Plant Physiol. (1986) 82, 443–447
0032-0889/86/82/0443/05/$01.00/0

Effects of KCN and Salicylhydroxamic Acid on Respiration of Soybean Leaves at Different Ages[1]

Received for publication March 3, 1986 and in revised form June 15, 1986

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ABSTRACT

Measurements of respiration were made on leaf discs from glasshouse-grown soybean (*Glycine max* [L.] Merr. cv 'Corsoy') plants in the presence and absence of cyanide (KCN) and salicylhydroxamic acid (SHAM). O_2 uptake by mature leaves measured at 25°C was stimulated by 1 millimolar KCN (63%) and also by 5 millimolar azide (79%). SHAM, an inhibitor of the alternative oxidase and a selection of other enzymes, also stimulated O_2 uptake by itself at concentration of 10 millimolar. However, in combination, KCN and SHAM were inhibitory. The rate of O_2 uptake declined consistently with leaf age. The stimulation of O_2 uptake by KCN and by SHAM occurred only after a certain stage of leaf development had been reached and was more pronounced in fully expanded leaves. In young leaves, O_2 uptake was inhibited by both KCN and SHAM individually. The uncoupler, *p*-trifluoromethoxy carbonyl-cyanide phenylhydrazone, stimulated leaf respiration at all ages studied, the stimulation being more pronounced in fully expanded leaves. The uncoupled rate was inhibited by KCN and SHAM individually. The capacity of the cytochrome path declined with leaf age, paralleling the decline in total respiration. However, the capacity of the alternative path peaked at about full leaf expansion, exceeding the cytochrome capacity and remaining relatively constant. These results are consistent with the presence in soybean leaves of an alternative path capacity that seems to increase with age, and they suggest that the stimulation of O_2 uptake by KCN and NaN_3 in mature leaves was mainly by the SHAM-sensitive alternative path. The stimulation of O_2 uptake by SHAM was not expected, and the reason for it is not clear.

The presence of a cyanide-resistant alternative respiratory pathway in mitochondria and intact plant tissue is routinely concluded from an insensitivity of O_2 uptake to cyanide and a specific inhibition by hydroxamates in the presence of cyanide (7, 15, 18). Its presence or activity has been demonstrated in a wide variety of plant tissues, and the proportion of a plant's respiratory capacity that is cyanide-resistant has been shown to vary among species and from tissue to tissue (7, 15, 18). Aroids (21) have received much attention because of the unusually high level of cyanide-resistant respiration in their spadices. Storage organs are of interest because this pathway is either induced or regenerated in aged slices (18, 27).

Studies of leaf respiration, using both isolated mitochondria and intact tissues, have provided a range of results. The pattern of leaf respiration has been shown to change with leaf development and there are indications that the degree of resistance to cyanide, and therefore, the possible contribution of the alternative pathway to total respiration, is either constant (1) or increases with maturation (7). O_2 uptake rates in bean (*Phaseolus vulgaris*) leaves decreased by about 70% by d 14 (1). A similar decrease in leaf respiration with maturation had been reported earlier (11, 14). This decline in leaf respiration has been attributed to the degeneration and consequent decrease in the biochemical efficiency of the mitochondria (11, 26). Earlier reports indicated that, whereas the respiration of young leaves is inhibited, that of mature leaves is either resistant or even stimulated by inhibitors of the Cyt oxidase (19, 20). It is noteworthy, however, that these earlier observations of respiratory stimulation were made before the discovery of specific inhibitors of the alternative pathway and therefore do not provide complete information regarding the possible involvement of the alternative pathway in these responses.

The present study is part of an effort to characterize alternative pathway respiration in soybean tissues. Published measurements of cyanide-resistant or alternative pathway respiration in soybean tissues are sparse and almost invariably have been restricted to imbibing seeds (24, 29), embryonic axes (23), and cultured cells (8, 22). Hrubec *et al.* (13) reported a 30% cyanide resistance in mitochondria isolated from young leaves, with malate and glutamate as substrates and with no residual respiration. To our knowledge, this is the only published report in the current literature on the involvement of the alternative pathway in soybean leaf respiration. In this report, we present results indicating stimulation of O_2 uptake by respiratory inhibitors in mature soybean leaves, and which suggest the existence of an alternative pathway capacity that increases with leaf maturation.

