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Occurrence of Alternative Respiratory Pathway in Freshly Excised Apple Root Tissue

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Abstract. **A cyanide-resistant alternative pathway was found to exist in root tissue of apple (*Malus domestica* Borkh.). In the absence of potassium cyanide (KCN), an inhibitor of cytochrome electron transport, the alternative pathway did not contribute to overall root respiration. However, in the presence of KCN or carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an uncoupler, active participation of the alternative pathway was detected. Inhibition of O_2 uptake by salicylhydroxamic acid (SHAM) was observed in the presence of antimycin A (AA) or sodium azide (NaN_3), but to a lesser degree than when KCN was present. The degree of inhibition by SHAM was greatest in the presence of KCN, followed by AA and then NaN_3. The antioxidant *n*-Propyl gallate (PG) was found to be an effective inhibitor of the alternative pathway. The site of inhibition in apple root tissue by PG is very similar to that of SHAM. Sodium benzoate, another antioxidant and free radical scavenger, and tetraethylthiuram disulfide (disulfiram), a copper chelator, did not inhibit the alternative pathway in apple root tissue.**

A respiratory pathway insensitive to cyanide has been found in many plant species and generally is called the alternative pathway (9). Salicylhydroxamic acid (SHAM) has been shown to be an inhibitor of this pathway (19). This alternative pathway is not associated with phosphorylation (1, 13, 29) and usually does not function unless the cytochrome pathway is either restricted or saturated (5). It is agreed generally that the location of the branch point for the alternative pathway in the cytochrome system is at or near coenzyme Q (1, 13, 29).

Most data concerning cyanide insensitive respiration have been derived from studies dealing with fruits or storage organs (11, 24). Effects of flooding on the shift of electron flow between cyanide-sensitive and alternative respiratory pathways in roots of ornamental tree species also has been reported (4). However, very little information is available on the nature of respiration in the roots of fruit trees under various conditions. This study was initiated to examine the features and significance of the 2 major respiratory pathways (cyanide-sensitive and cyanide-resistant) in apple root tissue. By using the Bahr/Bonner equation (2), as modified for plant tissue by Theologis and Laties (25), we determined the maximum capacity of apple root tissue for each pathway and estimated the relative contribution of each pathway to the total respiration with or without the specific inhibitors of the cytochrome-mediated or cyanide-resistant electron transport path.

Materials and Methods

Plant materials and treatments. Open-pollinated 'York Imperial' apple seedlings grown in the greenhouse (about 22°C) were used in this study. Seedlings were allowed to grow in flats of sand for 3 months. Nutrient solution (6) was applied to seedlings twice per week. Roots from these seedlings were used to study effects of inhibitors on respiration. Inhibitors of cytochrome-mediated respiration—potassium cyanide (KCN), antimycin A (AA), and sodium azide (NaN_3)—and cyanide-resistant respiration—SHAM, 3,4,5-trihydroxybenzoic acid propylester [*n*-propyl gallate (PG)], tetraethylthiuram disulfide (disulfiram), and sodium benzoate—were used.

Respiration measurement. Secondary roots were harvested and washed several times with distilled water and 10 mM N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES) buffer at pH 7.0. Roots were cut into 1.0-cm lengths and 0.2 g (fresh weight) were placed in a standard Gilson respiration flask containing 3 ml of 10 mM HEPES buffer with the appropriate respiratory inhibitors at pH 7.0. A filter paper wick and 0.2 ml of 10% KOH were placed in the center well. When cyanide was present, the center well solutions were those described by Robbie (18). Respiration was measured with a Gilson differential respirometer. All measurements were conducted at 25°C. The rate of O_2, uptake was calculated on a fresh weight basis and expressed as $\mu l\ O_2$ at STP/$g \cdot hr^{-1}$ (28).

Titration experiments. Titration of apple root tissue respiration with SHAM and KCN or other inhibitors was conducted according to the method of Bahr and Bonner (2), as modified for plant tissues by Theologis and Laties (25). The equation describing total tissue respiration is $V_T = \rho \cdot g(i) + V_{cyt} + V_{res}$ where V_T represents the total respiration rate; V_{cyt} is the contribution by the cytochrome pathway; V_{res} is the rate of the residual respiration uninhibited by the combined treatment of KCN and SHAM together; ρ is the fraction of the alternate pathway which is operating, and g(i) represents the maximal contribution by the alternate pathway at given concentrations of an alternate pathway inhibitor.

