Plant Physiol. (1991) 97, 1545–1550
0032-0889/91/97/1545/06/$01.00/0

Received for publication May 16, 1991
Accepted July 24, 1991

Alternative Respiration and Heat Evolution in Plants[1]

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ABSTRACT

The alternative respiratory pathway dissipates most of the chemical energy of respiratory substrates as heat. We have shown that this heat can be quantified by microcalorimetry and is a measure of alternative pathway activity *in vivo*. The alternative pathway is known to increase in aged potato (*Solanum tuberosum*) slices and in chill-stressed leaves. Aging of potato slices for 24 hours was accompanied by an almost fourfold increase in the rate of heat evolution. This heat increase was resistant to KCN but could be blocked by an alternative pathway inhibitor, salicylhydroxamic acid (SHAM). In cucumber (*Cucumis sativus*) leaves subjected to chilling stress (between 4 and 16°C), the rate of heat evolution was inversely related to temperature. As in aged potato slices, the increased rate of heat evolution in cucumber leaves was blocked by SHAM, but not by KCN. Nitrogen or the combination of SHAM and KCN blocked most of the heat evolution in both aged potato slices and chill-stressed cucumber leaves. Calorimetric measurements of the alternative pathway corresponded to respiration measurements performed using an oxygen electrode.

The discovery of cyanide-resistant respiration in plants was made more than 50 years ago (24, 29). This alternative pathway, which is common to all plants, branches from the Cyt *c* pathway at the level of ubiquinone and is nonphosphorylating after this branching point (10, 26). Thus, the alternative pathway bypasses energy conservation site II (between the *b* and *c* Cyt) and site III (between Cyt *a* and a_3). Because the alternative pathway does not create an electrochemical gradient, it dissipates most of the chemical energy of its respiratory substrates as heat. The cyanide-insensitive reduction of oxygen takes place on the inner mitochondrial membrane (26). A nuclear-encoded, alternative terminal oxidase protein was recently purified from the mitochondria of *Sauromatum guttatum* Schott (6–8). Although the activity of the alternative pathway is dependent upon developmental stage, tissue type, and physiological status, it is not uncommon for plants to burn 30 to 40% of their carbon through the alternative pathway (9, 21). In spite of continuous interest in the alternative pathway, the function of this seemingly bioenergetically wasteful process remains a mystery, except in thermogenic species, in which alternative pathway-evolved heat is used to attract insect pollinators (20). The "energy overflow" hypothesis of Lambers (16) and Palmer (25) suggests that the alternative pathway dissipates excess reducing power in situations in which energy and ATP are not limiting, or when the Cyt pathway is saturated.

Current methods for estimating alternative pathway activity are based on measurements of oxygen uptake in the presence of the Cyt pathway inhibitor, cyanide, and specific inhibitors of alternative pathway such as SHAM[2] and tetraethylthiuram disulfide (disulfuram) (15). A much more elaborate method is based on the discrimination of stable oxygen isotopes by alternative pathway and Cyt pathway (9).

In spite of the ease of operation and data collection, calorimetry has rarely been used in plant biology. Calorimetry has been employed to study plant responses to salinity (4), as a method for selecting vigorous plants (2), and as a tool for calculating respiratory efficiency in plants (3).

This paper demonstrates that calorimetric detection of alternative pathway-generated heat can be effectively used to measure alternative pathway activity in intact plant tissues. For our work, we chose two plant systems in which the alternative pathway and its regulation has been particularly well studied: aged potato slices and chill-stressed leaves (see "Discussion"). In addition, the contribution of the alternative pathway and Cyt pathway to the total heat evolution from cucumber leaves and potato slices was investigated.

MATERIALS AND METHODS

Plant Material

Potato tubers (*Solanum tuberosum* L.) were purchased locally and stored at 4°C. Cylinders of tissue 10 mm in diameter were cut with a cork borer and sliced with a razor blade into 1-mm thick discs. The slices were washed three times in distilled water and used directly or allowed to age. Aging took place on a rotary shaker at 25°C in 1 L flasks containing 50 mL of 0.1 mM $CaSO_4$. To prevent bacterial growth, chloramphenicol (50 μg/mL) was added to the incubation medium.