MATERIALS AND METHODS

Plant Material. Soybean (*Glycine max* (L) Merr. cv 'Corsoy') plants were grown in a glasshouse in 25-cm plastic pots containing either a 2:1:1 mixture of soil, peat, and sand or a 1:1:1 mixture of soil, sand, and perlite. Day temperatures ranged from 30 to 35°C and, night, from 20 to 25°C. Supplemental radiation was supplied by high pressure sodium lamps providing a 14-h photoperiod. One week after emergence, seedlings were thinned to one plant per pot. Plants were watered daily with tap water and flooded once a week with a solution of a general purpose (20-20-20) commercial fertilizer (Peters Fertilizer Products). Plant position on the benches was altered weekly to minimize any possible glasshouse positional effects.

Sampling. Leaves of either the third or the fifth node were

[1] Journal Paper No. J12207 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Projects No. 2496 and 2682. Supported in part by United States Department of Agriculture Cooperative State Research Service Special Grant 59-2191-1-2-010-0 and by Pioneer Hybrid International.

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harvested near the middle of the day to ensure that respiratory substrates did not limit the rate of respiration and to avoid the interference of diurnal fluctuations of the activity of the alternative pathway (1). The harvested leaves were transported to the laboratory in a beaker of chilled water. Before discs were punched out, leaves were rinsed repeatedly with chilled distilled H_2O. In most cases, only the terminal leaflets were used. Leaflets from three separate plants were pooled per replication, and four replications were used.

Leaf age was calculated in days, and 'day zero' was established as the day on which the leaflets of the selected leaf unfolded. The leaflets reached full expansion in 8 to 9 d.

Measurement of Respiratory Characteristics. Discs, each having an area of 0.3 cm^2, were punched from the sampled leaflets with a stainless steel punch. The discs were rinsed in chilled distilled H_2O. Twenty leaf discs were transferred to a transparent glass cuvette. After 15 min temperature equilibration at 25°C in 5 ml distilled H_2O, the rate of O_2 uptake (control rate) was measured using Clark-type O_2 electrodes and Yellow Spring Instruments Model 53 O_2 meters. Inhibitors were then added and the discs stirred for 25 to 30 min. Maximal inhibition or stimulation by either KCN or SHAM[3] appeared gradually over the course of a 25 to 30 min period. The solution was reaerated, then the rates in the presence of the inhibitors were determined. FCCP rates stabilized in about 3 to 5 min. Similar results were obtained in preliminary studies when distilled H_2O or buffered solution (1) was used as the measuring medium. The electrodes were calibrated against air-saturated distilled H_2O. KCN, NaN_3, SHAM, and FCCP were used at final concentrations of 1 mM, 5 mM, 10 mM, and 1 μM, respectively. KCN was dissolved in distilled H_2O, NaN_3, FCCP in 95% ethanol, and SHAM in 2-methoxyethanol. After measurements, samples were oven-dried at 80°C for at least 48 h to allow expression of the rate of O_2 uptake per g dry weight.

RESULTS

O_2 uptake rates of leaf discs of mature soybean leaves are presented in Table I. O_2 uptake, expressed either on a leaf-area or dry-weight basis, was strongly stimulated by KCN (63%) and NaN_3 (79%). Quite unexpectedly, SHAM, an inhibitor of the alternative path and a selection of known enzymes (4, 16), also stimulated O_2 uptake by itself (36%). However, KCN in combination with SHAM was inhibitory, the order of addition of KCN and SHAM did not seem to influence the results. These experiments were repeated several times on many batches of leaves, and the results were consistently reproducible. The combined effects of KCN and SHAM did not completely eliminate O_2 uptake, the residual component varying from 42 to 44% of the initial rates.