Chemicals. KCN was obtained from Fisher. SHAM and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were pur-

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chased from Aldrich. AA, NaN_3, PG, disulfiram, and HEPES were purchased from Sigma. Sodium benzoate was obtained from Eastman.

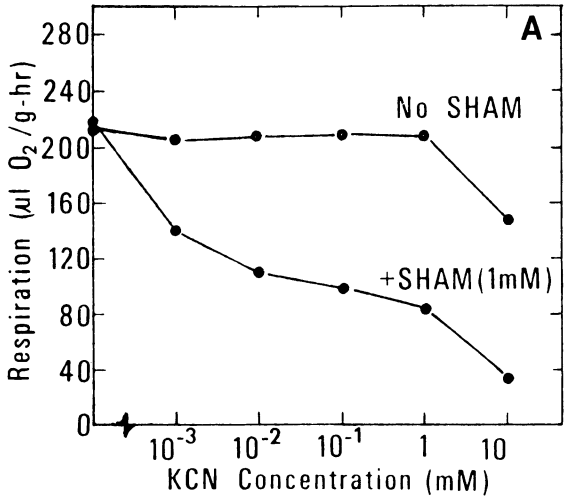
Additional details of methodology are given in the table and figure legends where applicable.

Results and Discussion

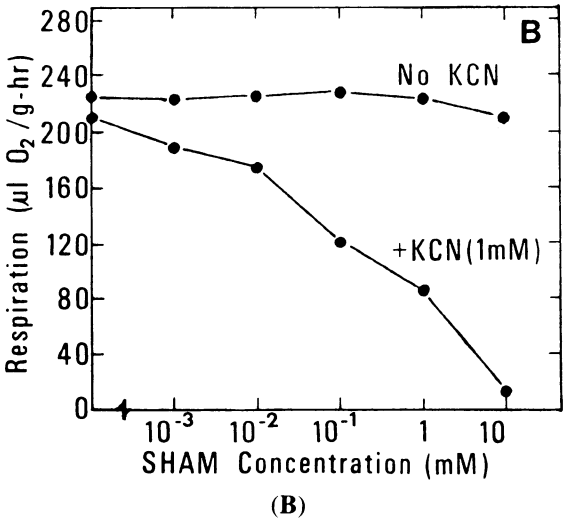
Effect of KCN and SHAM on respiration

KCN at ≤ 1 mM caused no inhibition of respiration (Fig. 1A), suggesting that a cyanide-resistant alternative path exists in these tissues. KCN at 10 mM resulted in 27% inhibition. Synergistic inhibition of respiration occurred with increasing concentration of KCN in the presence of 1 mM SHAM. An 84% inhibition of respiration was observed as KCN reached 10 mM in the presence of 1 mM SHAM, leaving 16% residual respiration which was resistant to the combination of KCN and SHAM (Fig. 1A).

SHAM at ≤ 10 mM did not inhibit root tissue respiration (Fig. 1B). SHAM inhibited O_2 consumption by apple root tissue only in the presence of 1 mM KCN. The combination of 10 mM SHAM and 1 KCN mM resulted in almost complete inhibition of O_2 consumption with minimal residual respiration (Fig. 1B). In the presence of 1 mM KCN, SHAM at 10 mM may have evoked a nonspecific inhibition that suppressed oxidative activities other than those involved in alternate respiration. Rich et al. (16) and Parrish and Leopold (14) have shown that substituted hydroxamates inhibit redox enzymes and lipoxygenase activity.



(A)



(B)

Fig. 1. (**A**) The effect of increasing concentration of KCN on the respiration of apple root tissue in the presence or absence of SHAM (1 mM). LSD (5%) = 9.6. (**B**) The effect of increasing concentrations of SHAM on the respiration of apple root tissue in the presence or absence of KCN (1 mM). LSD (5%) = 28.7.

