell respiration became less sensitive to cyanide; after 3 h of growth, 70% of oxygen uptake proceeded via the cytochromic pathway, and cells were able to recover total cyanide sensitivity only after 6 h and 10 h respectively. Cyanide-insensitive respiration increased when conidia were incubated at 43°C. After 4 h of growth, the total inhibition of respiration by KCN was only about 30% and it remained unchanged during 8 h of incubation. However, the recovery of cytochromic respiration was still observed and 90–100% of oxygen uptake was inhibited after 15 h of growth. In conidia incubated at 46°C, less than 5% of respiration remained cyanide-sensitive after 8 h of incubation. No recovery

of cytochromic respiration could occur during a span of 15 h of incubation.

Discussion. We have found that both rates of respiration and germ tube outgrowth were strongly enhanced in conidia at 37–41 °C compared to 25–30 °C. At 25 °C, the general pattern of oxygen uptake was the same as that measured by others[13,14,15], while at 37 °C it followed the rate of outgrowth, attaining its maximum by the end of that stage 1 h earlier than at 25 °C. At its peak of activity at 37 °C, the anticipated oxygen uptake was close to that measured at 25 °C. However, 30% of the high respiration at 37 °C was found to be rerouted toward its cyanide-insensitive or alternate oxidase pathway. It is known that the partitioned electron flow during respiration is regulated so that 'the more efficient cytochrome chain operates at maximal activity and the less efficient alternate oxidase accomodates the surplus'[5]. This suggests that in conidia treated with moderate heat (37–41 °C), the stimulated respiratory rate leads to a transient saturation of the cytochromic pathway, which is compensated by the operation of the alternate oxidase or SHAM-sensitive pathway. The optimal proportion of rerouting (30% alternate oxidase activity) was obtained at 37–41 °C, in parallel with the highest rates of germination. A higher participation (70%) of the alternate pathway in 43 °C treated conidia was also found in the 'poky' strains of *N. crassa*[16]; it still allows a fair rate of emergence but slows down the elongation rate of the hyphal tube.

When the cyanide-sensitive pathway practically disappears, as occurs after 8 h at 46 °C, no germ tube can emerge any more. It has been shown[6] that the functioning of the cyanide-insensitive pathway leads to an acidification of the hyphal cytoplasm, probably by an increased compensatory glycolysis. We suggest that such acidification, noticeable in 46 °C overswollen conidia stained with permeating pH-indicators such as bromocresol green and alizarin yellow S (Turian, unpublished observations) might be related to the slowed mitochondrial reintrusion of protons related to the less efficient ATP synthetic activity allowed by that pathway. Also, the fact that such overswollen conidia are devoid of the 'proton sink' of an emerging acid tip, might contribute to the maintenance of the generalized, nonpolarized internal acidity.

How then could the temperature-modulated ratio of the two types of respiration optimize the rate of germ tube outgrowth? For now, we can only suggest that at the maximal rate of respiration reached at 37–41 °C, the transient and moderate activity of the alternate oxidase might stimulate the rate of germ tube outgrowth through its early acidification of the cytosol in the swollen conidia. This would be further enhanced by the vectorial extrusion of protons from the mitochondria fronting the site of germ tube outgrowth through stimulated ATPase[9], and further relay by the increased activity of the cytochromic pathway (proton translocation by cytochrome oxidase[17]). Such polarized acidification at the site of germ tube outgrowth[8] would be counterbalanced by the relative alkalinization of the spore body cytosol resulting from the proton reintrusion required for enhanced ATP synthesis in the fully recoupled mitochondria of the germinated conidia.

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Zone immunoelectrophoresis assay applied to α_1-acid glycoprotein secretion by isolated rat hepatocytes

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Summary. A method for measuring proteins in low concentrations applying the zone immunoelectrophoresis assay is reported. The low detection limit makes it possible to measure α_1-acid glycoprotein in rat serum and also to quantify the secretion of this protein after concentration of the incubation media containing less than 10^7 isolated rat hepatocytes. The method is simple and consumes very small quantities of antiserum.

Key words. Rat serum; rat hepatocytes; α_1-acid glycoprotein; zone immunoelectrophoresis.

An in vitro system of isolated rat hepatocytes is well suited to the study of the synthesis and secretion of proteins from liver without interfering with the catabolic process. But although it is easy to measure the rate of secretion of some major proteins such as serum albumin, it is far more difficult to determine the amount of minor proteins such as α_1-acid glycoprotein (α_1-AGP). The usual techniques, such as the electroimmunodiffusion method of Laurell[1] and the radial immunodiffusion of Mancini[2] are not sensitive enough. Methods such as radioimmunoassay allow the detection of proteins in very low concentrations, but call for the use of radioactively labelled compounds and a specific spectrometer. Recently, Vesterberg[3] has