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Respiration in Cells and Mitochondria of Male-Fertile and Male-Sterile *Nicotiana* spp.[1]

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ABSTRACT

Three cytoplasmic male-sterile *Nicotiana* cultivars together with corresponding male-fertile progenitors and restored lines were investigated in order to find possible correlations between respiratory characteristics and male sterility. Oxygen consumption measurements were performed on cells from suspension cultures as well as on mitochondria isolated from green leaves. Inhibitors, which have been reported to specifically block either the cytochrome (KCN) or the alternative (propyl gallate and salicylhydroxamic acid [SHAM] respiratory pathways, were used in order to measure the capacity and activity of the two pathways. One of the inhibitors, SHAM, was found unsuitable to measure the activity of the alternative pathway due to the lack of specificity of SHAM for this pathway. A great difference in the capacity of the alternative pathway was detected between the two types of cell materials tested. Mitochondria isolated from green leaves showed a capacity of the alternative pathway of 5 to 20% of total mitochondrial repiration, while the capacity of cells from suspension cultures generally ranged from 50 to 80%. In addition to this, *in organello* synthesis of mitochondrial proteins revealed differences between mitochondria isolated from green leaves and from cell suspensions. No correlation, however, could be found between respiratory characteristics and male sterility.

CMS,[2] a widespread phenomenon among higher plants, is utilized by plant breeders in the production of hybrid seeds. Mitochondria are known to contain the cytoplasmic factor involved in CMS, and specific alterations in both DNA and proteins have been detected in male-sterile lines (16, 18). To detect the basic causes of the aberrant reproductive development, which are still not known, CMS has been studied both structurally and biochemically. For example, tapetal cell degeneration has been noted by Bino (4, 5) in *Petunia* and by Warmke and Lee (35) in *Zea mays*. Biochemical changes in male-sterile plants have been reviewed by Kaul (18). Whether such alterations are the cause or the result of abnormal development in sterile plants is difficult to establish. Musgrave *et al.* (26) reported on a major difference in respiratory behavior between male-fertile and male-sterile plants from several species. All sterile plants investigated were found to lack the alternative respiratory pathway, while fertile lines showed a capacity of this pathway of about 20% of the total respiration.

The existence of an alternative respiratory pathway in higher plants is well documented (21, 23, 31). It can be detected through its resistance to cyanide and sensitivity to inhibitors such as SHAM, propyl gallate and disulfiram. Lambers (20) suggested that the alternative pathway functions as an overflow mechanism when the cytochrome pathway is saturated. An additional terminal oxidase responsible for the activity of this alternative respiratory pathway has been identified (6, 11, 12).

This study was performed to investigate whether a difference in the capacity of the alternative respiratory pathway could be detected between male-fertile and alloplasmic male-sterile *Nicotiana* materials. The genetic background of CMS is well defined in the chosen materials making comparative measurements between male-sterile cultivars and male-fertile progenitor lines possible. Besides this, cultivars restored to fertility were included in the study. The *Nicotiana* materials used have been analyzed earlier for variations in mitochondrial DNA and proteins (15).

MATERIALS AND METHODS

Plant Material

Three sets of materials were used. Each set consisted of four lines: a male-fertile *Nicotiana tabacum* cultivar, a male-fertile cytoplasmic donor species (*N. repanda*, *N. suaveolens*, or *N. debneyi*), the male-sterile cultivar that resulted from an introgression of the nucleus of the male-fertile *N. tabacum* cultivar into the cytoplasm of the respective donor species, and a corresponding cultivar with restored fertility. In each of the three sets, the nuclear background of the male-sterile, male-fertile, and restored cultivars of *N. tabacum* was identical, except for the presence, in restored lines, of genetic material originating from the respective donor species. These *Nicotiana* materials and their suitability for studies of CMS were described in more detail by Håkansson *et al.* (15).

