

Alternative Respiratory Path Capacity in Plant Mitochondria: Effect of Growth Temperature, the Electrochemical Gradient, and Assay pH¹

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ABSTRACT

Influence of growth temperature on the capacity of the mitochondrial alternative pathway of electron transport was investigated using etiolated corn (*Zea mays* L.) seedlings. These seedlings were grown to comparable size in either a warm (30°C) or a cold (13°C) temperature regime, and then their respiration rates were measured as O₂ uptake at 25°C. The capacity of the alternative pathway (KCN-insensitive O₂ uptake) was found essentially to double in shoots of cold-grown seedlings. This increased capacity slowly developed over several days growth in the cold, but was lost within 1 day when the seedlings were exposed to a warm regime. When mitochondria were isolated from the shoots of these seedlings, a greater potential for flow through the alternative path was observed in mitochondria from the cold-grown seedlings with all substrates used (an average increase of 84%). Using exogenous NADH as the substrate, the effect of the electrochemical gradient on measurable capacities of the cytochrome and alternative pathways was investigated in mitochondria from both etiolated seedlings and thermogenic spadices. The uncoupler FCCP (*p*-trifluoromethoxycarbonyl cyanide phenylhydrazone) was used to diminish the electrochemical gradient when desired. In corn (*Zea mays* L.) shoot and mung bean (*Vigna radiata* L.) hypocotyl mitochondria, which have relatively low capacities of the alternative pathway, increased flow through the cytochrome chain in the absence of the electrochemical gradient was found not to influence the potential for flow through the alternative path. However, in mitochondria from skunk cabbage (*Symplocarpus foetidus* L.) and voodoo lily (*Sauromatum guttatum* Schott) spadices, which have high capacities of the alternative pathway, increased flow through the cytochrome chain in the absence of the gradient occurred at the expense of flow through the alternative pathway. These results suggest that in mitochondria of thermogenic spadices, the combined capacities of the cytochrome and alternative paths exceed the capacity of the exogenous NADH dehydrogenase. The effect of assay pH on measurable capacities of the cytochrome and alternative paths was determined over a pH range of 5.6 to 8.8 using exogenous NADH as the mitochondrial substrate. When the electrochemical gradient was present, it limited the electron transport rate and little effect of assay pH was observed. However, when formation of the gradient was prevented through inclusion of FCCP, measurable capacities of the cytochrome and alternative paths were found to be greatly influenced by pH. This experiment also revealed that the potential for respiratory control is largely dependent upon the assay pH.

The role of the mitochondrial alternative pathway of electron transport in plant thermogenesis is well established (14). Electrons and protons that would normally flow to Cyt oxidase branch to the alternative pathway *via* the quinone pool when the Cyt chain is saturated. The alternative path *per se* is not proton-motive and thus does not contribute to generation of the electrochemical gradient across the inner membrane (16). The energy that potentially could have been conserved by phosphorylation is lost as heat. This heat is used in some plant species as a vital component of the pollination process. In eastern skunk cabbage (*Symplocarpus foetidus* L.), which flowers in the early spring, heat generated by the alternative pathway in the spadix is used to maintain a warm environment within the enclosing spathe. This temperature regulation is maintained for several days, allowing for successful pollination under adversely cold conditions (10). In contrast, heat produced via the alternative pathway in the spadix of the voodoo lily (*Sauromatum guttatum* Schott) is used to volatilize insect-attracting amines. In voodoo lilies, this heat generation is accurately timed and occurs for only a few hours at the onset of an elaborate pollination scheme (14).

In plant tissues that are not strikingly thermogenic, the alternative pathway is usually present, but at a lower level. The role of the alternative pathway in these tissues has been long debated, although a general consensus is emerging that it can function as a mechanism for enabling metabolism to continue when the cellular energy charge is high (4, 11, 14). In this regard it is useful, in that it allows for regeneration of cytosolic and mitochondrial matrix NAD when cellular ATP levels are high. A role for the alternative pathway in helping plants endure cold stress has also been suggested. In wheat seedlings, that are of too small a mass to be capable of thermogenesis (17), mitochondria of winter varieties have been shown to have a higher capacity for flow through the alternative pathway than those of spring varieties (13). Presence of the alternative pathway has been correlated with the ability of some wheat seedlings to withstand frost (2). In this paper we present results with corn seedlings, showing that the capacity of the alternative pathway can increase with growth at cooler temperatures. In addition we have investigated, in mitochondria of both thermogenic and nonthermogenic species, the influence that the electrochemical gradient and assay pH have on measurements of the capacity of the alternative pathway.

