Inhibition of \( \text{O}_2 \) Consumption Resistant to Cyanide and Its Development by \( \text{N} \)-Propyl Gallate and Salicylhydroxamic Acid

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ABSTRACT

Kinetics of inhibition of cyanide-insensitive \( \text{O}_2 \) uptake by \( \text{n} \)-propyl gallate (PG) and salicylhydroxamic acid (SHAM) were determined in fresh slices from ethylene-treated tubers of \( \text{Solanum tuberosum} \) 'Norchip' and with mitochondria and lipoxygenase (EC 1.13.11.12) isolated from these tubers. PG and SHAM appeared to be inhibiting identical sites in mitochondria but at disparate sites in slices. The apparent \( K_i \) for SHAM was similar in mitochondria and slices. However, the apparent \( K_i \) for PG in mitochondria was about 40-fold lower than the \( K_i \) for PG inhibition of lipoxygenase activity. The amount of lipoxygenase associated with mitochondria increased when tubers were treated with ethylene. PG, but not SHAM, inhibited aging-induced development of cyanide-insensitive respiration. The latter two phenomena are in accord with the hypothesis that lipid metabolism is required for the development of the alternative pathway.

SHAM\(^2\) and related compounds have routinely been used to document the presence of a cyanide-resistant respiratory pathway in a large number of tissues (8). Unfortunately, hydroxamic acids are by no means specific inhibitors. Rich et al. (12) have documented inhibition of tyrosinase and peroxidase by SHAM; however, both of these enzymes are inhibited by cyanide. Parrish and Leopold (11) have demonstrated that the cyanide-resistant \( \text{O}_2 \) consumption by ground soybean endosperm which is inhibited by SHAM is attributable to lipoxygenase activity. Hence, the ability of SHAM to uniquely inhibit a single enzyme, i.e., the 'alternative oxidase,' in \( \text{vivo} \) is questionable.

A large number of workers have implied that an alternative oxidase is active in aged white potato tuber slices (7) and in whole tubers that have been treated with ethylene or other volatile compounds (5). The results presented here and by Siedow and Girvin (13) suggest that neither SHAM nor PG, a purported inhibitor of lipoxygenase (11), can be used to define the enzyme system(s) involved in cyanide-resistant \( \text{O}_2 \) uptake in potatoes. However, a kinetic analysis using both inhibitors is used in this report to demonstrate that in ethylene-treated potato tuber tissue, lipoxygenase is not the alternative oxidase.

MATERIALS AND METHODS

Locally grown potato tubers ( \( \text{Solanum tuberosum} \) L. 'Norchip') were preconditioned at room temperature for about 10 d following harvest or cold (\( 7^\circ\)C) storage. Tubers were used either immediately after the preconditioning or treated for 24 h with 10 \( \mu \)l/ethylene plus 100% \( \text{O}_2 \) to initiate cyanide-resistant respiration. Slices (1.5 g) 1 mm thick and 5 mm in diameter were cut from ethylene-treated or untreated control potatoes and the respiratory rate was measured in 10 mm K-phosphate (pH 5.5) in a Gilson respirometer. The pH of inhibitors was adjusted to 5.5 prior to use.

In some experiments, cyanide-resistant respiration was induced in the potato by aging slices from untreated potatoes in an aerated 0.1 mm CaSO\(_4\) solution. At times, the aging solution contained various concentrations of PG. Following aging, slices were rinsed and soaked in distilled H\(_2\)O with numerous changes for 1 h prior to respiratory determinations. In this way, any PG in the aging solution was removed prior to respiratory measurements.

A washed mitochondrial preparation was made using 100 g of potato tissue, representing a composite sample of 2 to 3 tubers according to the procedure outlined by Bonner (2). The final mitochondrial preparations contained on the average 5 mg protein/ml. The protein content was determined by the method of Lowry et al. (10). Respiration was measured with a YSI model 53 Oxygen Monitor. Lipoxygenase was obtained by homogenizing 50 g of potato tissue in 50 ml cold 10 mm K-phosphate buffer (pH 7.2) for 1 min in a VirTis blender. The extract was passed through Miracloth and centrifuged at 20,000g for 20 min. The supernatant was passed through a Sephadex G-50 column, and the pooled protein fraction was used for the lipoxygenase assay. Lipoxygenase was assayed at pH 7.0 polarographically as described by Parrish and Leopold (11). The washed mitochondrial fraction was also used to determine the lipoxygenase activity associated with it using the methodology of Siedow and Girvin (13).

