Plant Physiol. (1982) 69, 1439–1443
0032-0889/82/69/1439/05/$00.50/0

Cyanide-Resistant Respiration in Suspension Cultured Cells of *Nicotiana glutinosa* L.

Received for publication February 27, 1981 and in revised form December 14, 1981

MICHAEL E. HORN[1] AND DAN MERTZ
Division of Biological Science, University of Missouri, Columbia, Missouri 62511

ABSTRACT

The respiration of dark-grown *Nicotiana glutinosa* L. cells in liquid suspension culture was found to be highly cyanide resistant and salicylhydroxamic acid (SHAM) sensitive, indicative of an active alternative respiratory pathway. This was especially true during the lag and logarithmic phases of the 14-day growth cycle. Mitochondria isolated from logarithmically growing cells exhibited active oxidation of malate, succinate, and exogenous NADH. Oxidation of all three substrates had an optimum pH of 6.5 and all were highly resistant to inhibition by cyanide and sensitive to SHAM. Respiratory control was exhibited by all three substrates but only if SHAM was present to block the alternative pathway and divert electrons to the phosphorylating cytochrome pathway. The cyanide-resistant oxidation of exogenous NADH has previously only been associated with *Arum* spadix mitochondria. Coemergence during evolution of the alternative respiratory pathway and the exogenous NADH dehydrogenase in plant mitochondria as a possible mechanism for removal of cytoplasmic NADH is proposed. Evidence is presented which suggests that mitochondrial assays should be performed at pH 6.5.

Cyanide-resistant respiration has been described in a wide assortment of plant species (10, 24). Most of the work has been performed with mitochondria isolated from intact plant tissues (mung bean shoots, skunk cabbage spadices, etc.). With the current interest in plant tissue culture as an experimental tool it was thought to be of importance to characterize the respiration of cultured plant cells. Wilson (28–30) has examined respiration of isolated mitochondria from cultured sycamore cells and found it to be partially resistant to inhibition by cyanide. RC[2] was rather low with a ratio of 2.0 or less. With the discovery of the hydroxamates, inhibitors of the alternate respiratory pathway (22), it has been reported that they are effective in inhibiting respiration resistant to cyanide or antimycin A in cultured cells of tobacco (21) and soybeans (16). In the present investigation, using suspension cultured cells of *Nicotiana glutinosa* L. and isolated mitochondria from these cells, it has been established that respiration is sensitive to the hydroxamate SHAM and the sensitivity varies over the course of the growth cycle. The high sensitivity of respiration to SHAM and the low RC found in mitochondria from these cultures suggests that mitochondrial ATP production is limited.

[1] Present address: Department of Agronomy, University of Illinois, 1102 S. Goodwin, Urbana, IL 61801; to whom reprint requests should be addressed.

[2] Abbreviations: RC, respiratory control; SHAM, salicylhydroxamic acid; RCR, respiratory control ratio.

MATERIALS AND METHODS

Culture of Pith Tissue. Callus cultures were established from pith segments of greenhouse grown *Nicotiana glutinosa* L. plants. The culture medium was MS medium (19) with 2.0 mg/L 2,4-D and 0.25 mg/L kinetin. To establish a cell suspension approximately 1-g pieces of callus were transferred to flasks containing 50 ml of liquid MS medium and grown in the dark on a gyratory shaker at 110 rpm at 20 to 22°C. The cells were subcultured every 14 days by transferring 0.5 g fresh weight of cells to 50 ml fresh medium. All work was performed aseptically in a laminar flow hood. For experiments involving mitochondrial isolation, 1.5 g of cells were transferred to 150 ml medium in 500 ml flasks. After 8 d growth, three flasks yielded 100 to 150 g fresh weight of tissue.

Oxygen Monitor Apparatus and Respiratory Measurements. For O_2 uptake studies, a temperature-controlled (28°C) Yellow Springs Model 52 Biological Oxygen Monitor was used. The reaction volume was 3.0 ml. The reaction mixture for mitochondrial experiments consisted of 0.25 M sucrose, 0.01 M Hepes buffer, 0.005 M $MgCl_2 \cdot H_2O$, 0.01 M KH_2PO_4, and 0.1% BSA (fraction V; Sigma). The pH was adjusted to 6.8 unless otherwise stated. The inhibitors SHAM and antimycin A were dissolved in 95% ethanol. Preliminary studies showed that a final concentration of 1.6% alcohol caused no significant change in either the qualitative or quantitative activity of the mitochondria within a 10-min period.

