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# Cyanide-insensitive Respiration

## I. THE STEADY STATES OF SKUNK CABBAGE SPADIX AND BEAN HYPOCOTYL MITOCHONDRIA*

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SUMMARY

**The effect of aromatic hydroxamic acids on skunk cabbage spadix and bean hypocotyl mitochondrial respiration has been measured. It is shown that the measurement of rate *versus* inhibitor concentration leads to the determination of the fluxes of reducing equivalents in each of the two pathways of electron transport in both State 3 and State 4. The alternate, cyanide-insensitive pathway is regulated by the activity of the normal cytochrome pathway. The conclusions about fluxes in State 3 are confirmed by the quantitative effect of hydroxamic acids on the ADP to oxygen ratio. The alternate pathway may be involved in regulation of NADH levels in the plant cell and in heat production by the skunk cabbage spadix.**

The respiration of citric acid cycle substrates by the mitochondria isolated from many higher plant species is not fully inhibited by concentrations of cyanide high enough to completely block cytochrome oxidase (1). This phenomenon, cyanide-insensitive respiration, was first observed in slices of *Sauromatum* spadix by van Herk (2). Although many biochemists have studied cyanide-insensitive respiration (3–8), it was not until the work of Bendall and Bonner (1) that the correct explanation was established. These investigators showed that cyanide- and antimycin A-insensitive respiration was mediated by an additional electron transport pathway consisting of the same set of dehydrogenases as the normal respiratory chain, but entirely by-passing the cytochromes via a second oxidase.

The existence of two electron transport pathways in the same mitochondria raises the question of the relative activity of each pathway in the absence of inhibitors. Since both enzyme systems catalyze identical reactions, direct measurement of their rates was not possible. Recently, Schonbaum *et al.* (9) showed that aromatic hydroxamic acids were specific inhibitors of the cyanide-insensitive oxidase system. This result made possible the determination of the relative rates in the two pathways of skunk cabbage spadix and bean hypocotyl mitochondria reported here.

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### MATERIALS AND METHODS

Mitochondria were prepared from indicated plant tissues by the method of Ikuma and Bonner (10), modified to include 10 mM morpholinopropanesulfonic acid buffer in the grinding and wash media. Spadices of skunk cabbage (*Symplocarpus foetidus*) were collected from natural growths in several counties of eastern Pennsylvania. Bean seedlings (*Phaseolus aureus* and *Vigna sinensis*) were grown in a dark chamber which was maintained at 28° and 60% relative humidity. The young seedlings, 4- to 6-days-old, were freed of roots, cotyledons, and leaves prior to homogenization.

Respiratory activity was measured polarographically in a medium containing 0.3 M mannitol, 10 mM phosphate, 10 mM KCl, and 5 mM $MgCl_2$ adjusted to pH 7.2. Succinate (7.0 mM) was used as the substrate. The ADP to oxygen values were calculated by the method of Chance and Williams (11).

All chemicals were reagent grade. The hydroxamic acids were kindly supplied by Dr. Gregory R. Schonbaum (St. Jude Children's Research Hospital, Memphis, Tennessee) and dissolved in dimethylformamide. Adsorption of the hydroxamic acids to the oxygen electrode reaction chamber was overcome by a thorough rinsing with 50% aqueous dimethylformamide between experiments.

### RESULTS

*Use of Thiocyanate and 8-Hydroxyquinoline*—Bendall and Bonner (1) reported that potassium thiocyanate, 8-hydroxyquinoline, and related metal ion chelators inhibited the cyanide-insensitive pathway with little or no effect on the cytochrome pathway. These compounds seemed to be possible tools for studying the relative rates of the two pathways. We examined their effects to see if it would be possible to completely block the cyanide-insensitive pathway and leave the cytochrome pathway unaffected. Titrations of the respiratory rate of skunk cabbage mitochondria in State 4 in the presence and absence of cyanide are shown in Fig. 1. As shown by their ability to completely inhibit the total State 4 rate, both thiocyanate and 8-hydroxyquinoline acted on the cytochrome pathway at concentrations only slightly higher than that needed to block the cyanide-insensitive respiration. These compounds were, therefore, unsuitable as specific inhibitors of the cyanide-insensitive pathway.