Cucumber (*Cucumis sativus* L., cv Marketmore 76) plants were grown in an environmentally controlled growth chamber at 24°C, 75% RH with a 16 h photoperiod (600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) provided by a combination of incandescent and cool white fluorescent lights. Four-week-old plants were equilibrated at 24°C in continuous light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 24 h. The plants were then exposed to different temperatures for 8 h in continuous light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$) unless otherwise noted. Newly expanded leaves were used in all experiments.

[1] This study was supported in part by the New Jersey Commission for Science and Technology.

[2] Abbreviation: SHAM, salicylhydroxamic acid.

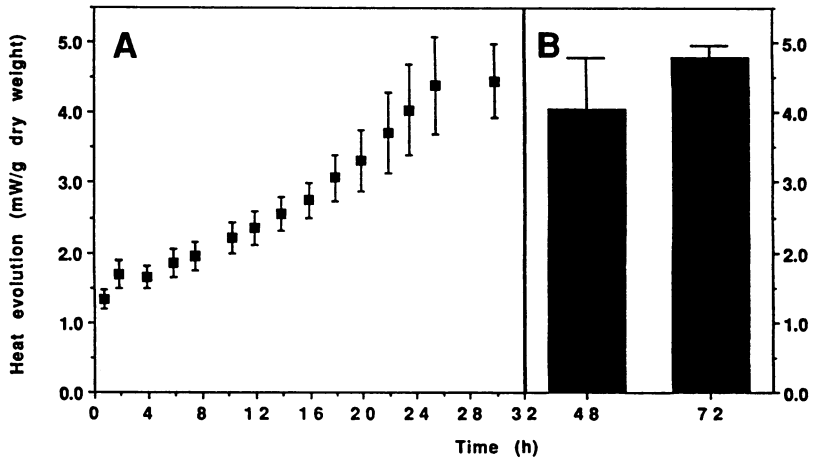

Figure 1. Heat evolution during the aging of potato tuber slices. Each data point is a mean of three replicate treatments ± SE. A, Time course of heat evolution, in calorimetric ampule, during aging. B, Heat evolution after 48 and 72 h of aging, outside of calorimetric ampule.

Calorimetric Measurements

Calorimetric measurements were made with a Hart Scientific model 7707 differential scanning microcalorimeter (Pleasant Grove, UT). Three simultaneous measurements of the rate of heat evolution in Watts ($W = J \cdot s^{-1}$) were made in 1 cm^3 ampules (10 mm diameter). Three cucumber leaf discs (10 mm diameter) or one potato disc were placed in each calorimetric ampule containing 100 μL of 30 mM Mops (pH 6.5) or 50 μL H_2O, respectively. All calorimetric measurements were performed at 20°C after 1 h equilibration using the isothermal operation mode.

Oxygen Measurement

Oxygen uptake was measured in darkness at 20°C in a cell containing 5 mL of air-saturated 30 mM Mops buffer (with or without respiratory inhibitors) using a YSI model 5300 oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, OH). Fifteen cucumber leaf discs (6 mm diameter) or three potato slices were used for each measurement.

All experiments included at least three replicates and were repeated at least three times with similar results.

Inhibitor Treatments

Cucumber leaf discs (4 mm in diameter) were used for SHAM experiments, whereas 10 mm discs were used for all other calorimetric measurements. Smaller leaf discs were used to improve the inhibitor uptake. Potato tuber slices were 1 mm thick × 1 cm diameter in all experiments. Twenty to 30 cucumber leaf discs or 1 potato tuber slice were placed in the calorimetric ampule after 1 h incubation in 30 mM Mops buffer (pH 6.5) containing 10 or 15 mM SHAM. Leaf discs (10 mm in diameter) and potato slices were incubated in 30 mM Mops buffer (pH 6.5) containing KCN (1 mM) for 15 min. These concentrations of respiratory inhibitors did not cause visible phytotoxicity for the duration of the experiment. One hundred microliters of incubation solution were added to each ampule to keep tissue moist. Control leaf discs and potato tuber slices were kept in Mops buffer (pH 6.5) without inhibitors for the same time as the experimental treatments. When both inhibitors were used, SHAM was added 45 min before KCN (1 h total incubation before the tissue was placed in the calorimeter). For nitrogen treatments, ampules containing the plant tissue were flushed with nitrogen and sealed in a chamber.