The reaction mixture for the respiratory measurements of whole cells consisted of 0.09 M sucrose, 50 mM Mes (pH 6.5). The buffer and pH were determined to be optimum for these cells (11).

Mitochondrial Isolation. The mitochondrial isolation procedure was similar to that described by Charbonnier and Bervillé (4). The tissue was placed in a chilled mortar containing cold grinding medium (1:1, w/v) and a small amount of cold, wet quartz sand. The grinding medium was composed of 0.4 M sucrose, 0.05 M KH_2PO_4, 0.005 M EDTA, and 1.0 mg/ml BSA. The pH was adjusted to 7.2 with 1 M KOH. In some experiments, 0.005 M β-mercaptoethanol was added to the grinding medium; however, β-mercaptoethanol had no noticeable effect. The tissue was then homogenized for about 30 s using a chilled pestle. The homogenate was squeezed through a layer of Miracloth into centrifuge tubes. The homogenate was centrifuged at 10,000*g* for 15 s (once speed was reached) in a Sorvall refrigerated centrifuge utilizing an SS-34 rotor. The supernatant was then centrifuged for 2 min (once speed was reached) at 34,000*g*. The pellet was suspended in 1.0 ml of cold 0.25 M sucrose (pH 7.2). A 0.1-ml aliquot was removed for protein determination (14) and a 0.1-ml aliquot of 1 mg/ml BSA in 0.25 M sucrose (pH 7.2) was added back bringing the total volume to 1.0 ml.

For comparative studies of isolation procedures, the methods of Day and Hanson (5) and Bonner (3) were followed. For the purification of mitochondria, the procedure of Douce *et al.* (6) was used. Corn shoot mitochondria were isolated using the method of Day and Hanson (5) and 8-d-old etiolated shoots grown in vermiculite at 29°C.

Table I. *Effect of Cyanide and SHAM on Respiration during the Growth Cycle of Cultured Cells of Nicotiana glutinosa L*

The reaction mixture contained 100 to 300 mg of washed cells, 0.03 g/ml sucrose, and 50 mM Mes buffer (pH 6.5). Final volume was 3.0 ml. Values are averages of two trials.

Day	Basal[a] Rate	1 mM KCN	Change	2 mM SHAM	Change	KCN and SHAM	Change
			%		%		%
2	1.2×10^{-4}	1.3×10^{-4}	+6.6	1.1×10^{-4}	−11.0	4.2×10^{-6}	−96.5
4	1.3×10^{-4}	1.4×10^{-4}	+7.6	9.8×10^{-4}	−24.9	2.6×10^{-6}	−98.0
6	1.0×10^{-4}	8.3×10^{-5}	−17.5	3.9×10^{-5}	−60.8	4.2×10^{-6}	−95.8
8	2.9×10^{-5}	2.1×10^{-5}	−26.6	9.9×10^{-5}	−65.7	0	−100.0
11	2.7×10^{-5}	2.2×10^{-5}	−18.6	2.7×10^{-5}	−1.8	9.5×10^{-7}	−96.5
13	1.9×10^{-5}	1.7×10^{-5}	−12.2	2.5×10^{-5}	+30.8	1.5×10^{-6}	−92.3

[a] μmol O_2/min·mg fresh weight.

RESULTS

Effects of Inhibitors on Whole Cell Respiration. The effects of cyanide and SHAM on dark-grown *N. glutinosa* L. cells were investigated (Table I). During the course of the 14-d growth cycle, 1 mM KCN slightly promoted O_2 uptake during lag phase (day 0–4); thereafter KCN became inhibitory, reaching a maximum of 27% at day 8. After the log phase of growth had ended at day 8 a slight decline was observed in the amount of inhibition by cyanide. A somewhat different inhibition was noted with the inhibitor SHAM (2 mM). During the logarithmic growth phase inhibition by SHAM increased from 25% at day 4 to a maximum of 66% at day 8. After day 8 the inhibitory effects of SHAM decreased until day 13 at which time SHAM promoted O_2 uptake. However, by day 13 the rate of respiration was very low; therefore, a slight increase in the rate of O_2 uptake translated into a large percentage. In the presence of cyanide and SHAM, respiration during the entire growth period was inhibited by 92% or higher, indicating that all but a small fraction of the O_2 uptake was attributable to the mitochondria.