Contribution of cytochrome and alternative pathways to total respiration by apple root tissue

Effect of cytochrome chain inhibitors (KCN, AA, NaN_3) on respiratory activity as measured with KCN and SHAM. Titration of respiration in coupled and uncoupled apple root tissue with SHAM in the presence or absence of KCN are shown in Fig. 2 and Table 1. SHAM at concentrations as high as 4 mM did not inhibit respiration in the absence of KCN (Fig. 2A). In the presence of 0.01 mM or 0.1 mM KCN, respiration was inhibited by 4 mM SHAM 63% and 71%, respectively (Fig. 2A). In the absence of KCN and CCCP, ρ was 0, indicating that the alternative pathway did not contribute to respiration (Table 1). However, in the presence of 0.01 mM KCN, the value of ρ was shifted to 0.9, indicating 90% engagement of the alternative pathway under these conditions. The cytochrome pathway also was reduced by KCN, whereas the alternate pathway increased, suggesting that when cytochrome is restricted, respiratory electrons are diverted to the alternative oxidase and extend participation of the alternative pathway as reported previously (11). CCCP, a potent uncoupler of oxidative phosphorylation, allows electron transport to continue but prevents the phosphorylation of ADT to ATP. It characteristically stimulates the rate of O_2

Table 1. A comparison of respiratory components (V_T, V_{cyt}, ρ·g(i), V_{res}) of coupled or uncoupled apple root tissue as measured with KCN and SHAM. Data were derived from Fig. 2, according to equation $V_T = \rho \cdot g(i) + V_{cyt} + V_{res}$.

0.01 mM CCCP	0.01 mM KCN	ρ	Respiration (µl O_2/g·hr^{-1}) V_T	ρ·g(i)	V_{cyt}	V_{res}
−	−	0.0	295.9	0.0	205.1	90.8
−	+	0.9	283.6	166.1	23.7	93.8
+	−	0.5	328.3	59.5	235.5	33.3
+	+	1.0	170.4	118.9	21.8	29.7
LSD (5%)			11.0	32.5	24.7	24.2

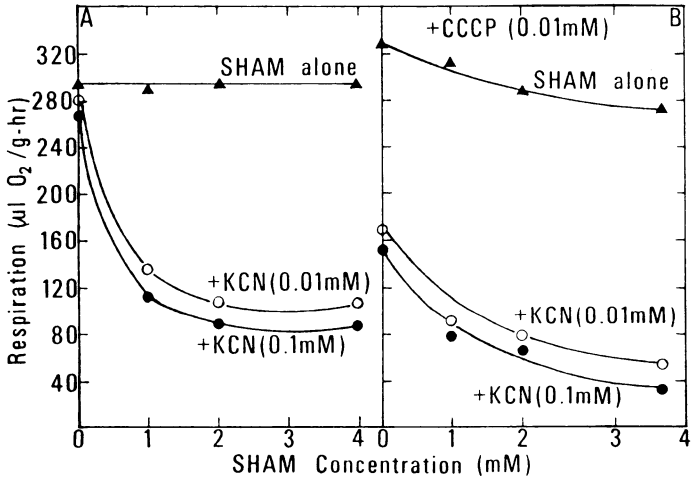


Fig. 2. Effect of SHAM with or without KCN on coupled (A) or uncoupled (B) apple root tissue respiration. LSD (5%) for A = 10.1; B = 15.7.

uptake. Theologis and Laties (25) found that expanding the respiratory activity in potato slices with CCCP could lead to the engagement of the alternative pathway of respiration. CCCP caused a slight increased in O_2 consumption and increased V_T in apple root tissue (Table 1). In the presence of the uncoupler, respiration was inhibited by SHAM alone (Fig. 2B). A synergistic inhibition was observed when KCN was added with SHAM. In the presence of CCCP and absence of KCN, ρ was 0.5 (Table 1); thus, 50% of the alternative pathway was operational. With addition of 0.01 mM KCN, ρ = 1 was obtained, indicative of maximum alternative pathway operation. It has been suggested by Theologis and Laties (25) that the uncoupler may enhance glycolysis, which in turn raises the activity of tricarboxylic acid cycle, thereby saturating the cytochrome chain and promoting engagement of the alternative pathway. It also was found that V_T decreased in the presence of KCN in both coupled and uncoupled root tissues. Addition of the uncoupler also resulted in lower V_{res} in apple root tissue (Table 1).