Previous reports (19) that the level of resistance to cyanide is related to the age of the tissue raised the possibility that soybean leaf tissue might display a similar response. Results of experiments undertaken to explore this possibility are presented in Figures 1 to 3. In all cases, the rate of O_2 uptake decreased consistently with leaf age, dropping about 70% over the growth period studied. This result agrees with others (25) that show a steady decline of soybean leaf respiration with maturation. A similar pattern of O_2 uptake was also observed by Azcon-Bieto *et al.* (1), using both intact bean leaves and leaf slices. O_2 uptake rates of young (2–4-d-old for KCN and 2–9-d-old for SHAM) leaves were inhibited by both KCN and SHAM individually, but were inhibited less as the leaves developed and respiration declined. Around age 5 d, stimulation of O_2 uptake by cyanide appeared and persisted as a plateau until the 18th d before declining, and by the 32nd d the effect, while still stimulatory, was significantly less than in the earlier periods. At the time O_2 uptake stimulation by KCN was observed, the terminal leaflet had already reached 80% of maximum area (data not shown). The response to SHAM (Fig. 2) followed a pattern similar to that of the KCN response, although stimulation by SHAM appeared about 5 d later. A similar trend was evident when the sensitivity of the leaves of a whole plant was estimated by sampling all leaves at the same time (data not shown).

The combination of KCN and SHAM was inhibitory at all ages in this study, and the residual rate, in absolute terms, was rather constant at all ages (Fig. 3).

O_2 uptake was stimulated by FCCP at all ages studied (Figs. 4 and 5). Stimulation by FCCP was considerably less in younger leaves than in fully expanded leaves. The uncoupled rates were inhibited by KCN and SHAM, individually, at all ages. The sensitivity of O_2 uptake to SHAM was high in young leaves and decreased with age (Fig. 5). This pattern of response was not expected and did not complement that obtained with KCN in the presence of FCCP (Fig. 4).

The presence of FCCP did not alter the general pattern of respiratory decline during leaf maturation. The reason for the more precipitous drop in the control rates in Figure 4 compared to Figures 1 to 3 is not apparent except to note that different

[3] Abbreviations: SHAM, salicylhydroxamic acid; FCCP *p*-trifluoromethoxy carbonylcyanide phenylhydrazone; V_{alt}, alternative pathway capacity; V_{cyt}, cytochrome pathway capacity.

Table I. *Effect of Inhibitors on the Respiration of Soybean Leaf Discs*

Results are means of four independent determinations with a set of three plants each. Each determination was performed in triplicate. Values shown are means ± SE. Leaflets of the third trifolium (14-d-old) were used. All other factors were as described in "Materials and Methods."

Sequential Additions	Rate of O_2 Uptake		Stimulation (+) or Inhibition (−)
	$\mu mol\ m^{-2}\ s^{-1}$	$\mu mol\ g^{-1}\ dry\ wt\ min^{-1}$	%
Experiment 1:			
None	0.57 ± 0.07	2.23 ± 0.29	
+ KCN	0.93 ± 0.09	3.64 ± 0.34	+63.2
+ KCN + SHAM	0.25 ± 0.04	0.99 ± 0.16	−73.0
Experiment 2:			
None	0.59 ± 0.04	2.30 ± 0.23	
+ SHAM	0.80 ± 0.05	3.12 ± 0.21	+35.6
+ SHAM + KCN	0.25 ± 0.06	0.98 ± 0.20	−68.6
Experiment 3:			
None	1.01 ± 0.18	3.01 ± 0.23	
NaN_3	1.81 ± 0.14	5.40 ± 0.54	+79.4