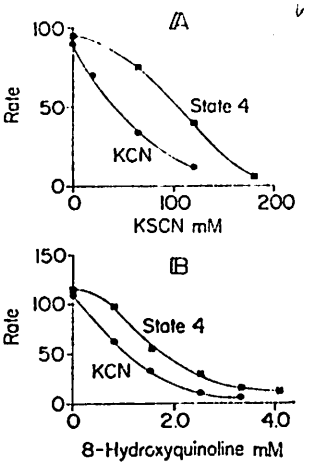


FIG. 1. Effect of thiocyanate and 8-hydroxyquinoline on respiration of skunk cabbage mitochondria. *A*, inhibition of State 4 and cyanide-insensitive respiration by thiocyanate (KSCN). *B*, inhibition by 8-hydroxyquinoline. Rates in nanoatoms of $O_2$ per min per mg of protein. Substrate is succinate (7.0 mM); cyanide when present is 0.3 mM. Mitochondria were incubated 2 min with 180 μM ATP prior to substrate addition; approximately 0.4 to 0.6 mg of protein per ml.

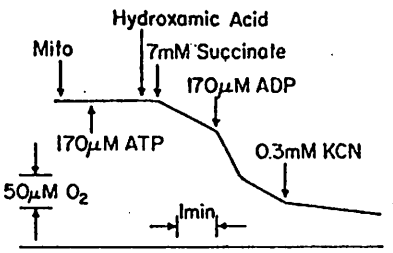


FIG. 2. Typical experiment used to determine effect of hydroxamic acids on respiratory activity of skunk cabbage mitochondria. Mitochondria are incubated with ATP for 2 min prior to addition of hydroxamic acid and substrate. This avoids an undesirable lag in reaching the first steady state. Mitochondrial concentration is about 0.4 to 0.6 mg of protein per ml.

The test of an inhibitor's usefulness in studies of cyanide-insensitive respiration lies in its ability to give a pattern of inhibition such that, when plotted as described below for the aromatic hydroxamic acids, a straight line is obtained. Only inhibitors specific for the alternate pathway will do so. Wilson (12) has reported on the use of pericidin A and thienoyltrifluoroacetone in studies of cyanide-insensitive respiration. The specificity and suitability of these inhibitors cannot be determined from the data he has presented.

*Titration of Succinate Oxidation with Hydroxamic Acids*—Aromatic hydroxamic acids were shown by Schonbaum *et al.* (9) to inhibit specifically the cyanide-insensitive pathway with little effect on the cytochrome pathway. In addition, no inhibition was seen with potato tuber mitochondria which lack the cyanide-insensitive pathway. We have extended these studies by measuring the State 3, State 4, and cyanide-insensitive respiratory activities of skunk cabbage mitochondria as a function of the concentration of aromatic hydroxamic acid. Experiments of the type shown in Fig. 2 were carried out on several preparations of mitochondria and with several different substituted hydroxamic acids. The results cited here are for *m*-iodobenzhydroxamic acid. Other hydroxamic acids appeared to differ only in their $K_i$ values (Table I).

Fig. 3 shows the dependence of the respiratory rate of the various steady states of skunk cabbage mitochondria on hydroxamic acid concentration. In the presence of 0.3 mM cyanide essentially complete inhibition was obtained. The reciprocal plot of such data was linear and gave the $K_i$ values cited in Table I. In both State 3 and State 4 the respiratory rate reached a non-zero plateau value at high hydroxamic acid concentrations. This rate represented the activity of the cytochrome pathway alone since the cyanide-insensitive pathway was completely blocked.

TABLE I

*Inhibitor constants for hydroxamic acids*

The inhibitor constants were determined from plots of the reciprocal of the respiratory rate in the presence of 0.3 to 0.5 mM cyanide against hydroxamic acid concentration. Other hydroxamic acids, including benzhydroxamic and salicylhydroxamic have been shown to be specific inhibitors as well. The constants reported here were all obtained with a single preparation of mitochondria. The $K_i$ values vary with the preparation, compare Fig. 3*C* with 3*D*.

| Compound | $K_i$ |
|---|---|
| | μM |
| *m*-Chlorobenzhydroxamic acid..... | 150 |
| *m*-Iodobenzhydroxamic acid..... | 37 |
| 2-Naphthylhydroxamic acid..... | 270 |