Effect of cytochrome chain inhibitors as measured with AA and SHAM. AA, a specific inhibitor of complex III in the electron transport chain (17, 23), was substituted for KCN in titration with SHAM. AA at both 0.01 and 0.1 mM stimulated respiration (Fig. 3A). However, AA inhibited respiration in the presence of SHAM (1 to 4 mM). SHAM did not affect respiration in the absence of antimycin A, even at 4 mM. This indicates that the respiratory flux was through the cytochrome path. In the presence of 0.01 or 0.1 mM AA, respiration was inhibited by 4 mM SHAM 32% and 41%, respectively (Fig. 3A), compared to 63% and 71% inhibition in the presence of 0.01 or 0.1 mM KCN (Fig. 2A). In the absence of AA, ρ = 0 was obtained, with no contribution of alternative pathway to V_T (Table 2). When 0.01 mM antimycin A was present, activity of the V_{cyt} pathway of apple root tissue decreased from 116.0 to 46.0 μl $O_2/g \cdot hr^{-1}$ and ρ increased from 0.0 to 0.5, indicating 50% engagement of the alternative pathway (Table 2). SHAM at 1.0 to 4.0 mM progressively inhibited the respiration of uncoupled root tissue (Fig. 3B), but not that of coupled root tissue (Fig. 3A). The elevated respiration of uncoupled tissue decreased to a level comparable with that of coupled tissue by addition of 4.0 mM SHAM (Fig. 3). In uncoupled tissue without AA, ρ = 0.6 (Table 2) indicated 60% engagement of the alternative path.

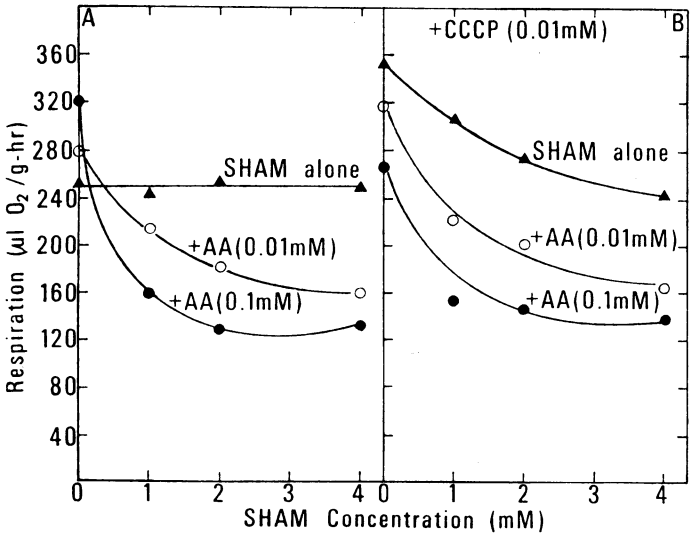


Fig. 3. Effect of SHAM with or without AA on coupled (A) and uncoupled (B) apple root tissue respiration. LSD (5%) for A = 28.6; B = 25.1.

Table 2. A comparison of respiratory components (V_T, V_{cyt}, ρ·g(i), V_{res}) of coupled or uncoupled apple root tissue, as measured with AA and SHAM. Data were derived from Fig. 3, according to equation $V_T = \rho \cdot g(i) + V_{cyt} + V_{res}$.

0.01 mM CCCP	0.01 mM AA	ρ	Respiration (μl $O_2/g \cdot hr^{-1}$) V_T	ρ·g(i)	V_{cyt}	V_{res}
−	−	0.0	249.5	0.0	116.0	133.5
−	+	0.5	287.4	95.7	46.0	145.7
+	−	0.6	350.1	70.8	140.0	139.3
+	+	1.0	318.9	118.0	64.0	136.9
LSD (5%)			29.0	13.7	17.2	NS

When 0.01 mM antimycin A was present in the uncoupled tissue, V_{cyt} was reduced from 140.0 to 64.0 μl $O_2/g \cdot hr^{-1}$ and ρ = 1 was obtained, suggesting that the alternative path was operating at maximum capacity. V_{res} generally was higher in the presence of AA than in the presence of KCN (Tables 1 and 2). This may indicate that AA exerted less inhibition than did KCN. The weaker synergistic effect of AA and SHAM also has been reported for potatoes and sweet potatoes (26, 27). It was suggested that a bypass might be operating around the antimycin-sensitive site to allow the flow of electrons to the cytochrome path (26).