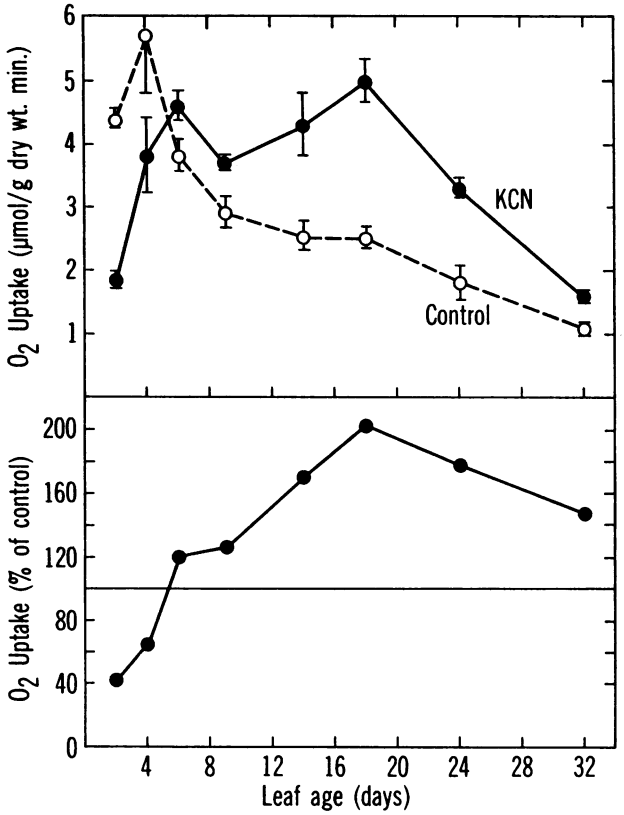

FIG. 1. Effect of KCN on respiration of soybean leaf discs as a function of leaf age. Data points represent the mean of four independent determinations with a set of three plants each. Each determination was performed in duplicate. Leaflets of the fifth trifolium were used. Bars indicate SE. All other factors were as described in "Materials and Methods."

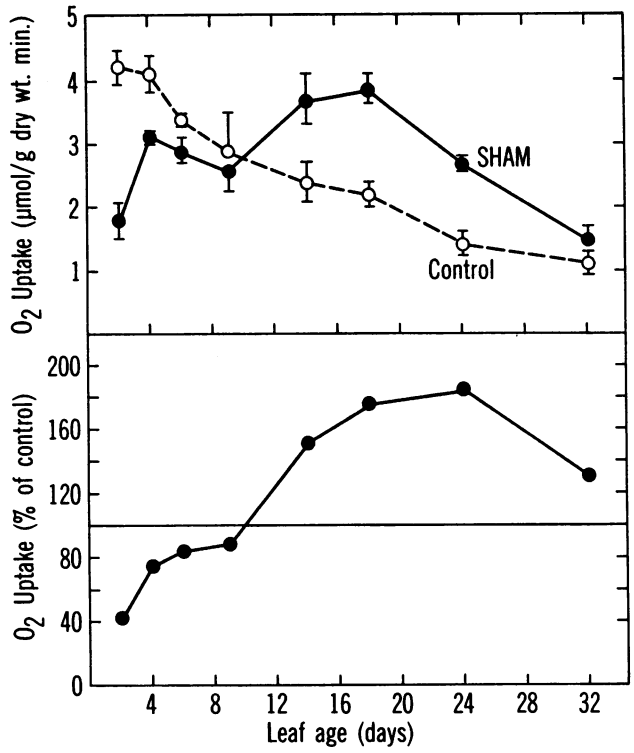

FIG. 2. Effect of SHAM on respiration of soybean leaf discs as a function of leaf age. For further information, see the legend to Figure 1.