The effects of cyanide, antimycin A, azide, and rotenone were compared in the presence and absence of SHAM (Table II). Antimycin A (5 g/L) and rotenone (0.1 mM) were only slightly inhibitory to cell respiration while cyanide and azide (5 mM) inhibited respiration by 22 and 61%, respectively. These results show that the respiration of cultured tobacco cells is more or less similar to that of differentiated tissue.

Mitochondrial Isolation. Several methods of isolation were tested in efforts to obtain a mitochondrial preparation which would exhibit a high RCR. Of the several procedures used, none gave an RCR greater than 2.0 and 2 mM SHAM had to be present for any RC to be observed (Table III). Purification of mitochondria on a discontinuous sucrose gradient (6) did not improve the RC values. Respiratory control values obtained from mitochondria isolated from etiolated corn shoot are shown in Table III for comparative purposes. Using corn shoot mitochondria, succinate oxidation exhibited good RCR in the absence of SHAM. Thus, it seems that the apparent low RCR of mitochondria from suspension cultures was not due to the method of isolation used but was an actual measure of RC potential *in vivo* as also found by Wilson (29).

Table III. *RCR Values and ADP:O Ratios of Mitochondria for Various Substrates in the Presence and Absence of SHAM*

RCR was determined using 179 μM ADP. All values are the range determined from many experiments.

	Basal Rate	Respiratory Control Ratio		ADP:O
		SHAM absent	SHAM present	
	nmol O_2/min·mg protein			
N. glutinosa mitochondria				
Malate	76–85	1.0	1.25–1.68	2.4
Succinate	60–70	1.0	1.20–1.35	1.7
NADH	90–100	1.0	1.10–1.35	1.8
Zea mays shoot mitochondria				
Succinate	48–50	2.4–2.6	2.4–2.6	1.3

pH Studies. Malate, succinate, and NADH were readily oxidized by mitochondria from 8-d-old *N. glutinosa* cells. Experiments were performed to determine the optimum pH for the oxidation of each substrate in the presence and absence of 1 mM cyanide and 2 mM SHAM. Hepes buffer (10 mM) was used throughout the pH range tested (4.5–7.5) even though the buffering effectiveness of Hepes has a lower limit of pH 6.5 (9). The pH of the reaction mixture was checked after each experiment and found to be within ± 0.1 pH units from the initial pH.

Malate. The oxidation of 10 mM malate (Fig. 1) gave a linear

Table II. *Effect of Cyanide, Antimycin A, Azide, and Rotenone on Respiration of 8-Day-Old Cells of Nicotiana glutinosa L*

The reaction mixture was identical to Table I with 100 mg of washed 8-d-old cells. Antimycin A and rotenone were added in 0.05 ml of 95% ethanol. Values shown are averages of two trials.

Inhibitor	Basal Rate[a]	+ Inhibitor	Inhibition	+ 2 mM SHAM	Inhibition
			%		%
KCN, 1 mM	1.16×10^{-4}	8.99×10^{-5}	22.5	1.94×10^{-5}	83.3
Antimycin A, 5 μg/ml	3.44×10^{-5}	3.34×10^{-5}	2.9	0	100.0
Azide, 5 mM	6.83×10^{-5}	2.65×10^{-5}	61.2	5.70×10^{-6}	91.7
Rotenone, 0.1 mM	5.17×10^{-5}	4.95×10^{-5}	4.3	2.01×10^{-5}	61.2
SHAM, 2 mM	3.79×10^{-5}			1.30×10^{-5}	65.7[b]

[a] μmol O_2/min·mg fresh weight.

[b] This represents the sole effect of SHAM.