RESULTS

Heat Evolution and Respiration in Potato Tuber Slices

The rate of heat evolution from potato tuber slices aged inside the calorimetric ampules at 20°C (briefly opened every 2 h to maintain an adequate oxygen supply) increased steadily for the first 24 h (Fig. 1A). During this 24 h of aging, the rate of heat evolution increased 3.3-fold and remained constant for an additional 48 h (Fig. 1B). The heat evolved by potato slices aged for 24 h in water containing 0.1 mM $CaSO_4$ and no chloramphenicol was similar (data not shown).

To investigate if the alternative pathway is a major contributor to heat evolution during aging, alternative pathway and Cyt pathway inhibitors were applied to both fresh and aged potato tuber slices. Rates of heat evolution in fresh potato tuber tissue were unaffected by the addition of either KCN, a Cyt pathway inhibitor, or SHAM, a specific inhibitor of the alternative pathway (Fig. 2A). However, 10 mM SHAM reduced the rate of heat evolution from aged potato tuber slices by 54%, whereas 1 mM KCN caused only a 14% reduction. In contrast, 10 mM SHAM had virtually no effect on heat evolution from fresh potato slices. Nitrogen or the combination of KCN and SHAM reduced the rates of heat evolution from the fresh slices by 54 and 84%, respectively, compared with the control. When applied to potato tuber slices aged for 24 h, these treatments completely blocked aging-associated increases in heat evolution and reduced total heat evolution by 86 and 95%, respectively.

Oxygen uptake by fresh and aged potato tuber slices (Fig. 2B), measured at 20°C, paralleled the heat evolution data (Fig. 2A). Aging for 24 h resulted in a threefold increase in oxygen uptake by potato tuber slices. In addition, KCN, SHAM, and their combination had similar effects on potato oxygen uptake and the rate of heat evolution in both fresh and aged tuber slices. KCN partially inhibited oxygen uptake and the rate of heat evolution in the fresh slices, whereas SHAM was ineffec-

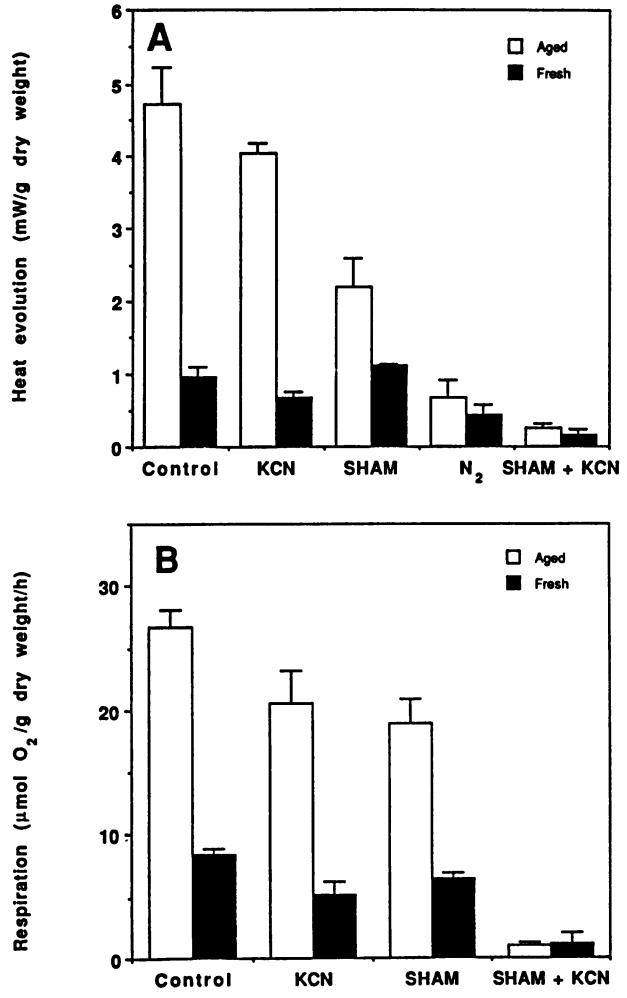

Figure 2. Effect of 10 mM SHAM, 1 mM KCN, and nitrogen on the rate of heat evolution (A) and oxygen uptake (B) of fresh (■) and 24 h-aged potato tuber slices (□). Each bar is a mean of five replicate treatments ± SE.

tive (Fig. 2A, B). In contrast, SHAM inhibited a substantial portion of oxygen uptake and heat evolution in aged slices.