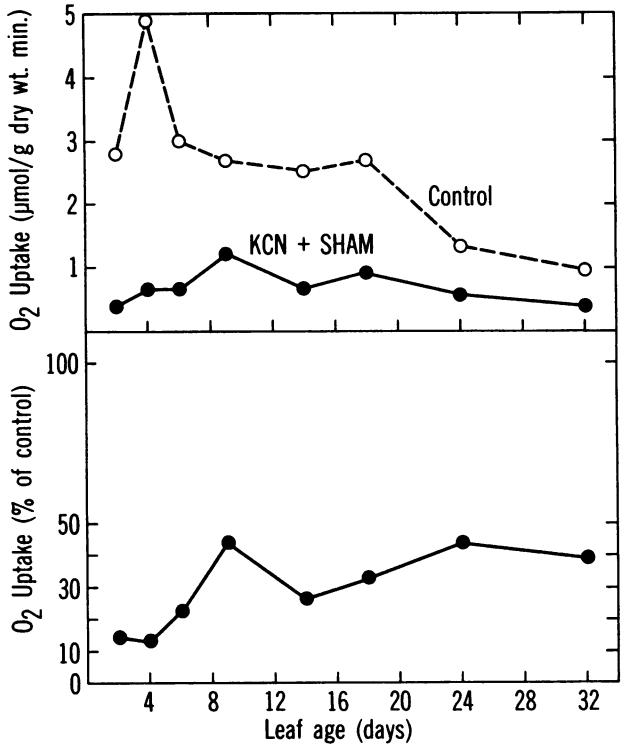

FIG. 3. Effect of KCN on respiration of soybean leaf discs in the presence of 10 mM SHAM as a function of leaf age. For further information, see legend of Figure 1.

batches of plants were used in the studies reported in the two groups of figures. The results shown in Figure 4 are summarized in Table II. It should be pointed out that while the values reported for the capacity of the Cyt path (V_{cyt}) in Table II may be of some theoretical interest, they should be regarded only as approximations. The capacity of the Cyt path would normally be estimated as the rate of respiration in the presence of an uncoupler and SHAM minus the residual rate. This method was considered unsuitable in this case given the unusual effect of SHAM in the presence of FCCP (Fig. 5). The capacity of the Cyt path was therefore estimated as inhibition by KCN in the presence of the uncoupler FCCP. This method was based on the assumption, perhaps unjustified, that uncoupling with FCCP led to a respiration rate that fully saturated both pathways or at least approached saturation. In the absence of an absolute way of ascertaining the maximal rate of electron transport (17), and because the alternative path is engaged only when the Cyt path is saturated or restricted (2, 18, 27), O_2 uptake rates in the presence of FCCP were taken as representing the maximum respiratory capacity. Day and Lambers (6) observed that in bean, wheat (*Triticum aestivum* L.), and maize (*Zea mays* L.) the potential respiratory rates, measured with isolated mitochondria, were similar to the root rates measured in the presence of FCCP. It seems of interest that the decline in total respiration with leaf age was closely paralleled by a decrease in the estimated capacity of the Cyt path (V_{cyt}). In contrast, the capacity of the alternative path (V_{alt}) peaked at about full leaf expansion, exceeding V_{cyt}. Thus, the ratio V_{cyt}/V_{alt} declined with leaf maturation.

DISCUSSION

The results obtained from the present study indicate that O_2 uptake by mature soybean leaf tissue is stimulated by inhibitors

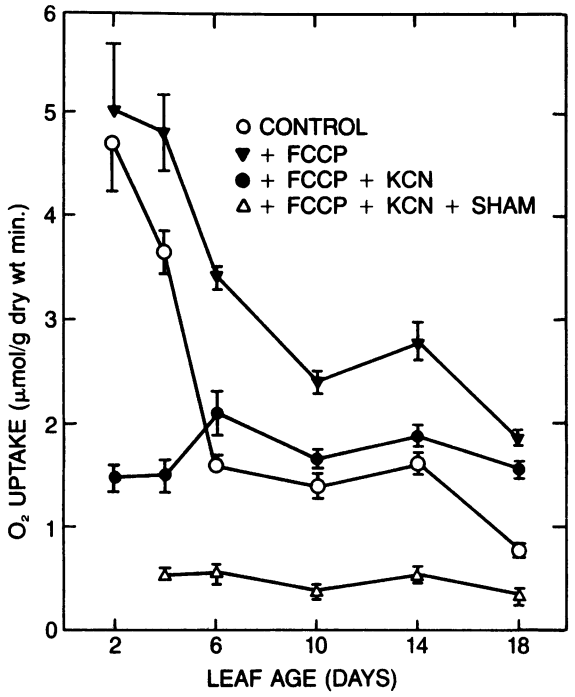

FIG. 4. Effects of KCN and KCN + SHAM on respiration of soybean leaf discs in the presence of 1 μM FCCP as a function of leaf age. Control rate represents O_2 uptake in the absence of inhibitors. For further information see legend of Figure 1.