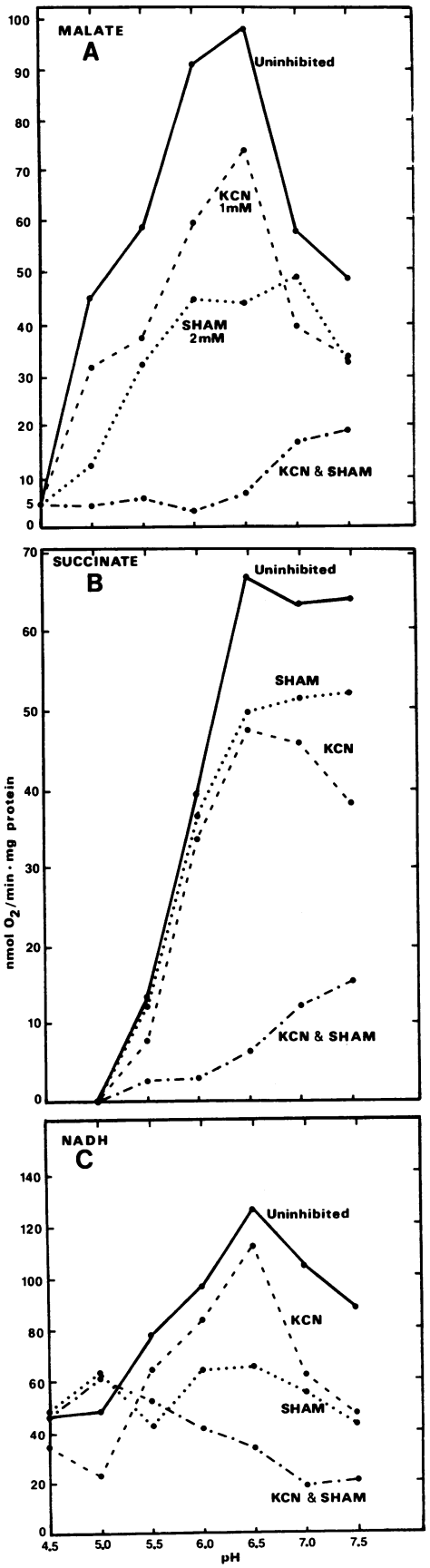

FIG. 1. Effect of pH on substrate oxidation by tobacco mitochondria in the presence of 1 mM cyanide, 2 mM SHAM, and cyanide + SHAM. The rates are state 2 (no ADP present).

rate of O_2 uptake until anaerobiosis was reached. Since no metabolite such as pyruvate was needed to relieve inhibition by the malate oxidation product oxaloacetate, it is assumed that there exists an endogenous mechanism for removal of oxaloacetate (unpublished data). The optimum pH for the oxidation of malate was 6.5 (27). Inhibition by cyanide was around 30% through the entire pH range. Inhibition by SHAM in general, was near 50% in the middle of the pH range and then dropped to 15% at pH 7.0. Inhibition in the presence of cyanide and SHAM was 90% or higher from pH 5.0 to 6.5 but somewhat less (71%) at 7.0.

Succinate. The oxidation of 10 mM succinate was negligible at pH 4.5 and 5.0 but increased rapidly thereafter until the optimal pH of 6.5 was reached (Fig. 1). Inhibition by cyanide ranged from 30 to 40% from pH 6.0 to 7.5 while the inhibition by SHAM increased from 7% at pH 6.0 to 26% at pH 6.5 before decreasing to 19% at 7.0 to 7.5. Inhibition in the presence of cyanide and SHAM was around 80% except at pH 6.0 and 6.5 where it increased to 92 and 90%, respectively.

NADH. The rates of oxidation of NADH were the highest for any substrate tested (Fig. 1). The optimum pH for oxidation was 6.5 at which point cyanide inhibition was only 11%. With higher pH, the cyanide inhibition increased to 40 to 45%. Inhibition by SHAM was quite variable with a slight promotion of O_2 uptake at pH 4.5 and 5.0. With higher pH SHAM inhibition ranged from 33 to 50%. The inhibition in the presence of cyanide and SHAM increased from pH 5.5 to reach a maximum of 82% at pH 7.0.