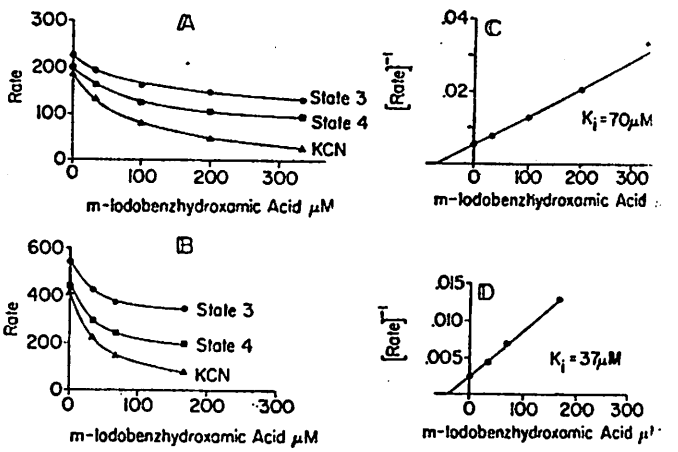


FIG. 3. Dependence of respiratory rates of skunk cabbage mitochondria on hydroxamic acid concentration. *A* and *B* represent different preparations of mitochondria. Each point represents an experiment of the type shown in Fig. 2. Rate is in nanoatoms $O_2$ per min per mg of protein. *C* and *D* are reciprocal plots of the inhibition in the presence of cyanide.

*Analysis of Titration Data*—It is clear then that there are two pathways of electron transport in these mitochondria. In the absence of inhibitors and at saturating substrate concentration the total rate, $V_T$, must equal the sum of the actual rates in each pathway. In the presence of cyanide the observed rate will be due entirely to the alternate pathway and will be the maximum rate possible for that pathway, $V_{alt}$. As the concentration of hydroxamic acid is increased, the alternate pathway activity must decrease. The titration data in the presence of cyanide define a function $g(i)$ which describes the maximum possible rate of the alternate pathway as a function of hydroxamic acid concentration.

If we postulate that the contribution of the alternate pathway to the total ratio is $\rho g(i)$ where $\rho$ is a constant between one and zero, that the cytochrome pathway rate is not affected by change in the alternate rate, the following equation for the total rate results.

$$V_T = \rho g(i) + V_{cyt}$$

A plot of $V_T$ in either State 3 or State 4 against the function $g(i)$ should give a straight line with slope $\rho$ and intercept $V_{cyt}$ at $g(i) = 0$. If changes in the alternate pathway rate did affect the cytochrome pathway rate, then a straight line would not necessarily be obtained. Fig. 4 shows the data of Fig. 3 replotted in this way. These data, and those of other preparations not shown, are fit well by a straight line.

Table II lists the steady state parameters of several skunk cabbage mitochondrial preparations. The variation in the magnitudes of the rates is attributed to variation in plant material during the growing season.

*Value of* $\rho$—The slope of the straight lines in Fig. 4 are clearly different in State 3 and State 4. There was some variation from one preparation to another but the State 4 $\rho$ was always higher than the State 3 $\rho$. The average values were 0.65 in State 4 and 0.55 in State 3. This result has two implications: (*a*) that the alternate pathway is not fully active in the absence of cyanide, and (*b*) that its activity is dependent on the state of the cytochrome pathway.

*Effect of Hydroxamic Acids on ADP to Oxygen Ratio*—The studies of Hackett and Haas (13) indicated that the alternate pathway included phosphorylation site I but not sites II and III, facts that were quantitatively confirmed by Storey and Bahr (14). The ADP to oxygen ratio, therefore, depends on the substrate used and on the relative activity of the two pathways. The alternate pathway contributes to oxygen utilization but, with succinate as substrate, no phosphorylation. The ADP to oxygen ratio at high hydroxamic acid concentrations reflects the inherent stoichiometry of the cytochrome pathway; in the absence of an inhibitor, the ADP to oxygen ratio is lowered due to the activity of the alternate pathway (Fig. 5). The fractional contribution to the total State 3 respiration attributable to the cytochrome pathway is given by the ratio of the ADP to oxygen ratio at no inhibitor to that at high inhibitor concentrations. The cytochrome pathway State 3 rates determined from the changes in ADP to oxygen ratios are in good agreement with the rates determined in Fig. 4. Although Wilson (15) has reported ADP to oxygen ratios of 0.5 with succinate in the presence of cyanide in mitochondria from suspension-cultured cells of sycamore, the agreement seen here on the basis of an assumption of no phosphorylation in the presence of cyanide argues that in fact Storey and Bahr's measurements in skunk cabbage are correct, at least for that tissue.