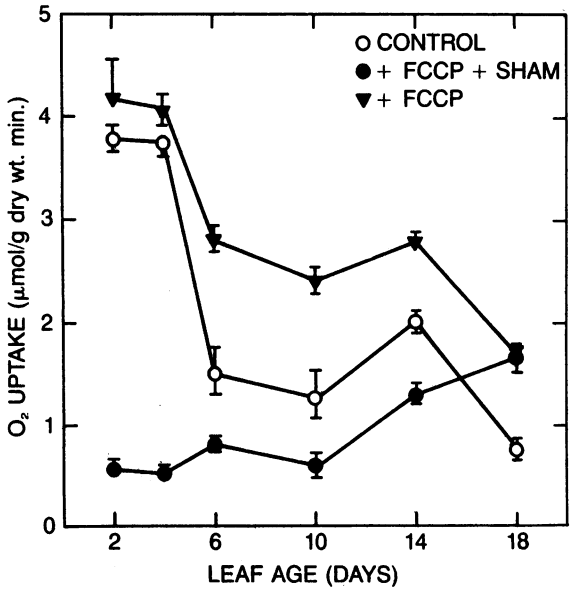

FIG. 5. Effect of SHAM on respiration of soybean leaf discs in the presence of 1 μM FCCP as a function of leaf age. For further information see legend of Figure 1.

of both the Cyt and alternative-path terminal oxidases. The stimulation of respiration by inhibitors of Cyt oxidase has been observed previously for mature leaves of carrot, *Daucus carota* (20), mustard, *Sinapis alba*, and belladonna, *Atropa belladonna* (19). The interaction between KCN and SHAM in inhibiting O_2 uptake by soybean leaf tissue is consistent with the presence of an alternative path (5, 7, 16) and further suggests that KCN and NaN_3 were stimulating O_2 uptake mainly by the SHAM-sensitive alternative path. The stimulation of O_2 uptake by SHAM alone, as far as we know, is an unknown phenomenon for leaf tissue,

Table II. *Estimation of the Capacities of Respiration Pathways in Soybean Leaves*

Rates shown were calculated from the data of Figure 4. Residual respiration (O_2 uptake in the presence of FCCP + KCN + SHAM) was subtracted from all values. V_t is the rate of O_2 uptake in the absence of FCCP and inhibitors. V_{cyt} is the capacity of the Cyt path, estimated as inhibition in the presence of FCCP and KCN. V_{alt} is the capacity of the alternative pathway, estimated as O_2 uptake in the presence of FCCP and KCN.

Leaf Age	V_t	Respiratory Capacities		V_{cyt}/V_{alt}
		V_{cyt}	V_{alt}	
d		*μmol/g dry wt·min*		*ratio*
4	3.12	3.28	0.98	3.35
6	1.02	1.31	1.54	0.85
10	1.04	0.72	1.30	0.55
14	1.10	0.91	1.36	0.67
18	0.42	0.30	1.21	0.25

although it has recently been observed in roots of other legume plants (9). Propyl gallate, a potent inhibitor of alternative oxidase, also stimulated O_2 uptake (data not shown).

According to existing information, cyanide stimulation of O_2 uptake should occur only in tissues with a large potential capacity for the alternative path. To estimate the relative potential capacities of the pathways during leaf development, the effect of KCN and SHAM in the presence of the uncoupler FCCP was determined. The effect of FCCP is to abolish the electrochemical proton gradient across the inner mitochondrial membrane and thereby allow maximum electron flux through the respiratory pathways (10). Inhibition of O_2 uptake by either KCN or SHAM in the presence of the uncoupler would therefore allow the capacities of the pathways to be estimated.