The Effect of Azide, Antimycin A, and Cyanide on Malate Oxidation. Antimycin A and azide were compared with cyanide for their effectiveness in inhibiting electron transport through the Cyt chain (Fig. 2). Azide and cyanide inhibited malate oxidation 41 and 35%, respectively, whereas antimycin A caused a 23% inhibition. In the presence of SHAM, cyanide and antimycin A respiration was inhibited by 94 and 92%, respectively, while azide produced only a 78% inhibition. In contrast with the cell respiratory studies, azide did not inhibit Cyt oxidase or the Cyt chain to the extent of antimycin A or cyanide. Again, these data show that mitochondria from cultured tobacco cells are basically similar in many respects to those from differentiated tissue.

DISCUSSION

We have shown that the respiration of *N. glutinosa* suspension cultured cells was highly cyanide-resistant during the lag and logarithmic phases of growth. In addition, SHAM was a significantly more effective respiratory inhibitor than cyanide during the same growth phases. We conclude therefore that respiration was mediated mainly via the alternative respiratory pathway. It has been suggested that lipoxygenase is responsible for much of the cyanide-resistant O_2 uptake in some systems (7, 8, 20) but inasmuch as this enzyme is mainly associated with lipid degradation it is hard to conceive of lipoxygenase playing a significant role during exponential growth (26).

Our data supports the overflow hypothesis (13) in which it is postulated that the alternative pathway is active during periods where there is an abundance of carbohydrate relative to the demand. In our system, the growth medium initially contained 3% sucrose and then declined during the two week growth cycle.

Mitochondria isolated from cells in the logarithmic growth phase exhibited malate oxidation that was also highly cyanide-resistant and SHAM-sensitive, and the inhibitory characteristics were quite similar to whole cell respiratory inhibition *only* if the mitochondrial assay was performed near pH 6.5 (compare Table I with Fig. 1A). Moreover, all three mitochondrial substrates had optimum oxidation rates at pH 605 (Fig. 1; also Ref. 16). Because most mitochondrial respiratory assays are performed at alkaline pH (7.2–7.5) this may be a significant problem.

The chemiosmotic hypothesis (17) states that mitochondria have active proton pumps which pump protons in an outward manner

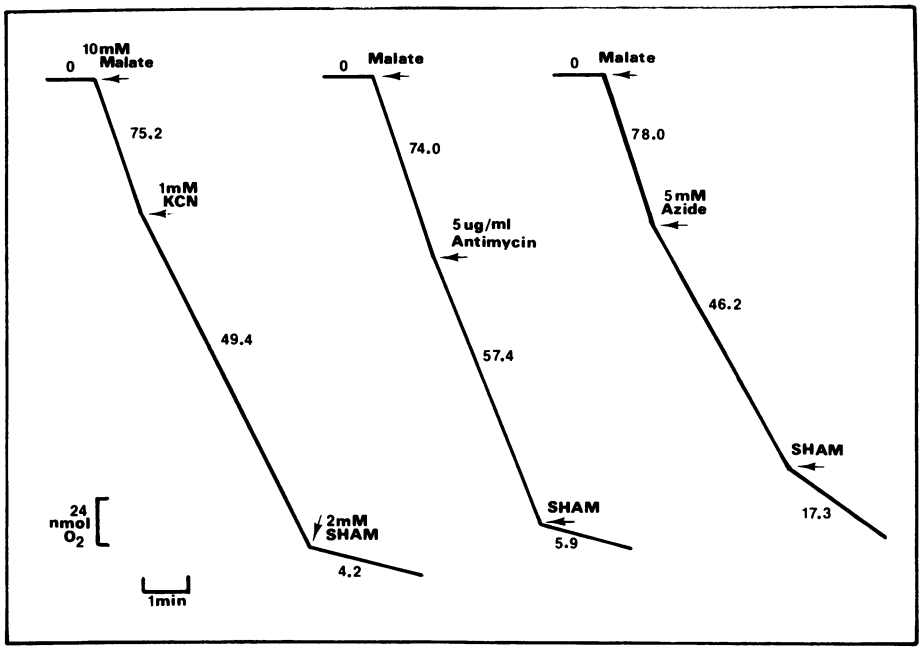

FIG. 2. Effects of cyanide, antimycin A, and azide on malate oxidation by tobacco mitochondria. Arrows indicate the addition of inhibitors. The rates are state 2 (no ADP present).