Effect of cytochrome chain inhibitors as measured with NaN_3 and SHAM. NaN_3 is another cytochrome oxidase inhibitor which inhibits at the same site as KCN in the cytochrome electron transport chain (8). NaN_3 was used as a substitute for KCN in titrations with SHAM because of its much lower volatility. Special manometric techniques (12), required when using cyanide, were not necessary with NaN_3. The results of titrating root tissue with SHAM in the presence or absence of NaN_3 are shown in Fig. 4 and Table 3. 0.01 mM NaN_3 stimulated O_2 consumption 18.0% in the absence of SHAM. In combination with SHAM, NaN_3 inhibited O_2 consumption. In the presence of NaN_3, titration with SHAM resulted in reduction of V_{cyt} from 64.0 to 36.0 μl $O_2/g \cdot hr^{-1}$ and a shift of ρ from 0.0 to 0.8, indicating 80% engagement of the alternative path (Table 3). Relatively high V_{res} were obtained, suggesting that NaN_3 exerted less inhibition on O_2 consumption than did KCN or antimycin A in the presence of SHAM. These data indicate that NaN_3 is a much weaker synergistic inhibitor than KCN or antimycin A (Fig. 2, 3, 4 and Tables 1, 2, 3).

Effect of alternative pathway inhibitors (PG, sodium benzoate, and disulfiram) on respiratory activity as measured with KCN and PG. PG is an antioxidant and has been shown to be a very effective inhibitor of the alternative pathway (21, 22). It also is an excellent inhibitor of lipoxygenase (15, 22). An attempt was made to substitute PG for SHAM in titrations with KCN to estimate the contribution of the CN-resistant pathway to the total respiration of apple root tissue. The results are presented in Fig. 5 and Table 4. PG alone had no effect on O_2 consumption (Fig. 5). In the presence of KCN, electron flow was primarily via the alternative pathway and respiration was found to be inhibited markedly by increasing concentration of PG. This suggests that PG is functioning on the same pathway as SHAM. These results were consistent with those found with fresh slices of ethylene-treated potato tuber tissue (10) and mung bean mitochondria (22). As mentioned before, analysis of titration data (Table 4) indicated that ρ was zero and the alternate pathway did not operate in the absence of KCN. In the presence of 0.01 mM KCN, ρ was 0.8, indicating 80% engagement of the alternate pathway. The rate of the cytochrome pathway was reduced at the same time from 144.7 to 29.9 μl $O_2/g \cdot hr^{-1}$. Similar results

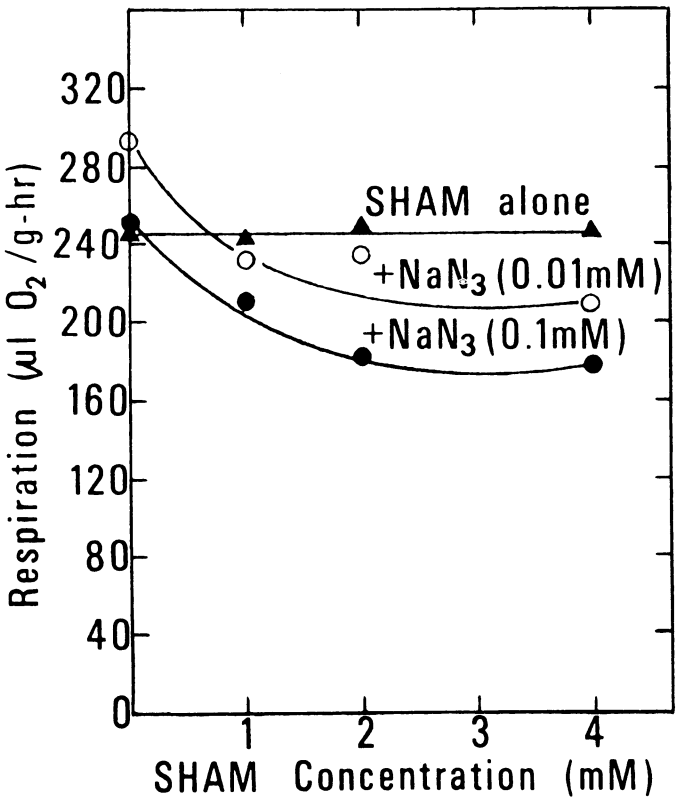


Fig. 4. Effect of SHAM with or without NaN_3 on apple root tissue respiration. LSD (5%) = 20.8.