MATERIALS AND METHODS

Corn seedlings (*Zea mays* L. Mo17 × B73) were grown in the dark in moist vermiculite. They were grown either at 30 ± 2°C for 3.5 to 4 d or at 13 ± 2°C for about 14 d at which age the seedlings were of comparable size. Mitochondria were isolated from the shoots using a modification of the procedure of Day

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and Hanson (5). Insoluble PVP (5% w/v) was included in the grinding medium, and 50 mM Tes was used rather than 50 mM KH_2PO_4 as buffer. Corn mitochondria isolated in the presence of PVP tended to have a higher measurable capacity of the alternative pathway.

Mung bean seedlings (*Vigna radiata* L.) were grown in the dark at $28 \pm 2^\circ\text{C}$. These seedlings were grown on perforated stainless steel sheets suspended at the surface of water-filled plastic trays. Mitochondria were isolated from the hypocotyls of 4- to 5-d-old seedlings according to Bonner (3), except that 20 mM Mops³ was included as a buffer in the grinding and wash mediums. The washed mung bean mitochondria were then purified on sucrose gradients according to Douce *et al.* (6).

Skunk cabbage spadices (*Symplocarpus foetidus* L.) were collected in eastern Pennsylvania, and voodoo lily spadices (*Saurorhynchum guttatum* Schott) were obtained from corms purchased commercially. Washed mitochondria were isolated from the entire floral region of the spadix in skunk cabbage, and from just the appendix region of the voodoo lily spadix. Voodoo lily mitochondria were isolated from spadices before the day of flowering (14). Both these types of mitochondria were isolated with the same procedure used to obtain mung bean hypocotyl mitochondria, except that the BSA concentration was increased to 5% (w/v) and the EDTA concentration to 2 mM.

Isolated mitochondria were suspended in a small volume of SRM consisting of 250 mM sucrose and 30 mM buffer (10 mM each Mes, Tes, and Tricine). Mitochondrial protein was estimated with the procedure of Lowry *et al.* (12) using BSA (fraction V) as the standard. O_2 utilization was measured at $25 \pm 1^\circ\text{C}$ using either a Yellow Springs or a Rank Brothers electrode. O_2 content of air-saturated water was estimated according to Estabrook (9). FCCP (2.0 mM) and *n*-PG (2.0 M) were dissolved in (100% v/v) ethanol. SHAM was prepared either at a concentration of 500 mM in (100% v/v) ethanol or at 2.0 M in (100% v/v) DMSO. Although DMSO is a more convenient solvent, our experience has shown that it influences generation of the electrochemical gradient, even at a concentration as low as 0.05% (v/v).

RESULTS AND DISCUSSION

Effect of Seedling Growth Temperature on the Capacity of the Cyt and Alternative Pathways in Corn Shoots. A number of experiments were conducted to evaluate the effect of seedling growth temperature on the capacity of the alternative pathway. In the first experiment (Table Ia), the KCN-insensitive rate of O_2 uptake was 15% (of the initial rate of respiration) greater in cold-grown than in warm-grown shoots, while the capacity of the Cyt chain (SHAM-insensitive respiration) was 5% less. When the residual O_2 uptake rate (the rate in the presence of both inhibitors) was subtracted before estimation of the capacity, the alternative path was 21% greater in cold-grown shoots and the capacity of Cyt chain remained essentially unchanged. If the actual rates of O_2 uptake are considered, rather than their percentage of the initial rate, somewhat different conclusions are drawn. The residual respiration rate was found to be similar in shoots of warm- and cold-grown seedlings. However, the capacity of both the alternative and Cyt pathways increased in shoots of cold-grown seedlings.

In a second experiment where *n*-PG was used to inhibit the alternative path rather than SHAM, KCN-insensitive respiration was 17% greater in cold-grown shoots while the estimated capacity of the Cyt chain (*n*-PG-insensitive respiration) was 12% less.

³ Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; SRM, standard reaction medium; *n*-PG, *n*-propyl gallate; SHAM, salicylhydroxamic acid; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazine.

If the residual rate was subtracted, then the alternative path capacity was 15% greater in cold-grown shoots while the capacity of the Cyt chain was 14% less. When the actual O_2 uptake rates were considered, again the residual respiration rate was found not to vary between shoots of warm- and cold-grown seedlings. The capacity of the alternative pathway was greater, while that of the Cyt path remained unchanged.

Shoot segments consisting of both coleoptile (along with the enclosed young leaves) and hypocotyl regions were used in these experiments. The observed greater capacity of the alternative pathway in cold-grown shoots could potentially be present in both of these regions, or it could be a localized response. As shown in Table Ic, KCN-insensitive respiration increased 9% ($0.14 \mu\text{mol O}_2/\text{min} \cdot \text{g dry wt}$) in the coleoptile region of cold-grown shoots. This activity increased to a greater extent (32% or $0.40 \mu\text{mol O}_2/\text{min} \cdot \text{g dry wt}$) in the mesocotyl region. The results of all three experiments of Table I indicate that seedling growth temperature influenced the capacity of the alternative pathway, with a cooler growth temperature effecting an increased capacity for flow through the alternative pathway.

Another experiment was designed to determine if alternative path capacity could be induced or lost upon change of the growth temperature (Table II). When warm-grown seedlings were transferred to a cold regime, the capacity of the alternative pathway gradually increased with time from 22 to 31% of the initial respiration rate. In contrast, when cold-grown seedlings were transferred to a warm regime, the greater capacity for flow through the alternative pathway was rapidly lost (drops from 39 to 27% of the initial rate in 1 d). These changes in capacity for flow through the alternative pathway, with change in growth temperature regime, were also evident when the actual KCN-insensitive rates were considered.

Effect of Seedling Growth Temperature on the Capacity of the Cytochrome and Alternative Pathways in Isolated Corn Shoot Mitochondria. To ensure that the temperature responses observed with corn shoot segments reflect changes in capacity of mitochondrial electron transport chain pathways, we isolated mitochondria from the shoots of warm- and cold-grown corn seedlings (Fig. 1). In addition to the initial substrate-dependent rate of O_2 uptake, the capacities of the Cyt (SHAM-insensitive minus the residual rate) and alternative (KCN-insensitive minus the residual rate) pathways were measured both during (state 3) and after (state 4) phosphorylation.

The initial rate of substrate-dependent O_2 uptake in mitochondria can be limited by a number of processes, including substrate availability, substrate transport capacity, capacity of the dehydrogenase involved, the capacity of the electron transport chain, or by the magnitude of the electrochemical gradient (8). When estimating the capacity of the Cyt and alternative paths in isolated mitochondria, it is useful to establish which of these processes is limiting the initial rate of substrate-dependent O_2 uptake. For the results of Figure 1, all substrates showed phosphorylation-induced respiratory control. Thus under the assay conditions used, the electrochemical gradient was the factor limiting the initial rate during state 4, and representing data as a percentage of the initial rate is inappropriate. In general, the initial rates of substrate oxidation (either state 3 or 4) were less in mitochondria isolated from shoots of cold-grown seedlings. The RC_{ADP} is a ratio of the state 3 to the state 4 rate, and is a measure of how well phosphorylation is coupled, via the electrochemical gradient, with electron transport. The average RC_{ADP} ratio for the initial rates changed slightly from 2.24 in mitochondria from the shoots of warm-grown seedlings to 1.91 in those from cold-grown seedlings (the RC values represent an average for all substrates used).