during coupled electron transport. In both animal (2) and plant mitochondria (18) this proton pumping results in a ΔpH of considerable magnitude (ΔpH = 1.62, (18)). Assuming a cytoplasmic pH of 7.0–7.2 (23), it is quite possible that a part of this ΔpH is manifested as a localized pH drop in the immediate environment of the mitochondria, perhaps as low as pH 6.5. We suggest that pH 6.5 is a more reasonable reaction mixture pH.

The mitochondria from cells in the logarithmic phase of growth were loosely coupled to oxidative phosphorylation as indicated by the low RCR values. Significant RC was measured when SHAM was present, however. This suggests that part of the reason for the low RCR was the high engagement of the nonphosphorylating alternative respiratory pathway. Presumably, the blocking of this pathway by SHAM increased the proportion of electrons flowing through the phosphorylating Cyt pathway and resulted in measurable RC.

Exogenous NADH was readily oxidized by the mitochondria in this study with O_2 uptake rates higher than those recorded with malate and succinate as substrates. With few exceptions, most plant mitochondria can readily oxidize exogenous NADH. Only in special cases, however, is this oxidation highly cyanide-resistant (*e.g. Arum* spadix mitochondria) (12) as it was shown to be in the present study. In this respect, our data differed greatly from that of Wilson (29) in which mitochondria isolated from *Acer pseudoplatanus* suspension cultures were inhibited 90% by cyanide. Our data fit better, however, with the accepted general scheme of the plant mitochondrial electron transport chain in which electrons from exogenous NADH are passed to ubiquinone and from there to either the Cyt or the alternative respiratory pathway (1, 12, 25).

The ability of mitochondria in some situations (*e.g. Arum* spadix, cultured cells) to pass electrons from exogenous NADH to the alternative oxidase raises some interesting possibilities. Both the alternative respiratory pathway and the exogenous NADH dehydrogenase are found only in plant mitochondria. It is conceivable therefore that both enzymes evolved together for the purpose of removing excess reducing equivalents from the cytoplasm without the concomitant production of ATP. This may be the case during those periods of rapid growth, *e.g.* logarithmic phase, when intermediates of the TCA cycle are being removed for amino acid biosynthesis and must be replenished by a rapid carbon flux through glycolysis, thereby producing cytoplasmic NADH. Thus, the supply of carbon skeletons may be the limiting factor in rapid growth and are therefore increased at the expense of ATP synthesis (13). The data presented here support this hypothesis inasmuch as the period of high cyanide-resistant respiration is the period of maximum synthetic activity. Electron flux from cytoplasmic NADH to the alternative oxidase should not result in ATP production since the alternative pathway is generally thought to be nonphosphorylating. It should be mentioned however, that a recent report provided good evidence of a phosphorylation site on the alternative respiratory pathway (31).

Acknowledgment—A special thanks is extended to Dr. Don Miles, University of Missouri, for the use of the oxygen electrode used in this study.