Isolation of Mitochondria

Isolation of washed mitochondria for respiratory measurements was carried out according to Schwitzguebel and Siegenthaler (29) with the following modifications. Young leaves were harvested from 10 to 14 week old plants grown in the greenhouse. The leaves with a total fresh weight of 20 to 25 g were ground in several batches with a mortar and pestle

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[2] Abbreviations: CMS, cytoplasmic male sterility; SHAM, salicylhydroxamic acid.

together with extraction medium. Mitochondria were suspended in a small volume of 0.3 M mannitol buffer, containing 0.1 mM EDTA, 20 mM Hepes (pH 7.2) and 0.1% BSA (w/v).

Protein and Chl Estimation

Protein and Chl content were measured in the final mitochondrial suspension, as well as in the chloroplast fraction. Proteins were measured using the Bio-Rad protein assay based on the dye-binding method of Bradford (7) and Chl was estimated by the method of Arnon (1).

Cell Suspensions

Cell suspensions, derived from all the different *Nicotiana* lines, were grown in NO_3/NH_4 medium (27) except *N. repanda* which was grown in a modified MS medium (25) containing 0.1 mg/L 2,4-D, 1.0 mg/L NAA, and 0.5 mg/L BAP but no casein hydrolysate. The cells were cultured at 27°C in the dark in 25 mL of medium. Cell suspensions were subcultured every third day by adding 25 mL of fresh medium, swirling, and dividing the suspension into two flasks.

For respiration measurements, cells were washed through a sieve (1 mm pore size) using 0.1 M potassium phosphate buffer (pH 6.3) and then collected by centrifugation at 350*g*. The cells were resuspended in buffer, collected on a mesh (0.1 mm pore size), and transferred to Whatman No. 1 filter paper in a Büchner funnel.

Dry weight was determined after drying cells at 60°C for one day in a forced draft oven.

Respiratory measurements

Oxygen uptake was measured with a Clark O_2-type electrode (Yellow Springs Instrument Co, Yellow Springs, OH) at 25°C. The electrode was calibrated using air-saturated phosphate buffer (pH 7.2) in which the O_2-concentration was assumed to be 240 μM. The concentration of inhibitors needed for each *Nicotiana* line, to obtain total inhibition of a respiratory pathway, was determined by titration experiments. Propyl gallate, dissolved in absolute ethanol, and SHAM, dissolved in DMSO, were added in volumes resulting in final concentrations of less than 0.5% (v/v) of ethanol or 1% (v/v) DMSO. Respiration rate was measured 3 min after addition of the inhibitor. Electron flow through the different pathways was calculated according to van der Plas *et al.* (34), based on the equation $V_T = \rho V_{alt} + V_{cyt} + V_{res}$ (33). V_T is the total oxygen consumption in the absence of inhibitors. V_{alt}, the capacity of the alternative pathway, is the rate of oxygen consumption after addition of KCN (corrected for residual oxygen uptake). V_{cyt}, the capacity of the Cyt pathway, is the respiration after addition of SHAM or propyl gallate (corrected for residual oxygen uptake). The activity of the alternative pathway in the absence of inhibitors is ρV_{alt} and it is calculated as V_T minus V_{cyt} (corrected for residual respiration). That portion of the capacity of the alternative pathway which is engaged in the absence of inhibitors ($\rho V_{alt}/V_{alt}$) is indicated by ρ. V_{res} is the oxygen uptake that remains after adding inhibitors for both the Cyt and the alternative pathways. The symbol V_T' was used to represent that portion of total oxygen uptake which is actual respiration (33). V_T' is calculated as V_T minus V_{res}. When V_{cyt}, V_{alt}, and ρV_{alt}, which are always presented after correction for V_{res}, are given as percentages of total respiration, they were calculated as parts of V_T'. V_{res} was calculated as a percentage of V_T.

Measurements on isolated mitochondria were performed in the reaction medium described by Siedow and Bickett (32), with the addition of 750 μM ADP. About 2.5 mg protein/mL was added to a 3-mL chamber and respiration was started by addition of 10 mM succinate. The concentrations of inhibitors needed for total inhibition were 0.25 mM KCN, 5 μM propyl gallate or 1 mM SHAM.

Respiration by cells in suspension culture was measured 2 d after subculture, using 1.3 g washed cells in 0.1 M potassium phosphate buffer (pH 6.3), in a 4-mL chamber. KCN was used at a concentration of 2 mM and SHAM at 2 mM. Propyl gallate was used at 5 μM, except for *N. debneyi* (15 μM) and *N. suaveolens* (125 μM).