The capacity of the Cyt chain changed with growth temperature in a substrate-dependent manner. With malate plus pyruvate

Table I. Effect of Growth Temperature on Respiration of Corn Shoots

Measurements were made on approximately 1 g fresh weight of shoots (using 5-mm segments) from 3.5- to 4-d-old seedlings at the warm temperature and seedlings of comparable size (about 2 weeks old) at the cooler temperature. O₂ uptake was measured polarographically at 25°C in 5.0 ml of water. After cutting, the tissue was stirred in the cuvette for 5 to 10 min prior to measuring O₂ uptake rates. The initial rates represented, are the rates before addition of any inhibitors. Rates in the presence of inhibitors were taken after 30 min exposure to the inhibitor in a stirred O₂ electrode cuvette, with the solution being aerated prior to measuring the rate. Incubation of the tissue for 30 min in the absence of inhibitors resulted in only a slight (5–10%) decrease in the respiration rate. Duplicate measurements were made on each sample with the solution being aerated between measurements. KCN and *n*-PG were used at a final concentration of 1 mM and SHAM at 10 mM. Values in parentheses represent the percent of the initial respiration rate. All data represent the means and average deviations of results obtained with at least three separate batches of seedlings (with four replicate samples from each batch).

Growth Conditions	Initial Rate	Inhibitor-Insensitive Rates		
		$\mu\text{mol O}_2/\text{min}\cdot\text{g dry wt}$ (% of initial rate)		
		KCN	SHAM	KCN + SHAM
a)				
Entire shoots		<i>Insensitive</i>	<i>Insensitive</i>	<i>Insensitive</i>
3½ at 30°C	1.92 ± 0.10	0.58 ± 0.05 (30)	1.59 ± 0.13 (83)	0.46 ± 0.07 (24)
13 d at 13°C	2.95 ± 0.30	1.33 ± 0.11 (45)	2.30 ± 0.11 (78)	0.53 ± 0.09 (18)
		KCN	<i>n</i> -PG	KCN + <i>n</i> -PG
b)				
Entire shoots		<i>Insensitive</i>	<i>Insensitive</i>	<i>Insensitive</i>
3½ d at 30°C	2.50 ± 0.20	0.62 ± 0.05 (25)	2.17 ± 0.21 (87)	0.50 ± 0.08 (20)
14 d at 13°C	2.71 ± 0.19	1.13 ± 0.04 (42)	2.02 ± 0.15 (75)	0.59 ± 0.05 (22)
		KCN		
c)				
4 d at 30°C		<i>Insensitive</i>		
Coleoptile	2.24 ± 0.40	0.62 ± 0.09 (28)		
Mesocotyl	2.60 ± 0.42	0.64 ± 0.08 (25)		
14 d at 13°C				
Coleoptile	2.03 ± 0.21	0.76 ± 0.11 (37)		
Mesocotyl	1.84 ± 0.38	1.04 ± 0.29 (57)		

Table II. Influence of Growth Temperature Regime on Induction or Loss of Cyanide-Insensitive Respiration in Corn Shoots

Except for changes in the growth temperature regime, experimental conditions are the same as in Table I.

Growth Conditions	Initial Rate	KCN-Insensitive Rate
Transferred from:		
28°C → 13°C		
3 d → 1 d	1.78 ± 0.16	0.39 ± 0.04 (22)
2 d → 5 d	2.35 ± 0.22	0.61 ± 0.10 (26)
1 d → 11 d	1.83 ± 0.10	0.57 ± 0.06 (31)
13°C → 28°C		
17 d → 0 d	1.78 ± 0.08	0.69 ± 0.03 (39)
13 d → 0.5 d	1.69 ± 0.06	0.59 ± 0.03 (35)
13 d → 1 d	1.47 ± 0.03	0.40 ± 0.06 (27)

(mal + pyr) or proline as substrates, little influence of growth temperature on the Cyt chain was observed. With succinate, growth at the cooler temperature resulted in an increased potential for flow through the Cyt chain. In contrast, the oxidation of exogenous NADH showed a decreased potential. As is well established, the measurable capacity for flow through the Cyt chain increases dramatically during state 3. The RC_{ADP} ratio for the capacity of the Cyt chain did not change between mitochondria from cold-grown (average RC with all substrates of 1.85) and warm-grown (average RC of 1.78) seedlings.