Heat Evolution and Respiration in Cucumber Leaves

Because the activity of the alternative pathway is known to increase during chilling stress, cucumber, a cold-sensitive plant, was exposed to different temperatures for 8 h. Thereafter, the rate of heat evolution from the cucumber leaf discs was measured at 20°C (Fig. 3). Leaves from plants incubated at 4, 8, and 12°C evolved significantly more heat than those incubated at 16, 20, 24, and 28°C. Below 16°C, the rates of heat evolution were inversely related to temperature. Cucumber leaves exposed to 4°C evolved 2.4-fold more heat than leaves exposed to 16°C. Young, fully expanded cucumber leaves evolved more heat than older leaves after being exposed for 8 h to both chilling (8°C) and normal (24°C) temperatures (data not shown). However, the relative effect of chilling temperature on the rate of heat evolution was similar in the leaves of all ages. As a result of this observation, we have chosen to use the youngest, fully-expanded leaves in our study on the effects of chilling stress on oxygen uptake and heat evolution in cucumber.

To study the involvement of respiration in heat evolution, air in the calorimetric ampule containing leaf discs excised from cucumber plants incubated at 8 or 24°C for 6 h was replaced by nitrogen. Nitrogen dramatically reduced the rate of heat evolution in leaves incubated at both 8 and 24°C, compared with the control. When KCN was added to the leaf discs excised from plants incubated at 8 and 24°C, the rate of heat evolution increased by 8 and 16%, respectively. Simultaneous addition of 10 mM SHAM and 1 mM KCN resulted in 8.2 and 6-fold reduction in heat evolution, respectively, when compared with nontreated leaves exposed to the same temperatures (Fig. 4).

Heat evolution from leaves exposed to 8°C was inhibited by SHAM to a much greater extent than heat evolution from leaves exposed to 24°C (Fig. 5). Inhibition of the alternative pathway by SHAM resulted in a complete repression of the cold-inducible component of heat evolution. The levels of heat evolved by leaf discs in the experiments using SHAM (Fig. 4) are lower, possibly because of the greater tissue damage resulting from the use of smaller leaf discs. In addition, smaller leaf discs were more easily submerged in the liquid, imposing limitations on oxygen diffusion.

To correlate heat evolution with alternative pathway and Cyt pathway activity, oxygen uptake by cucumber leaf discs excised from plants incubated at 8 and 24°C was measured in the presence or absence of KCN and/or SHAM (Fig. 6). Consistent with its effects on heat evolution, 10 mM SHAM caused 36% inhibition of oxygen uptake in leaves exposed to 8°C and only 15% inhibition in leaves exposed to 24°C. KCN did not significantly affect oxygen uptake in cucumber leaves exposed to 24°C. However, KCN inhibited oxygen uptake by

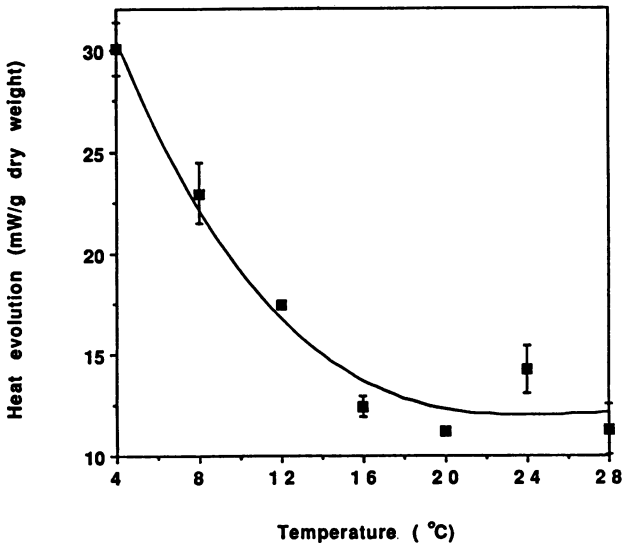

Figure 3. Effect of incubation at different temperatures on the rate of heat evolution from cucumber leaves. Plants were incubated at the temperatures shown for 8 h. Each data point is a mean of three replicate treatments ± SE.