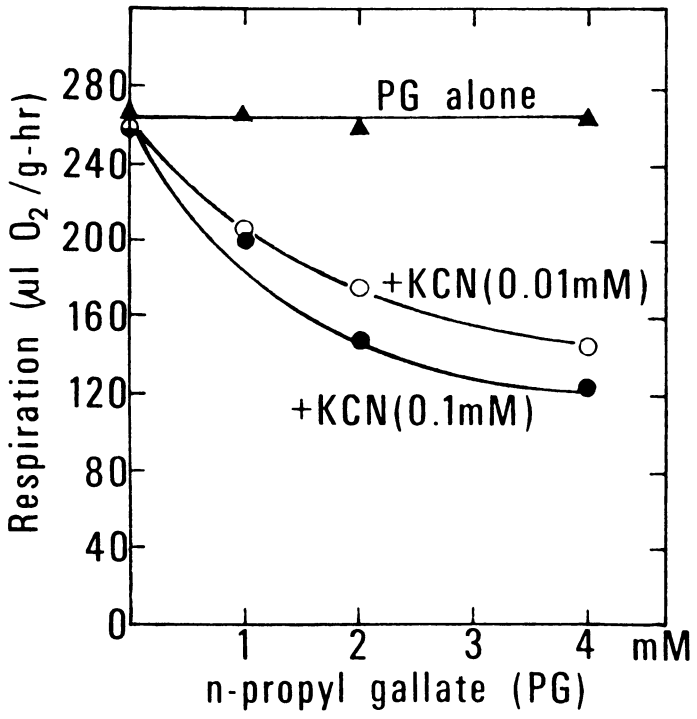


Fig. 5. Effect of PG with or without KCN on apple root tissue respiration. LSD (5%) = 17.6.

also were obtained with SHAM (Table 1). Jones and Wiest (10) reported that PG and SHAM appeared to be functioning at identical sites in mitochondria but at disparate sites in slices of ethylene-treated potato tuber tissue. It also was reported that SHAM and PG act at the same site to inhibit the alternative pathway in mung bean mitochondria (22). Multiple inhibitor experiments were conducted to compare sites of inhibition of the alternative pathway in apple root tissue, using both PG and SHAM in the presence of 0.5 mM KCN; results are presented as a Dixon plot (20) in Fig. 6. If 2 inhibitors bind in a mutually exclusive fashion to inhibit a given activity, then parallel lines will be obtained in a Dixon plot (20). A series of parallel lines were obtained by plotting the reciprocal of the alternative-pathway flux against various concentration of SHAM in the absence or presence of different constant concentrations of PG (Fig. 6). Thus, this Dixon plot indicates that SHAM and PG act at either the same or very similar sites in apple root tissue. Therefore, PG should serve as a useful alternative to SHAM in respiratory pathway studies of apple root tissue.

Effect of alternate pathway inhibitors as measured with KCN and sodium benzoate or disulfiram. Sodium benzoate has been shown to possess properties of an antioxidant and a free radical scavenger (3). An attempt was made to use sodium benzoate to substitute for SHAM in titration with KCN to determine if it inhibits O_2 consumption associated with the alternative respiratory pathway. Our results (data not shown) indicate that this free, nonesterified benzoic acid derivative does not have the ability to inhibit the alternative pathway in apple root tissue. This may be due to the fact that sodium benzoate lacks certain structural features required to cause the inhibition of the alternative pathway. Siedow and Bickett (21) concluded that a simple phenolate anion is the minimum structural feature needed for specific inhibition of the alternative pathway. Unlike PG or SHAM, sodium benzoate does not possess this characteristic and therefore lacks the capacity for inhibition.

Disulfiram, originally explored as a copper chelator, was shown to be a potent inhibitor of the alternative pathway in mitochondria of potato tubers (7). However, disulfiram did not inhibit the cyanide-resistant respiration in apple root tissue (data not shown). This may be because of its insufficient penetration into the tissue or because it dissipates in the cytosol as was found with tissue of red sweet potato (7).

This work has focused on the cyanide-insensitive pathway of respiratory electron transport—its presence, engagement, regulation, and interaction with the cytochrome system. Taken to-

Table 3. Activities of the cytochrome and alternative pathways as measured with NaN_3 and SHAM in apple root tissue. Data were derived from Fig. 4, according to equation $V_T = \rho \cdot g(i) + V_{cyt} + V_{res}$.