In Organello Protein Synthesis

Isolation of mitochondria from green leaves and cell suspensions of *N. debneyi*, *in organello* synthesis of mitochondrial proteins and SDS-gel electrophoresis were as described by Håkansson *et al.* (15).

RESULTS

Respiration of Isolated Mitochondria

Table Ia and Figure 1a show the O_2-consumption data for mitochondria isolated from green leaves, when propyl gallate was used as an inhibitor of the alternative respiratory pathway. The uninhibited oxygen uptake (V_T) was about the same for all lines, around 10 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, except for the restored line with *N. debneyi* cytoplasm which showed a slightly higher rate (Table Ia). The capacity of the alternative pathway (V_{alt}) was consistently low, with only one example (*N. repanda*) where a capacity above 20% of total respiration (V_T') was found (Fig. 1a). Male-sterile and restored lines showed a slightly lower V_{alt} (3–8%) compared to the cytoplasmic donor species (13–21%). The restored line carrying *N. suaveolens* cytoplasm was an exception to this with a V_{alt} of 16%. The capacity of the cytochrome pathway (V_{cyt}) was almost always 100%, which means that there was no electron flow down the alternative pathway in uninhibited respiration. Residual oxygen uptake (V_{res}), as calculated from Table Ia, varied from 6 to 13% with a mean value of 9%.

When SHAM was used as an inhibitor of the alternative respiratory pathway in the absence of KCN, the inhibition of respiration was usually higher than if SHAM was added after KCN. Such results indicate that the participation of the alternative pathway (ρV_{alt}) was larger than the capacity (V_{alt}) and thereby the fraction of the alternative pathway which was engaged (ρ) reached values greater than 1 (Fig. 1b). No difference in residual respiration, depending on if SHAM was added before or after KCN, was detected (data not shown).

Protein and Chl Estimations

The contribution of contaminating thylakoids to protein estimations in preparations of leaf mitochondria was calcu-

Table I. *Respiratory Characteristics of Nicotiana*

V_T = uninhibited oxygen uptake; V_{cyt} = respiration after addition of propyl gallate; V_{alt} = respiration after addition of KCN; V_{cyt} and V_{alt} are both corrected for residual respiration (V_{res}); $\rho V_{alt} = V_T - V_{cyt} - V_{res}$ = the activity of the alternative pathway in the absence of inhibitors; $\rho = \rho V_{alt}/V_{alt}$; n = number of experiments.