LITERATURE CITED

1. BENDALL DS, WD BONNER JR 1971 Cyanide-insensitive respiration in plant mitochondria. Plant Physiol 47: 236–245
2. BOHNENSACK R 1981 Control of energy transformation in mitochondria. Analysis by a quantitative model. Biochim Biophys Acta 634: 203–218
3. BONNER JR WD 1965 Mitochondria and electron transport. *In* J Bonner, JE Varner, eds, *Plant Biochemistry*. Academic Press, New York, pp 89–123
4. CHARBONNIER M, A BERVILLE 1980 Sunflower mitochondria: A procedure for the extraction of tightly coupled mitochondria. Plant Physiol 65: S-651
5. DAY DA, JB HANSON 1977 On methods for the isolation of mitochondria from etiolated corn shoots. Plant Sci Lett 11: 99–104
6. DOUCE R, EL CHRISTENSON, WD BONNER JR 1972 Preparation of intact plant mitochondria. Biochim Biophys Acta 275: 146–160
7. GOLDSTEIN AH, JO ANDERSON, RG MCDANIEL 1980 Cyanide-insensitive and cyanide-sensitive O_2 uptake in wheat. I. Gradient-purified mitochondria. Plant Physiol 66: 488–493
8. GOLDSTEIN AH, JO ANDERSON, RG MCDANIEL 1981 Cyanide-insensitive and cyanide-sensitive O_2 uptake in wheat. II. Gradient-purified mitochondria lack cyanide-insensitive respiration. Plant Physiol 67: 594–596
9. GOOD NE, D WINGET, W WINTER, T CONNOLY, S IZAWA, RMM SINGH 1966 Hydrogen ion buffers for biological research. Biochemistry 5: 467–477
10. HENRY M-F, EJ NYNS 1975 Cyanide-insensitive respiration. An alternative mitochondrial pathway. Subcell Biochem 4: 1–65
11. HORN ME 1980 Characterization of the electron transport chain in mitochondria from suspension cultured cells of *Nicotiana glutinosa* L. MA thesis. University of Missouri, Columbia
12. HUQ S, JM PALMER 1978 The involvement and possible role of quinone in cyanide resistant respiration. *In* G Ducet, C Lance, eds, Plant Mitochondria. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 225–232
13. LAMBERS H 1980 The physiological significance of cyanide-resistant respiration in higher plants. Plant Cell Environ 3: 293–302
14. LOWRY OM, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
15. MACRAE AR 1971 Effect of pH on the oxidation of malate by isolated cauliflower bud mitochondria. Phytochemistry 10: 1453–1458

16. MILLER CO 1979 Cytokinin inhibition of respiration by cells and mitochondria of soybean, *Glycine max* (L.) Merril. Planta 146: 503–511
17. MITCHELL P 1966 Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol Rev Camb Philos Soc 41: 445–502
18. MOORE AL, WD BONNER JR 1981 A comparison of the phosphorylation potential and electrochemical proton gradient in mung bean mitochondria and phosophorylating sub-mitochondrial particles. Biochim Biophys Acta 634: 117–128
19. MURASHIGE T, F SKOOG 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
20. PARRISH DJ, AC LEOPOLD 1978 Confounding of alternate respiration by lipoxygenase activity. Plant Physiol 62: 470–472
21. POLACCO J, M POLACCO 1977 Inducing and selecting valuable mutations in plant cell culture: A tobacco mutant resistant to carboxin. Ann NY Acad Sci 287: 385–400
22. SCHONBAUM G, WD BONNER JR, BT STOREY, JT BAHR 1971 Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Plant Physiol 47: 124–128
23. SMITH FA, JA RAVEN 1979 Intracellular pH and its regulation. Annu Rev Plant Physiol 30: 289–311
24. SOLOMOS T 1977 Cyanide-resistant respiration in higher plants. Annu Rev Plant Physiol 28: 279–297
25. STOREY BT 1976 Respiratory chain of plant mitochondria XVIII. Point of interaction of the alternate oxidase with the respiratory chain. Plant Physiol 58: 521–525
26. STUMPF PK 1976 Lipid metabolism. In J Bonner, JE Varner, eds, Plant Biochemistry, Ed 3. Academic Press, New York, pp 427–461
27. TOBIN A, B DJERDJOUR, E JOURNET, M NEUBERGER, R DOUCE 1980 Effect of NAD^+ on malate oxidation in intact plant mitochondria. Plant Physiol 66: 225–229
28. WILSON SB 1970 Energy conservation associated with cyanide-insensitive respiration in plant mitochondria. Biochim Biophys Acta 223: 383–387
29. WILSON SB 1971 Studies on the growth in culture of plant cells. XIII. Properties of mitochondria isolated from batch cultures of *Acer pseudoplatanus* cells. J Exp Bot 22: 725–734
30. WILSON SB 1971 Studies on the cyanide-insensitive oxidase of plant mitochondria. FEBS Lett 15: 49–52
31. WILSON SB 1980 Energy conservation by the plant mitochondrial cyanide-insensitive oxidase. Biochem J 190: 349–360