0.01 mM NaN_3	ρ	Respiration (µl $O_2/g \cdot hr^{-1}$)			
		V_T	$\rho \cdot g(i)$	V_{cyt}	V_{res}
−	0.0	244.0	0.0	64.0	180.0
+	0.8	287.4	75.1	36.0	176.3
LSD (5%)		24.8	22.1	16.1	NS

Table 4. Relative contribution of cytochrome and alternative pathway in apple root tissue estimated with KCN and PG. Data were derived from Fig. 5, according to equation $V_T = \rho \cdot g(i) + V_{cyt} + V_{res}$.

0.01 mM KCN	ρ	Respiration (µl $O_2/g \cdot hr^{-1}$)			
		V_T	$\rho \cdot g(i)$	V_{cyt}	V_{res}
−	0.0	268.0	0.0	144.7	123.3
+	0.8	249.8	100.6	29.9	119.3
LSD (5%)		10.3	18.7	16.4	NS

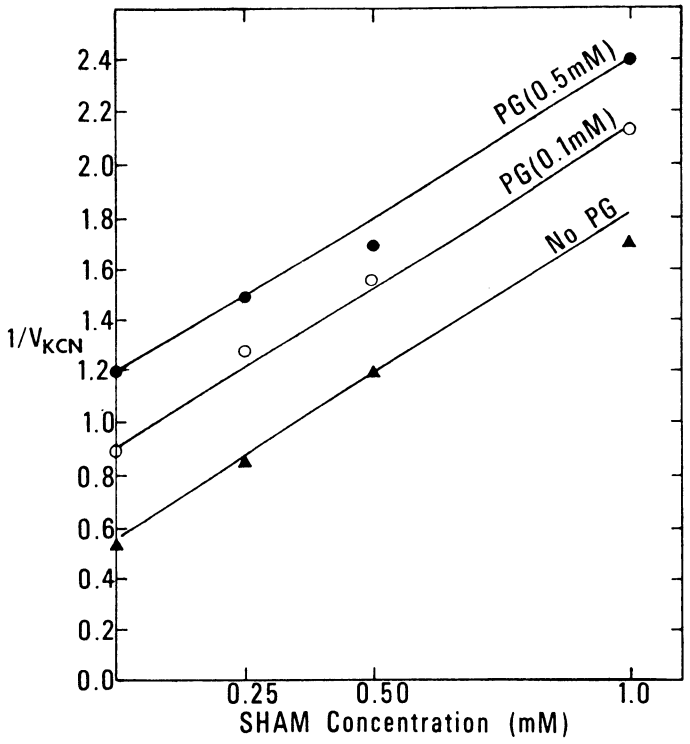


Fig. 6. Dixon plot of the rate of apple root tissue respiration in the presence of 0.5 mM KCN and various concentration of SHAM and PG as indicated. $1/V_{KCN}$ represents reciprocal of respiration rates ($\mu l\ O_2/g \cdot hr^{-1}$).

gether, the data obtained from freshly excised apple seedling root tissue showed that there was no inhibition of O_2 consumption when SHAM or KCN alone was applied. O_2 consumption was inhibited greatly in the presence of SHAM by KCN, an inhibitor of cytochrome electron transport, or inhibited to a lesser degree by AA or NaN_3. The observation that SHAM inhibits O_2 consumption only in the presence of KCN suggests that the inhibition being measured in these tissues mainly are effects on respiratory oxidations and shows that a cyanide-resistant pathway of respiration is present in these tissue. A bypass might be operating around the antimycin-sensitive site to allow the flow of electrons to the cytochrome pathway (26), therefore resulting in a lesser degree of inhibition. The engagement of alternative pathway in the absence of uncoupler (CCCP) in freshly excised apple seedling root tissue was virtually zero, suggesting that there is very little overflow of electrons to the alternative pathway. The elevation of respiratory activity by CCCP, an uncoupler, increases the degree of sensitivity of O_2 consumption to SHAM inhibition in the absence of cyanide, indicating an engagement of the alternative pathway. This may be due to the enhancement of glycolysis and the excessive substrate flux which exceeds the requirement of the cytochrome pathway (25). The ability of CCCP to stimulate respiration also suggests that there was unused cytochrome oxidase activity present in the tissue and that cytochrome pathway had not reached saturation. These data suggest that apple root tissue has the capacity for engaging the alternative pathway and the main effects of SHAM in these tissues are through its effects on the respiratory pathway. PG, a free-radical scavenger and antioxidant, is a very effective inhibitor of the alternative pathway (21, 22). PG alone had no effect on O_2 consumption in freshly excised apple seedling root tissue (Fig. 5). Electron flow in the presence of KCN was primarily via the alternative pathway and respiration was found to be inhibited markedly by increasing concentration of PG. This suggests that PG is functioning on the same pathway as SHAM. Results from multiple inhibitor experiments, expressed as a Dixon plot, showed that PG and SHAM act at either the same or very similar sites in freshly excised apple root tissue. Therefore, PG should serve as a useful alternative to SHAM in studying the respiratory pathway in freshly excised apple root tissue. Sodium benzoate and disulfiram did not inhibit cyanide-resistant respiration in apple root tissue. This may be due to the lack of certain structural features of these chemicals, insufficient penetration into the tissue, or dissipation in the cytosol. The residual respiration, which is not affected by cytochrome and alternative pathway inhibitor, was much higher in the presence of NaN_3 and SHAM than in KCN and SHAM. Although the nature of the residual respiration is not known, its magnitude appears to be a measure of the extent of inhibition of the 2 pathways. We conclude that, under normal conditions, the capacity of alternative pathway is present in freshly excised apple root tissue but is not operational. The alternative pathway is only engaged when the cytochrome pathway is either restricted by inhibitors of cytochrome electron transport or flooded by decontrolling glycolysis with uncouplers.