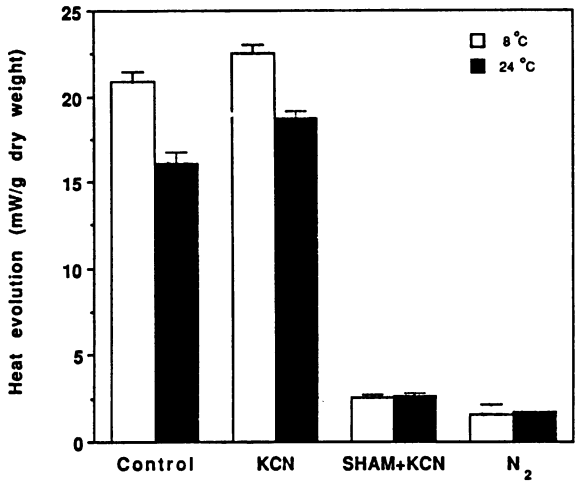

Figure 4. Effect of 1 mM KCN, the combination of 10 mM SHAM and 1 mM KCN, and nitrogen on the rate of heat evolution from cucumber leaves exposed to 8°C (□) and 24°C (■) for 6 h. Each bar is a mean of three replicate treatments ± SE.

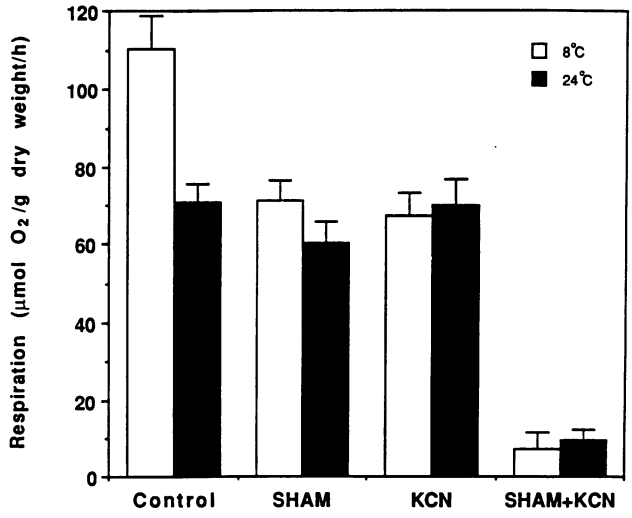

Figure 6. Effect of 10 mM SHAM and 1 mM KCN on oxygen uptake by cucumber leaves exposed for 8 h to 8°C (□) and 24°C (■). Each bar is a mean of five replicate treatments ± SE.

39% in leaves exposed to 8°C, in contrast with its stimulation of heat evolution in chilled leaves (Fig. 4). The combination of SHAM and KCN reduced O_2 uptake and blocked heat evolution.

DISCUSSION

It is well established that alternative pathway is induced in potato slices aged by exposure to air for 24 h. This induction is reflected in a three- to fivefold increase in oxygen uptake (11, 12, 23, 27). This rise in alternative pathway activity is accompanied by changes in the composition of mitochondrial membrane proteins (5). Monoclonal antibodies raised against alternative oxidase, a key enzyme in the alternative pathway, revealed a large increase in the levels of a 36 kD mitochondrial protein that paralleled the rise in alternative pathway activity during the aging of potato slices (11).

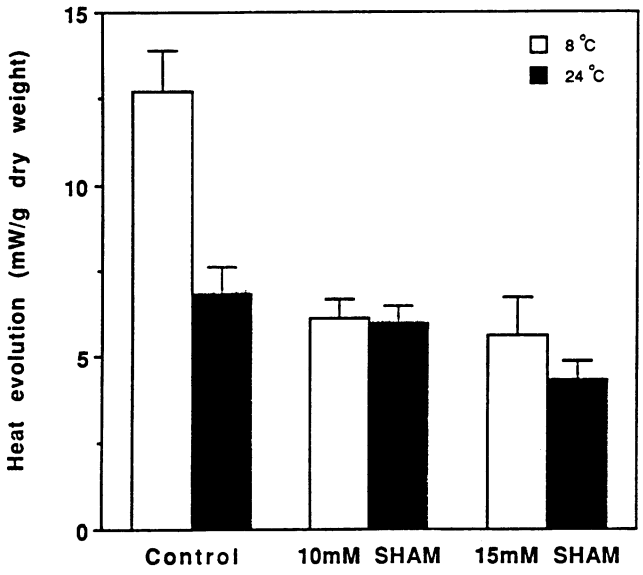

Figure 5. Effect of SHAM on the rate of heat evolution from cucumber leaves exposed to 8°C (□) and 24°C (■) for 8 h. Each bar is a mean of three replicate treatments ± SE.