*Activity of Alternate Pathway in Other Plant Tissues*—Skunk cabbage spadix mitochondria and *Arum maculatum* spadix mitochondria have received much attention due to the very high activity of the cyanide-insensitive pathway in the mitochondria of those tissues (1, 5, 6, 14). Other plant tissues display a smaller, but significant, activity. Table III presents data on several species examined in our laboratory. The cyanide-insensitive respiration is expressed as a percentage of the State 3 respiration. The mung bean and black-eyed pea hypocotyls were selected for further study on the basis of convenience in growing the plant material.

*Inhibition of Bean Hypocotyl Mitochondria by Aromatic Hydroxamic Acids*—In the presence of KCN (0.3 to 0.5 mM) black-eyed pea and mung bean mitochondria respire at 20% and 15% of their State 3 rates, respectively. Addition of *m*-iodobenzhydroxamic acid resulted in complete inhibition of the remaining respiration. The $K_i$ values for this inhibition were 27 $\mu$M and 17 $\mu$M, respectively, for the preparations of Fig. 6. Other preparations gave values up to twice as high. In the absence of cyanide the State 3 rate was unaffected by addition of hydrox-

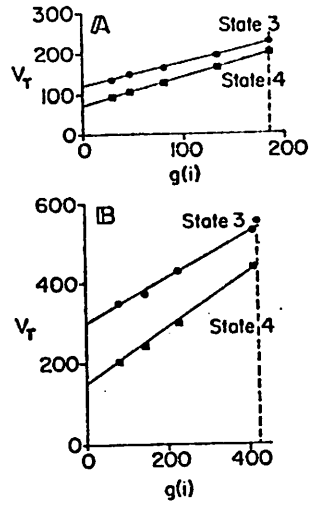


FIG. 4. The respiratory rate in States 3 and 4 *versus* $g(i)$. *A* and *B* are a replotting of the data of Fig. 3, *A* and *B*. $V_T$ and $g(i)$ are in nanoatoms of $O_2$ per min per mg of protein. The *dotted line* on the right indicates the maximum value of $g(i)$, $V_{alt}$.

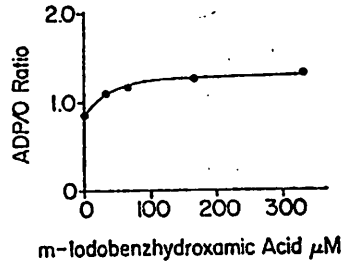


FIG. 5. Effect of hydroxamic acid on the ADP to oxygen ratio for succinate oxidation by skunk cabbage mitochondria.

TABLE II

*Steady state rates in skunk cabbage mitochondria*

Data were obtained from Fig. 4 and similar results. Preparations A and B are the same as in the previous figures. Note that although the rates vary with the preparation, parameters such as the respiratory control and $\rho$ are remarkably similar. All rates are in nanoatoms of $O_2$ per min per mg of protein.

| Preparation | Total State 3 | Total State 4 | Observed respiratory control | $V_{alt}$ | State 3 $\rho$ | State 4 $\rho$ | Cytochrome pathway rate | | Actual respiratory control |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | State 3 | State 4 | |
| A | 225 | 200 | 1.1 | 185 | 0.57 | 0.70 | 120 | 72 | 1.7 |
| B | 540 | 450 | 1.2 | 425 | 0.56 | 0.70 | 300 | 150 | 2.0 |
| C | 285 | 220 | 1.4 | 200 | 0.60 | 0.60 | 165 | 100 | 1.7 |

TABLE III

*Cyanide-insensitive respiration in various plant tissues*

The values below were determined from measurements on isolated mitochondria. The cyanide concentration used has been shown by experiments not reported here to be sufficient to saturate cytochrome oxidase. A small residual respiration via the cytochrome pathway continues to occur but at a level sufficiently low to be neglected. This probably accounts for the slight residual respiration of potato tuber mitochondria.

| Tissue | Percentage of State 3 respiration insensitive to 0.3 to 0.5 mM cyanide |
|---|---|
| White potato tuber (*Solanum tuberosum*).... | 1.0 |
| Alaska pea hypocotyl (*Pisum sativum*)..... | 10–15 |
| Black valentine bean hypocotyl (*Phaseolus vulgaris*)..... | 15–20 |
| Black-eyed pea hypocotyl (*Vigna sinensis*).. | 15–20 |
| Mung bean hypocotyl (*Phaseolus aureus*)... | 15–20 |
| Sweet potato tuber (*Ipomoea batatas*)..... | ≈50 |
| Skunk cabbage spadix (*Symplocarpus foetidus*)..... | 66–82 |
| *Arum maculatum* spadix..... | 100 |
| *Sauromatum guttatum* spadix..... | 100 |