RESULTS AND DISCUSSION

Respiratory rates of fresh slices from ethylene-treated tubers were determined in the presence or absence of various inhibitors (Table 1). \( \text{O}_2 \) consumption was not inhibited by 1 mm KCN, suggesting that a cyanide-resistant alternative pathway exists in these slices. KCN, in fact, slightly stimulated \( \text{O}_2 \) consumption—a phenomenon of unknown origin (possibly due to the Pasteur effect in the presence of KCN) that was reported previously (9). SHAM and PG by themselves had little effect on \( \text{O}_2 \) consumption. However, SHAM in conjunction with KCN has been used to indicate alternative pathway activity (1). Data presented in Table 1 suggest that PG is functioning on the same pathway as SHAM. This
Table 1. Rate of Respiration of Slices from Potato Tubers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O2 Uptake μl/g fresh wt·h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.4</td>
</tr>
<tr>
<td>KCN (1 mM)</td>
<td>63.2</td>
</tr>
<tr>
<td>SHAM (1 mM)</td>
<td>52.6</td>
</tr>
<tr>
<td>PG (0.5 mM)</td>
<td>53.4</td>
</tr>
<tr>
<td>KCN + SHAM</td>
<td>11.8</td>
</tr>
<tr>
<td>KCH + PG</td>
<td>8.6</td>
</tr>
</tbody>
</table>

conclusion has also been drawn for mung bean mitochondria by Siedow and Girvin (13).

Multiple inhibition experiments have long been utilized to deduce the mutualities of action of inhibitors (17). Yonemomi and Theorell (17) developed the following simplified but invaluable steady-state rate equation for enzyme kinetics in the presence of two inhibitors:

\[
\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{S V_m} + \frac{K_m I_1}{S V_m K_{I1}} + \frac{K_m I_2}{S V_m K_{I2}} + \frac{K_m I_1 I_2}{S V_m K_{I1} K_{I2}}
\]

where \( v \) = velocity of the reaction, \( V_m \) = maximal velocity, \( K_m \) = Michaelis constant, \( S \) = substrate concentration, \( I_1 \) and \( I_2 \) = inhibitor concentrations, \( K_{I1} \) and \( K_{I2} \) = dissociation constants of enzyme-inhibitor complexes and \( \alpha \) = a constant signifying the degree to which the binding of one inhibitor type influences the binding of a second inhibitor type. Equation 1, which was originally formulated for substrate-competitive inhibitors, is valid regardless of the mechanism by which the inhibitors act (i.e. competitive or noncompetitive with substrate). As implied by Cleland (3) and explicitly stated by Wong (16, p. 52): “These methods (including Yonemomi-Theorell plots) for distinguishing exclusive and nonexclusive binding are valid whether or not the pure-inhibition mechanisms for (different inhibitor species) are alike, e.g. they may both be competitive inhibitors against the substrate, both noncompetitive, or one competitive and one noncompetitive.” That inhibition by PG and SHAM is ‘pure’ (16) can be deduced from the linearity of Yonemomi-Theorell plots. Values of \( \alpha, K_{I1}, \) and \( K_{I2} \) can be obtained by multiple regression analysis of appropriate kinetic data if one further assumes that \( 1/V_m \ll K_m/S V_m \); \( \alpha \) is, for purposes of determining the site of action of the inhibitors, most important of the above parameters.

Equation 1 predicts that a plot of \( 1/v \) versus \( I_1 \) at constant \( S \) and \( I_2 \) will yield a straight line with a slope equal to:

\[
\frac{K_m}{S V_m K_{I1}} \left[ 1 + \frac{I_2}{\alpha K_{I1}} \right]
\]

A more simplified analysis, assuming that the binding of the second inhibitor species leads to a predicted slope of:

\[
\frac{K_m}{S V_m K_{I2}} \left[ 1 + \frac{I_2}{K_{I2}} \right]
\]