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The Occurrence of Mesocarpic Stone Cells in the Fruit of Cultivated Highbush Blueberry

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Abstract. **Stone cells of highbush blueberry (*Vaccinium corymbosum* L.) were distributed primarily toward the periphery of the fruit; they apparently differentiated from ground parenchyma shortly after anthesis. Secondary cell wall material continued to be accreted through harvest, with lamellations about 1μm in width. The lignified walls were heavily pitted, with pits contiguous with those of adjacent stone cells. The number of stone cells may be correlated positively to the length of the growth season for each cultivar.**

Stone cells, also termed sclereids, occur in the fruit of several species (6). These often are associated with the endocarpic region, but may be found throughout the pericarp. Because of their obvious economic importance, stone cells in the flesh of the pear fruit (*Pyrus communis* L.) have been studied extensively (2, 4, 5). Yarbrough and Morrow (1) reported that both endocarpic and mesocarpic sclereids occur in the fruit of several species of *Vaccinium*, including cultivars of *V. australe* Small. Some species had more or larger stone cells than other species and hence were "grittier" and less palatable. The objective of this study was to examine the ontogeny, composition, and distribution of mesocarpic stone cells in highbush blueberry.

Materials and Methods

Mature 'Earliblue', 'Collins', and 'Coville' plants were maintained on a Narragansett Silt Loam soil with a sawdust mulch at Kingston, R.I. The cultivars were early-, mid-, and late-ripening, respectively. Fruits were harvested at 6 color stages of maturity and ripeness during the 1977 growing season. The stages are as follows: 1) immature green (IG), with the largest berries a dark green color over 100% of their surface, about 8 days after full bloom; 2) mature green (MG), with the berries a light greenish-white color with the calyx just beginning to turn pink, about 28 days after full bloom; 3) green-pink (GP), with the surface coloration of these berries about 75% green and 25% pink; 4) pink-green (PG), with the fruit about 75% pink and 25% green in surface coloration; 5) blue-pink (BP), with the fruit surface about 75% blue and 25% pink; and 6) blue (B), with the berries blue on about 90% of their surface and about 10% pink coloration around the scar.

Ripe fruit were prepared for macroscopic investigation according to Crist and Batjer (2). Longitudinal and transverse freehand sections about 1.5 mm thick were dehydrated through an acetone/water (50%, 75%, 90%) series into acetone (100%) and finally into cedarwood oil. Cleared sections were suspended in glass cells and mounted in Canada balsam. Sections were photographed using Ektachrome film and bottom illumination.

Samples of fruit were fixed in Randolph's Modified Navashin Solution for microscopic investigation, dehydrated with an ethanol/t-butanol series and embedded in Paraplast Plus. Tissue was sectioned at 12 μm and stained in either Heidenhain's iron alum hematoxylin or in safranin O and fast green FCF, and then mounted in Canada balsam. Tissue was stained in 1% aqueous crystal violet, 0.001% aqueous methyl red; or treated with phloroglucinol/HCl, IKI/H^2SO_4, or chloroiodide of zinc according

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