	N. tabacum		Set 1			Set 2			Set 3		
	Kupchunos	SC58	*N. repanda*	CMS*	Restored*	*N. suaveolens*	CMS*	Restored*	*N. debneyi*	CMS**	Restored**
a. Mitochondria isolated from green leaves. All values are expressed in nmol $O_2 \cdot min^{-1} \cdot mg$ protein^{-1} ± SD.											
V_T	12.42 ± 1.35	9.70 ± 3.11	8.52 ± 0.84	13.02 ± 2.27	11.14 ± 1.57	9.59 ± 1.27	11.38 ± 3.79	11.91 ± 1.44	10.00 ± 2.89	11.55 ± 1.79	17.08 ± 2.36
V_{cyt}	11.99 ± 1.66	8.57 ± 3.48	7.70 ± 1.13	12.18 ± 2.64	10.57 ± 1.64	9.13 ± 1.43	10.56 ± 4.68	11.31 ± 1.69	8.37 ± 1.86	10.32 ± 1.86	17.24 ± 2.29
V_{alt}	0.78 ± 0.23	0.85 ± 0.76	1.55 ± 0.40	0.98 ± 0.42	0.61 ± 0.78	1.18 ± 0.63	0.66 ± 0.57	1.79 ± 0.69	1.16 ± 0.72	0.58 ± 0.52	0.36 ± 0.40
ρV_{alt}	0	0.12 ± 0.25	0	0	0	0	0	0	0	0	0
ρ	0	0.12 ± 0.25	0	0	0	0	0	0	0	0	0
V_{res}	0.96 ± 0.34	1.13 ± 0.28	0.85 ± 0.37	1.01 ± 0.36	0.84 ± 0.45	0.62 ± 0.20	0.99 ± 0.32	0.77 ± 0.41	1.02 ± 0.34	1.01 ± 0.52	1.32 ± 0.70
n	3	4	3	3	4	3	4	3	3	3	3
b. Cell suspensions. All values are expressed in μmol $O_2 \cdot min^{-1} \cdot g$ dry weight^{-1} ± SD.											
V_T	3.58 ± 0.79	4.50 ± 0.72	2.49 ± 0.40	3.49 ± 0.41	3.59 ± 0.38	3.01 ± 0.65	4.25 ± 0.25	4.22 ± 1.70	3.21 ± 0.99	4.14 ± 0.37	4.84 ± 1.12
V_{cyt}	2.30 ± 0.34	3.39 ± 0.59	1.79 ± 0.11	3.06 ± 0.15	3.03 ± 0.17	2.25 ± 0.52	3.33 ± 0.14	2.66 ± 0.57	2.27 ± 0.59	3.34 ± 0.42	3.23 ± 0.80
V_{alt}	1.76 ± 0.70	2.18 ± 0.25	0.46 ± 0.02	2.45 ± 0.32	2.02 ± 0.73	1.86 ± 0.52	3.03 ± 0.54	3.07 ± 1.09	2.29 ± 0.62	2.60 ± 0.45	2.61 ± 0.62
ρV_{alt}	0.72 ± 0.32	0.90 ± 0.28	0.26 ± 0.37	0.30 ± 0.43	0.38 ± 0.35	0.32 ± 0.24	0.70 ± 0.22	1.90 ± 1.69	0.43 ± 0.13	0.57 ± 0.43	1.21 ± 0.75
ρ	0.42 ± 0.22	0.40 ± 0.13	0.50 ± 0.71	0.10 ± 0.15	0.17 ± 0.16	0.18 ± 0.12	0.23 ± 0.08	0.40 ± 0.16	0.23 ± 0.09	0.27 ± 0.23	0.47 ± 0.26
V_{res}	0.24 ± 0.09	0.28 ± 0.14	0.28 ± 0.10	0.24 ± 0.09	0.22 ± 0.05	0.33 ± 0.11	0.28 ± 0.09	0.29 ± 0.16	0.27 ± 0.12	0.21 ± 0.12	0.27 ± 0.14
% Dry wt	2.7 ± 0.1	2.5 ± 0.1	3.6 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.3	2.6 ± 0.1	2.4 ± 0.2	2.7 ± 0.2	2.2 ± 0.2	2.3 ± 0.4
n	4	4	2	4	4	4	4	4	4	4	5

* Nucleus: *N. tabacum* var SC58. ** Nucleus: *N. tabacum* var Kupchunos.