With all substrates, the capacity for flow through the alternative pathway increased in mitochondria from cold-grown seed-

lings. The average measured capacity in mitochondria from warm-grown seedlings was 17.1 natoms O/min·mg protein, whereas the capacity in mitochondria from cold-grown seedlings increased to 31.4 natoms O/min·mg protein. In contrast to the Cyt chain where respiratory control was measurable, the capacity of the alternative pathway did not change between state 3 and state 4. This result may be an artifact of the assay procedure since the alternative path capacities were determined in the presence of KCN. Inhibition of electron flow through the Cyt chain also prevents the Cyt chain from contributing to generation of the electrochemical gradient (16). SHAM-sensitive respiration can also be used to estimate the capacity of the alternative pathway, and addition of SHAM should not greatly affect the level of the gradient. However in corn mitochondria, a slight uncoupling effect was observed upon addition of SHAM. Because of the low levels of the alternative pathway present, this made these estimates of the alternative pathway unreliable. This uncoupling effect has also resulted in an overestimation of the capacity of the Cyt chain, although the error is less because of the Cyt chain's higher capacity. The residual respiration rate was quite low (averaged 8.31 natoms O/min·mg protein) and did not differ in mitochondria from the shoots of warm- or cold-grown seedlings.

The results obtained with isolated corn mitochondria support those obtained with tissue segments, in showing that an increased potential for flow through the alternative pathway develops during growth at cooler temperatures. It is not possible from our results to determine how the increased potential for flow through the alternative path was achieved. It could have resulted from increased levels of the alternative pathway or from increased

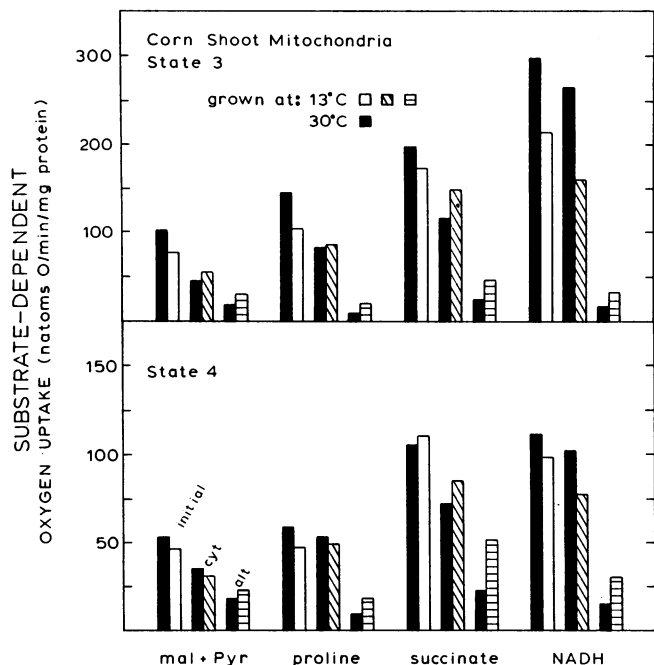


FIG. 1. Effect of seedling growth temperature on the capacity of the Cyt and alternative pathways in isolated corn shoot mitochondria. Seedlings from the two temperature regimes were grown to comparable size before mitochondrial isolation. O_2 uptake assays were conducted at 25°C in 2.0 to 2.2 ml SRM (pH 7.2) containing 1 mM $MgCl_2$, 1 mM KH_2PO_4 , and 1 mg/ml BSA. Concentration of substrates used was 10 mM malate (mal) plus 10 mM pyruvate (Pyr), 10 mM L-proline, 10 mM succinate, and 1 mM NADH. State 3 rates were maintained with inclusion of 0.6 mg hexokinase (Sigma Type III) and 50 mM glucose. State 4 rates are those observed following one state 3 rate (the state 3 rate was induced with addition of 200 nmol ADP). The initial rates represented are those rates in the presence of substrate before addition of any inhibitors (□, cold grown). The capacity of the Cyt chain (▨, cold grown) is the rate insensitive to 1 mM SHAM minus the residual rate after subsequent addition of 1 mM KCN. The capacity of the alternative path (■, cold grown) was estimated as the 1 mM KCN insensitive rate (minus the residual rate after subsequent addition of SHAM). The corresponding rates obtained with mitochondria from shoots grown at 30°C are represented by the solid bars. Mitochondrial protein averaged 563 μ g per assay. Data represent the mean of three experiments.

activation. It could also have been effected by the increased unsaturation of mitochondrial lipids which has been shown to occur with growth at cooler temperatures (15), since increased unsaturation of lipids could increase the potential for shuttling of electrons and protons to the alternative path *via* the quinone pool. The additional heat produced in these seedlings as a result of increased flow through the alternative pathway is certainly not enough for any sort of thermogenesis. However, it may be sufficient to help maintain fluidity of the inner mitochondrial membrane and thus lend some endurance to cold stress.