It seems quite likely that our results with FCCP and SHAM (Fig. 5) were influenced by some unspecific inhibitory action. A number of considerations complicate the interpretation of inhibitory effects of hydroxamates *in vivo*. Notable among these considerations are: (a) the presence of other hydroxamate-sensitive oxidases (4, 16), and (b) the possibility of other effects of hydroxamates on metabolism (9, 16). In this study, SHAM seemed to have some specificity for the alternative path as judged by its strong inhibition in the presence of KCN. However, the stimulation of O_2 uptake in mature leaves by SHAM alone would require either that SHAM causes the diversion of electron flux from the alternative path into an unsaturated Cyt path, an effect believed to be improbable (9, 27), or that SHAM has an additional effect either of uncoupling or of activation of extramitochondrial enzymes (9, 16). Along these lines, it is of interest to note that Lambers and his colleagues (15) have shown that high concentrations of SHAM (10–20 mM) can safely be used and even are required to give full inhibition of the alternative path in intact leaves and roots. De Visser and Blacquiere (9) observed SHAM simulation of respiration at low concentrations in roots of *Pisum sativum* and *Plantago* species, whereas they observed an inhibition at higher SHAM concentrations (25 mM). In titration experiments, we failed to detect inhibition of respiration by SHAM in the absence of KCN, even at SHAM concentrations as high as 30 mM (data not shown). However, there was a consistent decline in the stimulatory effect of SHAM at concentrations above 10 mM, and at 30 mM the stimulatory effect was completely lost. Maximal inhibition of respiration by SHAM in the presence of KCN was obtained at 10 mM.

The effect of KCN on O_2 uptake in the presence of FCCP gives information on the relative capacities of the two electron

transport pathways. The increase in V_{alt} occurred at the time of O_2 uptake stimulation by KCN and also when the stimulation by FCCP was most marked (Fig. 4). An important implication of these observations is that the rate of electron flux through the alternative path can exceed the maximum activity or capacity of the Cyt path in soybean leaf tissue. Thus, it might seem that the stimulation of O_2 uptake in mature soybean leaves in the presence of KCN and NaN_3 could be explained by the scheme put forward for mature *Chlorella* cells (12). This explanation is based on the assumption that the glycolytic rate is limited by the availability of phosphate acceptors (5) and, therefore, that the respiratory pathways are not saturated in the absence of inhibitors. This assumption is supported in this study by the stimulation of O_2 uptake by the uncoupler FCCP, although uncoupler stimulation could also conceivably result from the relief of respiration restrain in the electron transport path (3). It is clear, however, that the latter alternative would not lead to the observed results. The uncoupler was less effective in the young leaves, a response that is expected of young growing tissues. Day *et al.* (7) observed a similar response in *Lolium*. Presumably, with the high rate of O_2 uptake in young tissues, respiratory flux was already near maximal. This probably explains why the young leaves were sensitive to the inhibitors.

According to the proposed scheme for the stimulation of O_2 uptake in mature soybean leaves in the presence of KCN and NaN_3, the inhibition of the Cyt path, which is coupled to phosphorylation, diverts electron flow through the alternative path, thereby circumventing respiratory control. With the increased capacity of the alternative path, the increased electron flow can be accommodated, so total O_2 uptake is increased. A major difficulty with this scheme is the need to demonstrate that the apportioning of electrons to the alternative path is responsible for the respiratory stimulation. According to present thinking, however, the fact that the stimulation of O_2 uptake by KCN and NaN_3 is abolished by SHAM seems to provide good, though equivocal, evidence. The situation is further clouded by the consideration that, if the rotenone-sensitive NADH dehydrogenase and the alternative path operate in series, then electron transport through complex 1 (endogenous NADH to ubiquinone) would remain under respiratory control in the presence of KCN, since electrons branch to the alternative path from ubiquinone. It would seem therefore that the complete circumvention of respiratory control would require either that complex 1 must also be bypassed, a possibility inherent in the rotenone-resistant respiration so prevalent in plant tissues but a possibility on which some doubt has been cast (28), or that complex 1 must be disabled in some way in the presence of KCN. Thus, it is conceivable that while the alternative path itself is nonphosphorylating, the oxidation of tricarboxylic acid cycle substrates via the path in the presence of KCN may not be totally nonphosphorylating, at least for a certain period of time. However, even with the indication of a large potential capacity of the alternative path, our results did not indicate engagement of the alternative path in the absence of KCN, as shown by the stimulation rather than inhibition of O_2 uptake by SHAM alone. It remains to determine the relevance of the large alternative path capacity in mature soybean leaves.

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