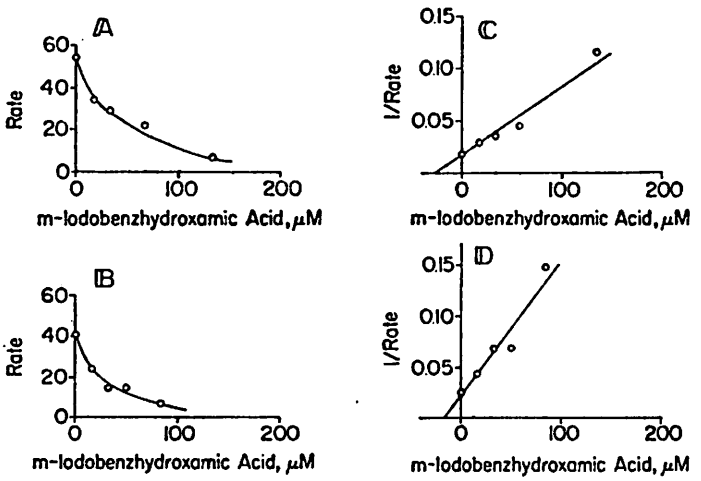


FIG. 6. Inhibition of respiration of bean hypocotyl mitochondria by *m*-iodobenzhydroxamic acid in the presence of cyanide. *A* and *B* are black-eyed pea and mung bean mitochondria, respectively. *C* and *D* are the reciprocal plots of the data in *A* and *B*. Cyanide concentration was 0.5 mM. The substrate was succinate at 7.0 mM.

amic acid whereas the State 4 rate was markedly inhibited. The inhibition of the State 4 rate was never complete.

Fig. 7 shows the titration data for bean hypocotyl mitochondria plotted as in Fig. 4. Again straight lines were obtained. In State 3, the increasing inhibition of the alternate pathway had no effect on the total rate, implying that it was not contributing to the State 3 rate. Conversely, in State 4 the slope of the line ($\rho$) was one. The alternate pathway was maximally active and contributed close to one-half of the total State 4 rate. The steady state fluxes of the two bean tissues are summarized in Table IV.

*Effect of Hydroxamic Acids on ADP to Oxygen Ratio*—With skunk cabbage mitochondria the changes in the ADP to oxygen ratio for succinate oxidation with increasing hydroxamic acid accurately measured the relative contribution of the cytochrome and alternate pathways in State 3. In the bean tissues, hydroxamic acids had no effect on the ADP to oxygen ratio (Fig. 8),

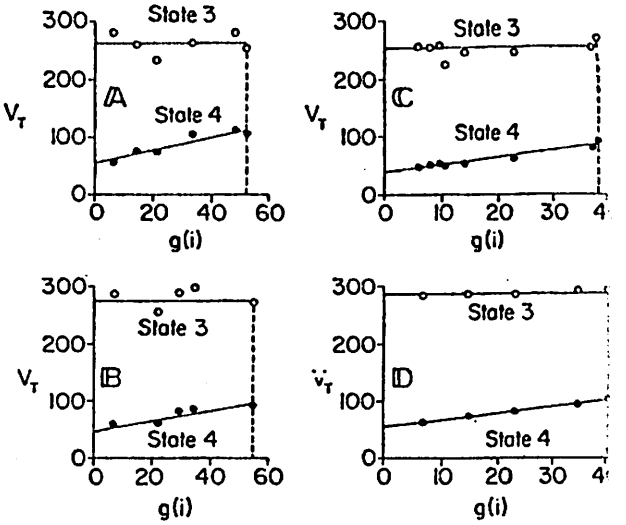


FIG. 7. The respiratory rate in States 3 and 4 *versus* $g(i)$. *A* and *B* are preparations of black-eyed pea hypocotyl mitochond *C* and *D* are preparations of mung bean hypocotyl mitochond Substrate is succinate at 7.0 mM. State 3 was obtained by addition of 180 μM ADP. Cyanide was 0.5 mM.