As heat is a major product of the alternative pathway, we employed calorimetry to estimate alternative pathway induction during aging of potato slices. Aging of potato slices was accompanied by a SHAM-sensitive, KCN-resistant, three- to fivefold increase in heat evolution (Figs. 1, 2A), which was similar in magnitude and timing to the previously described induction of alternative pathway (11, 23). Our measurements of oxygen uptake in fresh and aged potato in the presence or absence of respiratory inhibitors (Fig. 2B) showed a trend similar to that observed for heat evolution (Fig. 2A). However, whereas in the aged slices the oxygen uptake increased 3.2-fold compared with fresh slices, the rate of heat evolution increased fivefold. We suggest that this SHAM-sensitive rise in the heat evolution per unit of oxygen consumed observed during aging of potato slices is a reflection of increased alternative pathway activity.

In addition to aging, exposure to low temperatures also causes an increase in the activity of the alternative pathway. Lowering the ambient temperature significantly enhanced the alternative pathway in soybean leaves (17). The activity of the alternative pathway is also consistently higher in cold-adapted plants. Mitochondria of winter wheats exhibit five times greater capacity for electron flux through the alternative pathway than the mitochondria of spring wheats (18). Similarly, mitochondria isolated from two cold-resistant cultivars of corn had higher alternative pathway activity than the mitochondria of cold-susceptible cultivars (28). Arctic plants have a particularly high alternative pathway activity, which becomes even greater in chilling temperatures (19).

The most dramatic example of the stimulation of the alternative pathway by low temperatures is found in the reproductive structures of thermogenic plants, where alter-

native pathway-associated heat evolution is inversely proportional to ambient temperature (for review see ref. 20). For example, greater enhancement of alternative pathway activity at lower temperatures allows inflorescences of *Philodendron selloum* Koch to maintain temperatures of close to 40°C in air ranging from 4 to 39°C (22). Temperature regulation based on alternative pathway activation in cold also occurs in the thermogenic inflorescences of eastern skunk cabbage (*Symplocarpus foetidus* L.) (13, 14), *Arum maculatum* (1), and *Sauromatum guttatum* (I. Raskin, unpublished).

We observed that heat evolution in the leaves of cold-sensitive cucumber plants was inversely proportional to ambient temperature when plants were exposed to temperatures between 4 and 16°C for 8 h (Fig. 3). The effect of chilling on heat evolution was effectively reversed by SHAM (Fig. 5), suggesting that increases in the rate of heat evolution in chilled cucumber leaves are caused by increases in the activity of the alternative pathway. The rises in alternative pathway activity in response to chilling found in all plants that have been studied imply that the difference between thermogenic and nonovertly thermogenic plants is qualitative rather than quantitative.

Oxygen uptake measurements showed that leaves of cucumber plants incubated at 8°C respire at a higher rate than at 24°C. SHAM was as effective in blocking cold-inducible oxygen uptake (Fig. 6) as it was in blocking cold-inducible heat evolution (Fig. 5). Chemical blockage of one pathway of mitochondrial electron transport (alternative pathway or Cyt pathway) could cause electrons to utilize the excess transfer capacity of the parallel pathway (21). This may explain our observation that KCN slightly increased heat evolution in chilled cucumber leaves (Fig. 4) while inhibiting oxygen uptake (Fig. 6). Because the alternative pathway produces more heat per electron transferred to oxygen than the Cyt pathway, the fact that KCN induces heat evolution in plant tissues suggests the utilization of the excess capacity of the alternative pathway by the electrons, which would ordinarily move along the Cyt pathway. The fact that the combination of SHAM and KCN blocked heat evolution in potato tuber slices (Fig. 2) and cucumber leaf discs (Fig. 4) demonstrates that these inhibitors are effective in these tissues. It also suggests that respiratory electron transfer in the alternative pathway is responsible for a significant amount of the heat evolved from plant tissues.

Our results suggest that calorimetry may serve as a tool to study plant respiration, particularly the alternative pathway. Both calorimetry and oxygen uptake measurements are nondestructive and can be performed on the same tissue sample. The accurate determination of oxygen uptake per unit of heat evolution on a single plant tissue sample should produce an accurate assessment of *in vivo* alternative pathway and Cyt pathway activity, without the use of respiratory inhibitors.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R.W. Breidenbach and Dr. R.S. Criddle for helpful suggestions about calorimetry.

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