Effect of the Electrochemical Gradient on Measurable Capacities of the Cyt and Alternative Pathways in Isolated Mitochondria. It is clear that the level of the electrochemical gradient present greatly influences the measurable Cyt chain capacity, and it may also have an influence on flow of electrons through the alternative pathway. The oxidation of exogenous NADH is well suited for use in investigation of effects of the electrochemical gradient, since the gradient can be eliminated without substrate transport becoming a factor. Presented in Figure 2, are the measured capacities of the Cyt and alternative pathways in the presence and absence of saturating FCCP, using NADH as the

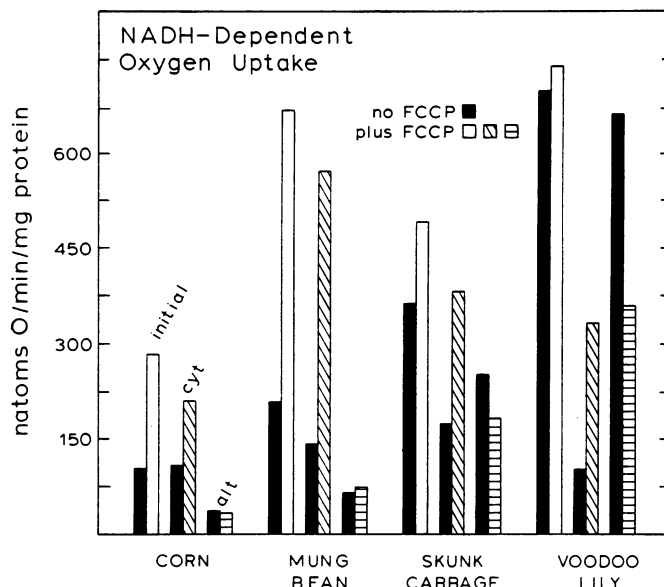


FIG. 2. Effect of the electrochemical gradient on measurable capacities of the Cyt and alternative paths in isolated mitochondria. Assays were conducted in 2.0 ml of SRM (pH 6.0), and were initiated upon addition of 1 mM NADH. The initial O_2 uptake rates in the presence of 1 μ M FCCP (□) are represented along with the capacities of the Cyt (▨) and alternative (■) paths. The solid bars represent the corresponding rates in the absence of FCCP. The corn and skunk cabbage results are the mean of two experiments, and the mung bean and voodoo lily results the mean of three. To allow for plotting of the data, all rates were normalized to the average initial rates (in the presence and absence of FCCP) for each type of mitochondria. Mitochondrial protein per assay averaged 423 μ g (corn), 429 μ g (mung bean), 1090 μ g (skunk cabbage), and 480 μ g (voodoo lily). Other conditions are the same as those in Figure 1.

substrate. FCCP was used to diminish the electrochemical gradient rather than phosphorylation because a considerable gradient is still maintained during phosphorylation (16). Mitochondria were isolated from four species which exhibit different levels of the alternative pathway. For each type of mitochondria higher concentrations of NADH did not increase the rate of O_2 uptake, indicating that the response was substrate saturated. If the initial rate of NADH oxidation is limited by the magnitude of the electrochemical gradient, then FCCP addition will result in increased oxidation. When the system is saturated with substrate, this increased oxidation rate is then limited either by the capacity of the dehydrogenase or by the capacity of the electron transport chain. The RC_{FCCP} is a ratio of the rate in the presence of FCCP to the initial rate, and is a measure of the maximum control that the electrochemical gradient can exert over the rate of electron transport. The initial NADH-dependent O_2 uptake rate in all four types of mitochondria showed some RC with FCCP. Corn and mung bean mitochondria exhibited good RC_{FCCP} with ratios of 2.78 and 3.20, respectively. Mitochondria of thermogenic tissues showed lower ratios, with mitochondria of skunk cabbage spadices having a ratio of 1.42, and those of voodoo spadices a ratio of 1.05. In contrast to the initial NADH-dependent O_2 uptake rates, the rates attributable to the Cyt chain show consistently good RC in mitochondria from both types of tissues. The RC_{FCCP} ratio for Cyt chain was 1.96 for corn, 4.04 for mung bean, 2.82 for skunk cabbage, and 3.27 for the voodoo lily. These results support others in the literature, in indicating the mitochondria with high capacities for flow through the alternative pathway have initial substrate-dependent O_2 uptake rates that exhibit poor RC (1).