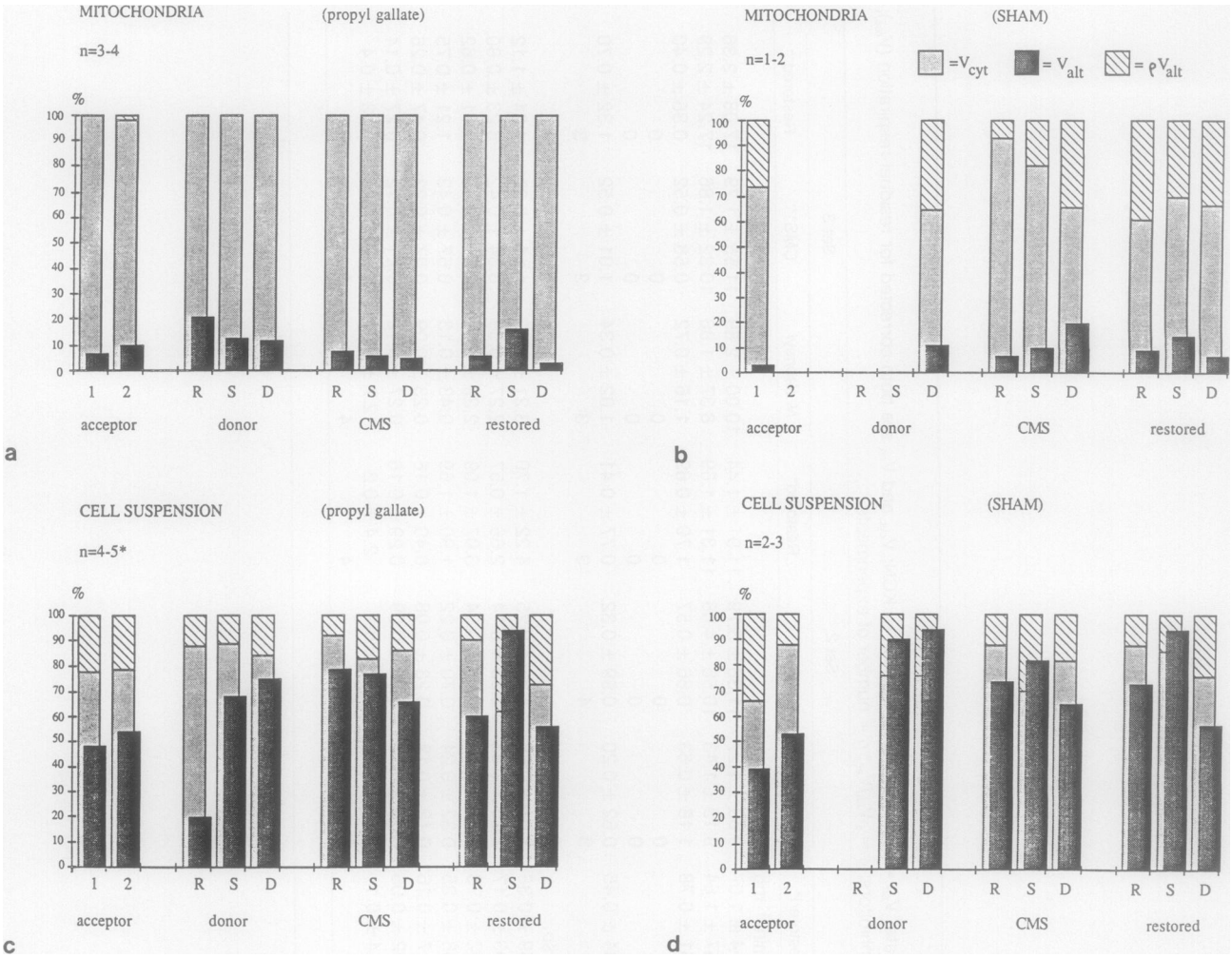

Figure 1. Capacity and activity of different respiratory pathways in *Nicotiana* as percentage of total mitochondrial respiration (V_T), using either propyl gallate (a, c) or SHAM (b, d) as inhibitor for the alternative pathway and KCN as inhibitor for the Cyt pathway. 1, *N. tabacum* var Kupchunos; 2, *N. tabacum* var SC58; R, *N. repanda*; S, *N. suaveolens*; D, *N. debneyi*. Donor: letters stand for species. CMS and Restored: letters stand for cytoplasm. R and S contain nucleus 2, D contains nucleus 1. Restored lines have additional nuclear genetic material from corresponding donor species. $V_{cyt} + \rho V_{alt} = 100\%$. See Table I for explanation of V_{cyt}, V_{alt}, and ρV_{alt}. * Donor R: $n = 2$.

lated for 17 different isolations. By measuring both protein and Chl content of membranes isolated from the chloroplast fraction, the thylakoid protein to Chl ratio was found to average 6 (SD ± 1) to 1. These data, together with data for the Chl content in mitochondrial preparations, were used to calculate the extent of contamination by thylakoids in the mitochondrial isolations. Contaminating proteins of thylakoid origin averaged 55% of total protein in mitochondrial preparations and varied from 38 to 77%.