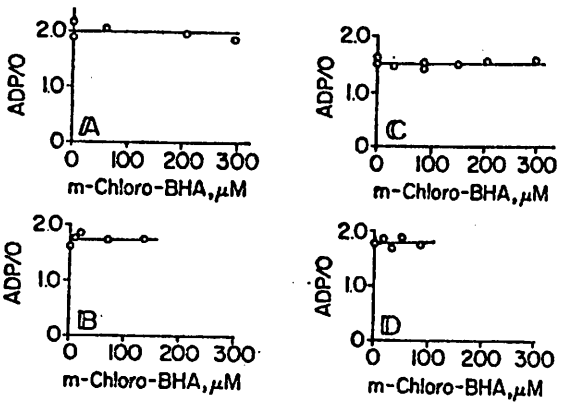


FIG. 8. The effect of hydroxamic acid on the ADP to oxyg ratio for succinate oxidation. *A* and *B* are black-eyed pea mit chondria. *C* and *D* are mung bean mitochondria.

corresponding to the lack of alternate pathway activity State 3.

## DISCUSSION

The alternate, cyanide- and antimycin A-insensitive pathw of electron transport is present in a wide variety of plant tissue It can contribute significantly to the over-all rate of electr transport from substrates to oxygen under certain condition This contribution can be determined from titrations of the d sired steady state rate of mitochondrial respiration with h droxamic acids. Such titrations are at present the only meth by which such data can be obtained.

The steady state oxidation of succinate by higher plant mit chondria can be described by simple equations involving t maximum rates of two competing electron transport pathway For skunk cabbage mitochondria in State 3 the total observe rate $V_T = 0.55\ V_{alt} + V_{cyt}$ (State 3), while in State 4 $V_T$ $0.65\ V_{alt} + V_{cyt}$ (State 4), where $V_{cyt}$ increases by a factor two on going from State 4 to State 3. In the bean hypocoty mitochondria in State 3, however, the entire flux of reducin equivalents from succinate is carried by the cytochrome path way, while in State 4 the alternate pathway is also fully active. These equations result in important conclusions regarding the

TABLE IV

*Steady state rates in bean hypocotyl mitochondria*

Data are obtained from Fig. 7. All rates are in nanoatoms of $O_2$ per mg of protein per min.

| Tissue | Total State 3 | Total State 4 | Observed respiratory control | $V_{alt}$ | State 3 $\rho$ | State 4 $\rho$ | Cytochrome pathway rate | | Actual respiratory control |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | State 3 | State 4 | |
| Black-eyed peas; Preparation A..... | 265 | 110 | 2.4 | 52 | 0.0–0.1 | 1.0 | 255–265 | 55 | 4.8 |
| Black-eyed peas; Preparation B..... | 275 | 95 | 2.9 | 54 | 0.0–0.1 | 0.9 | 265–275 | 46 | 5.0 |
| Mung bean; Preparation C..... | 255 | 85 | 3.0 | 38 | 0.0–0.1 | 1.1 | 245–255 | 42 | 6.7 |
| Mung bean; Preparation D..... | 288 | 102 | 2.7 | 40 | 0.0–0.1 | 1.1 | 280–288 | 57 | 5.1 |

mechanism of control of the alternate pathway activity to be discussed in the following paper.

The physiological role of the alternate pathway remains uncertain. Skunk cabbage spadices are known to be thermogenic. They live in late February and March in climates where the ambient temperatures can be below freezing for many days in a row. Direct measurements of spadix temperatures have indicated that they can maintain a 10–25° temperature differential relative to the air.[1] Consideration of the results of Poe and Estabrook (16) on the thermodynamics of coupled succinate oxidation by rat liver mitochondria and the flux data in Table II leads to the conclusion that the presence of the alternate pathway results in a 40–50% increase in the heat production per mole of succinate oxidized and in a two to 2½-fold increase in the rate of heat production per mg of mitochondrial protein.

While the thermogenic function may be important in skunk cabbage spadix and related tissues, it is difficult to see how this role is significant in the bean hypocotyl mitochondria. The alternate pathway is active only in State 4 in these mitochondria, resulting in a 1½- to 2-fold increase in the State 4 rate. Only when the plant cytoplasmic ADP level is low enough to limit the cytochrome pathway rate can we expect alternate pathway activity. The alternate pathway may be required either to increase the flux through the citric acid cycle or to increase the oxidation of cytoplasmic NADH in the absence of phosphate acceptor. Ikuma and Bonner (10) showed that external NADH could be oxidized by plant mitochondria. The alternate pathway may be involved in the regulation of a balance between the availability of reducing equivalents and of high energy phosphate in these plant cells.

[1] W. D. Bonner, Jr., unpublished observations.

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