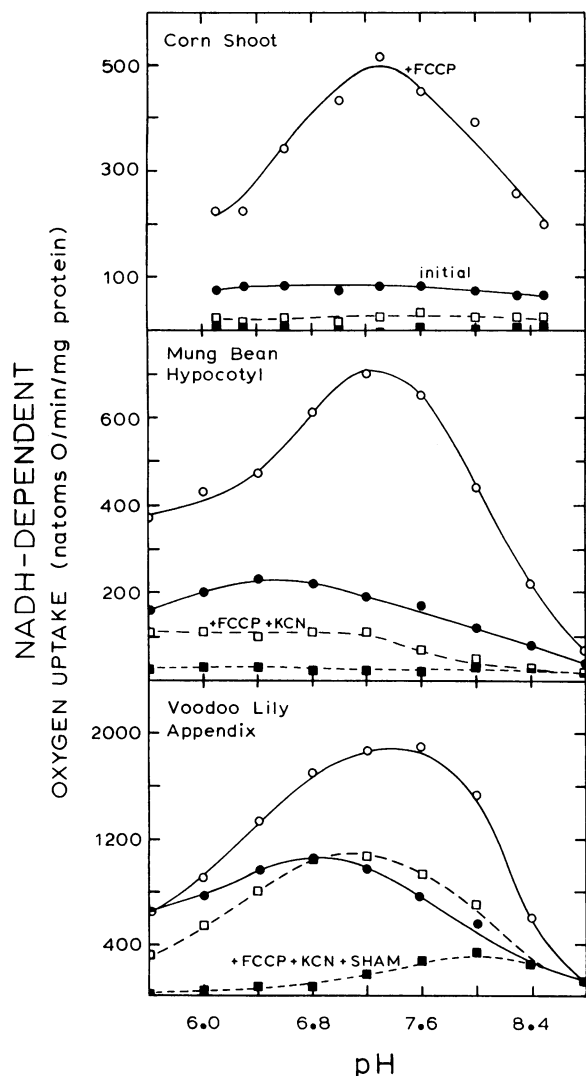


FIG. 3. Effect of pH on the capacity of the alternative pathway. O_2 uptake assays were conducted at 25°C in 2.0 ml of SRM, and were started upon addition of 1 mM NADH (the initial rates). The order of subsequent additions was 1 μ M FCCP, 1 mM KCN, and finally 1 mM SHAM. Mitochondrial protein per assay averaged 627 μ g for corn, 430 μ g for mung bean, and 74.2 μ g for the voodoo lily results. Each set of data represents the mean of three experiments.

The estimated capacity of the alternative pathway in Figure 2, is the KCN-insensitive rate minus the residual rate of O_2 uptake. Since with exogenous NADH, KCN addition results in loss of potential for gradient formation, little influence of FCCP on the estimated capacity for flow through the alternative pathway was expected. This was true of our results with corn and mung bean mitochondria, however the measurable capacity of the alternative pathway decreased in the presence of FCCP in skunk cabbage and in voodoo lily mitochondria. Similar results were obtained when SHAM-sensitive respiration was used to estimate the alternative pathway rather than KCN-insensitive respiration. In voodoo lily mitochondria, SHAM-sensitive respiration dropped from 545 natoms $O/min \cdot mg$ protein, to 372 in the presence of FCCP. In skunk cabbage the response was less dramatic, with SHAM-sensitive O_2 uptake dropping from 117 to 86 natoms $O/min \cdot mg$ protein. SHAM-sensitive O_2 uptake in mung bean mitochondria (42 natoms $O/min \cdot mg$ protein) did not change in the presence of FCCP (45 natoms $O/min \cdot mg$ protein). As noted before, in corn mitochondria SHAM addition causes a slight

uncoupling effect, which resulted in oxidation being stimulated by 17 natoms $O/min \cdot mg$ protein. However, if FCCP was present before SHAM addition in these mitochondria, then SHAM caused an inhibition of 64 natoms $O/min \cdot mg$ protein. The average residual respiration rate for each type of mitochondria was 12.5 natoms $O/min \cdot mg$ protein for corn, 38.1 for mung bean, 47.8 for skunk cabbage, and 47.0 for voodoo lily mitochondria.