Respiration in Cell Suspensions

Table Ib and Figure 1c show the data from respiration measurements on whole cells from cell suspensions, using propyl gallate as the inhibitor of the cyanide resistant pathway. Rates of uninhibited O_2-consumption (V_T) varied from 2.49 to 4.84 μmol $O_2 \cdot min^{-1} \cdot g$ dry wt^{-1}, with the highest value for the restored line with *N. debneyi* cytoplasm and the lowest rates for the cytoplasmic donor species (Table Ib). An active alternative respiratory pathway was detected in all lines. Its capacity ranged from about 20 to 95% of the total respiration (V_T') (Fig. 1c). The capacity of the Cyt pathway was found to be around 80 to 90% of total respiration (V_T') for most lines. Thus the activity of the alternative pathway in the absence of inhibitors was about 10 to 20% of V_T' (Fig. 1c) and ρ varied between 0.10 and 0.60 (Table Ib). Residual oxygen consumption, as calculated from Table Ib, was in the range from 4 to 12% with a mean of 7%.

When SHAM was used as the inhibitor for the alternative respiratory pathway in cell suspensions, similar results were obtained as for isolated mitochondria. SHAM was in most cases more inhibitory in the absence of KCN than was propyl gallate and therefore, higher values of ρV_{alt} were also obtained (Fig. 1d). The measured capacities of the alternative pathway (V_{alt}), using SHAM as an inhibitor (Fig. 1d), were in general agreement with those found for propyl gallate (Fig. 1c).

In Organello Protein Synthesis

The mitochondrial protein patterns resulting from *in organello* protein synthesis revealed variations between mito-

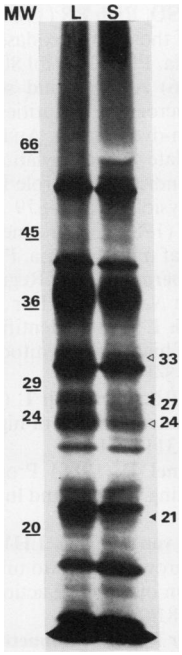

Figure 2. Polypeptides synthesized by mitochondria isolated from green leaves (L) and cell suspensions (S) of *N. debneyi*. Mol wt of marker proteins are indicated (kD). Each lane was loaded with labeled proteins corresponding to 100,000 cpm. Major differences in synthesized proteins are indicated with estimated mol wt (kD). (◁), Proteins that are synthesized in somewhat lower amounts in S compared to L; (◀), proteins that appear to be almost absent in S.

chondria isolated from green leaves and cell suspensions (Fig. 2). Several protein bands were either missing or appeared weaker in the lane with mitochondrial proteins from cell suspensions when compared to mitochondrial proteins from green leaves.

DISCUSSION

The capacity of the alternative respiration in mitochondria isolated from green leaves was, in most lines, found to be about 5 to 10% of total respiration (V_T'). This figure is very low, especially considering that the variation inherent in the techniques of measurement is about 5%. Although male-sterile lines showed a slightly lower capacity of the alternative pathway than male-fertile cytoplasmic donor species, restored lines were lower as well. Thus, no correlation between V_{alt} and CMS could be found for isolated mitochondria.

Respiration measurements of cell suspensions definitely showed the presence of an alternative pathway with a high capacity, but as for isolated mitochondria, no association between the capacity of this pathway and male-sterility could be found. These results are in contrast with those of Musgrave *et al.* (26), who reported that the alternative pathway was absent in male-sterile lines from several different plant species, while corresponding male-fertile materials revealed a capacity of about 20% of total oxygen uptake (V_T). However, more recent reports are consistent with the findings reported here. According to van der Plas *et al.* (34), no increase or decrease in V_{alt} was detected in male-sterile compared to male-fertile *Petunia* cell suspensions. Obenland *et al.* (28) performed respiration measurements on leaf and root tissues from male-sterile and male-fertile *Glycine max* and concluded that the presence of sterility had no influence on the capacity of the alternative pathway in this material.

No correlation was detected between total oxygen uptake and CMS. Van der Plas *et al.* (34) detected a higher V_T in suspension cultured cells of cytoplasmic male-sterile *Petunia* than in cells from male-fertile cultures. Even though our data on male-sterile *Nicotiana* showed a similar tendency, restored lines also showed high V_T and, therefore, the correlation to CMS could be ruled out.