Thus in seedling mitochondria, FCCP addition resulted in increased flow through the Cyt chain, that was reflected by an increased rate of substrate oxidation. However in spadix mitochondria, when FCCP addition effected increased flow through the Cyt chain, the rate of substrate oxidation did not increase proportionally. Instead, electrons were likely diverted from the alternative path into the Cyt chain. This diversion results in respiration rates that exhibit poor RC_{FCCP} ratios. Thus in spadix mitochondria, the combined capacities of Cyt and alternative paths appear to exceed the capacity of the exogenous NADH dehydrogenase. When increased flow occurs through the Cyt chain, it does so at the expense of flow through the alternative path. In seedling mitochondria where the capacity of the alternative pathway is relatively low, the activity of the exogenous NADH dehydrogenase is apparently sufficient to meet the demand of both pathways.

Effect of pH on the Capacity of the Alternative Pathway. When estimating the capacity of either the Cyt or alternative pathways, it is difficult to know at what pH the assay should be conducted. To get an indication of how assay pH affects these determinations, the effect of pH on the oxidation of exogenous NADH was measured in three types of mitochondria (Fig. 3). The initial rates of NADH-dependent O_2 uptake were relatively independent of pH. In corn mitochondria, there was almost no influence of assay pH on this rate. In mung bean mitochondria only a slight effect of pH was observed, with an optimum between 6.4 and 6.8. A much greater effect of assay pH on the initial rate of NADH-dependent O_2 uptake was observed in voodoo lily mitochondria, which exhibited an optimum near 6.8. Thus when the electrochemical gradient is present and limiting the rate of electron transport through the Cyt chain, this electron flow is relatively unaffected by assay pH.

When FCCP was added to diminish the electrochemical gradient, a more appropriate indication of the effect of pH on NADH oxidation was obtained. The +FCCP curves should represent either the effect of pH on the NADH dehydrogenase or the effect of pH on the electron transport chain, depending upon which was the limiting factor. Oxidation of exogenous NADH was substrate saturated in these experiments, in that further addition of NADH did not increase the rate. In all three types of mitochondria, the pH optimum of NADH-driven electron transport in the absence of the gradient was between 7.2 and 7.6. Similar optima for uncoupled NADH-dependent O_2 uptake have been reported for other mitochondria (7). It is evident from the mung bean and voodoo lily curves, that in the absence of the gradient, the optimum shifts to the alkaline side by approximately 0.8 pH units. The results also show, that the assay pH largely determines the value of the RC_{FCCP} ratio obtained. This is important in that the value obtained for the RC ratio is often used as a measure of mitochondrial quality. When KCN is added after FCCP, an estimate of the effect of pH on the maximum capacity for NADH-driven electron flow through the Cyt chain is obtained (the difference between the +FCCP and the +FCCP + KCN curves). The pH optimum for the Cyt chain was similar to that observed for the +FCCP rates, and was between 7.2 and 7.6 for all three types of mitochondria.

With the subsequent addition of SHAM, estimation of the effect of pH on the alternative pathway was obtained (the difference between the +FCCP + KCN and +FCCP + KCN + SHAM

curves). The capacity of the alternative pathway in corn mitochondria was relatively low, and essentially no effect of pH was observed. Flow through the alternative pathway was affected by pH in mung bean mitochondria, with little potential for flow above pH 8.4. In voodoo lily mitochondria, where the capacity of the alternative pathway was greatest, the effect of assay pH was more dramatic and showed an optimum between 6.8 and 7.2. The residual respiration rates (the +FCCP + KCN + SHAM curves) were low in all three types of mitochondria. An effect of pH on the residual rate was observed only in the voodoo lily mitochondria. The results of this experiment show that the assay pH can have a major effect on the value obtained for the capacity of the alternative path, especially when this capacity is presented as a percentage of the initial substrate oxidation rate.

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