The great difference in capacity of the alternative pathway in cell suspensions in comparison to mitochondria from green leaves is very striking. V_{alt} was about 5% of total respiration in mitochondria from leaves whereas V_{alt} for cell suspensions mainly ranged from 50 to 80%. Horn and Mertz (17) also found a high capacity (70%) of the alternative pathway in respiration measurements performed on *N. glutinosa* cell suspensions. When mitochondria were isolated from these suspensions, similar results were obtained. They concluded that their data support the overflow hypothesis (20), according to which saturation of the Cyt pathway will shift electron flow to the alternative pathway. Saturation can occur when there is a surplus of carbohydrates, which is the case in growth media for cell suspensions. It has also been suggested that elevated levels of endogenous ethylene could be the reason for the high values of V_{alt} found for cells in tissue culture (14). Most media for plant tissue culture contain high levels of auxin, which is known to stimulate ethylene synthesis. It would be interesting to investigate whether the great capacity of the alternative pathway in cell suspensions is correlated with a higher amount of the alternative oxidase that was identified by Elthon and McIntosh (11). In any case, suspension cultures could provide the cells of choice for those studying the biochemistry and physiology of the alternative pathway.

The *in organello* synthesis of mitochondrial proteins in green leaves and cell suspensions was performed to see if differences in protein synthesis could be correlated with the differences in capacity of the alternative pathway. In *Neurospora* the presence of the alternative pathway requires at least two nuclear genes (2). However, there is also evidence that a mitochondrically encoded regulatory factor is needed (10). Siedow and Berthold (31) described this factor as a repressor which prevents the expression of nuclear-encoded genes needed to develop an alternative pathway. Our results show that mitochondria from cells grown in suspension culture (the material with highest V_{alt}) fail to synthesize a couple of proteins and also synthesize lesser amounts of a few others, in comparison to mitochondria from green leaves. However, it still remains to be investigated if any of these proteins are correlated with the alternative respiratory pathway.

The rates of uninhibited oxygen uptake by isolated mitochondria from green leaves were found to be low compared to earlier studies where values from 46 up to 800 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ were reported (13, 19, 22, 29, 30). One reason for the low rates of oxygen uptake obtained in our study on *Nicotiana* could be that our measurements were performed on washed mitochondria, which are contaminated with broken thylakoids, rather than on mitochondria purified

on a gradient. To investigate how much thylakoid protein contamination contributed to the low values of oxygen consumption per mg protein, the ratio of thylakoid proteins to Chl was determined. A ratio of 6 to 1 was found, which is in agreement with earlier results showing a ratio of 7 to 1 (8, 9). The Chl content of the mitochondrial preparations indicated that about 40 to 75% of the protein in these preparations was of thylakoid origin. A correction for this protein contamination to the rate of oxygen uptake would increase rates to around 20 to 30 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, which is close to the lower values in earlier reports (13, 22, 29).

The results obtained when SHAM was used as an inhibitor of the alternative respiratory pathway indicated that the engagement of this pathway exceeded its capacity, which is logically impossible. Results such as these have been reported earlier (3, 22, 24) if the concentration of SHAM was higher than 2 mM. In our investigation values of ρ greater than 1 were obtained at concentrations of 1 to 2 mM SHAM. By titration experiments, these concentrations were found to be necessary for the complete inhibition of the alternative pathway. The residual respiration was found to be no higher when SHAM was added after KCN rather than before, and therefore incomplete inhibition of the alternative pathway in the former case can be ruled out as an explanation for obtaining ρ-values above 1. Instead, the most likely explanation seems to be that SHAM causes a nonspecific inhibition of the Cyt pathway. We conclude that SHAM is not suitable to use for determining the activity of the alternative pathway (ρV_{alt}) in the *Nicotiana* material. However, in measurements of the capacity of the alternative pathway (V_{alt}) SHAM is added after KCN, which means that the KCN sensitive pathway is already blocked and no unspecific SHAM inhibition can take place. This was confirmed by the fact that similar values of V_{alt} were found using either SHAM or propyl gallate; therefore, SHAM accurately measures the capacity of the alternative pathway.

The respiratory analysis of male-fertile and male-sterile *Nicotiana* materials revealed a great difference in the capacity of the alternative pathway between mitochondria isolated from green leaves and cells in suspension culture. However, no obvious correlation between respiratory characteristics and CMS was detected in either type of material.

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