Therefore, \( \alpha = 1 \) would suggest that the binding sites of \( I_1 \) and \( I_2 \) were independent. Similarly, if the binding of one inhibitor species precluded the binding of a second inhibitor species, i.e. if the enzyme could not bind both inhibitors at the same time, equation 1 would become:

\[
\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{S V_m} + \frac{K_m I_1}{S V_m K_{I1}} + \frac{K_m I_2}{S V_m K_{I2}}
\]

In this case, the slope of a plot of \( 1/v \) versus \( I_1 \) becomes simply \( K_m/S V_m \)-independent of \( I_2 \). Hence, varying the concentration of \( I_2 \) will produce a set of parallel lines. Comparison of equations 1 and 2 indicate that when binding of one inhibitor type precludes the binding of a second inhibitor type

\[
\frac{K_m I_1 I_2}{S V_m K_{I1} K_{I2}} = 0
\]

and thus, \( \alpha \) must equal \( \infty \). A qualitative analysis of equation 1 also suggests that if the two inhibitors interact attractively, i.e. if the binding of one inhibitor species facilitates binding of the second species, then one would expect the fifth term of equation 1 to be greater than that observed when \( \alpha = 1 \). Hence, the value of \( \alpha \) in this case must be between 0 and 1. Finally, if the binding of one inhibitor type hindered, but did not preclude, the binding of the second inhibitor type, the fifth term of equation 1 would be expected to be less than that observed when \( \alpha = 1 \). Hence, \( \infty > \alpha > 1 \). The above equations are greatly simplified from the real case for O2 consumption in vivo. However, while \( K_i \) values obtained may in fact be complex, rather than unique, dissociation constants, the value of \( \alpha \) will be useful in determining whether the inhibitors are acting at the same (\( \alpha = \infty \)) or unique (\( \alpha = 1 \)) sites.

The multiple inhibition experiment using both PG and SHAM in the presence of 1 mM KCN was conducted in order to test the hypothesis that these inhibitors are functioning at identical sites. Figure 1 presents a Yonemomi-Theorell plot (17) of the results. The point at which the lines intersect the abscissa indicate the apparent \( K_i \) for SHAM. Since the addition of PG significantly reduced the velocity of O2 consumption, but had no apparent influence on the \( K_i \) for SHAM, it would appear that PG in slices is acting at a site distinct from that influenced by SHAM, at least at the concentrations utilized. Equation 1 in the case of slices becomes:

\[
1/v = 0.0098 + 0.0647 I_1 + 1.1261 I_2 + 9.168 I_1 I_2
\]

where \( I_1 = \text{mM} \) SHAM concentration and \( I_2 = \text{mM} \) PG concentration. This equation accounts for 95% of the observed variance of the data. Assuming that \( 1/V_m \ll K_m/S V_m \), the \( K_i \) for SHAM is 0.15 mM, the \( K_i \) for PG is 0.009 mM, and \( \alpha = 0.81 \). These values

![Fig. 1. Yonemomi-Theorell plot of the rate of slice respiration in the presence of 1 mM KCN and various concentrations of SHAM and PG as noted in the figure. The slices were taken from tubers treated for 24 h with 10 μl/l ethylene.](https://example.com/fig1.png)
From this equation, the apparent $K_I$ for SHAM is 0.13 mM and the apparent $K_I$ for PG is 0.0002 mM. The fact that $\alpha$ appears to be large suggests that the binding of one of the inhibitors precludes the binding of the other. PG and SHAM apparently prevent each other's binding to the enzyme(s), presumably by acting at identical sites.

The results presented in Figure 2, when compared with those in Figure 1, suggest that PG may be acting on different sites in slices and isolated mitochondria. It is conceivable that PG, being a free-radical scavenger, is acting on several enzyme systems in the freshly cut potato slice as well as the mitochondrial alternative oxidase.

That PG appears to be capable of inhibiting at least two distinct sites of $O_2$ consumption in slices can be deduced from Figure 3, a Dixon plot of PG inhibition in the absence of SHAM. The non-linearity of the plot suggests that PG is inhibiting at least two sites of $O_2$ uptake with $K_I$ values of 6.2 $\mu$M and 0.13 mM.

Had PG inhibited the alternative oxidase in data presented in Figure 1, $\alpha$ should have been greater than 1.0. However, $\alpha$ was observed to be about 1.0, suggesting that PG was not inhibiting the same enzyme as SHAM (i.e. the alternative oxidase). The apparent discrepancy between results presented in Figure 1, and data suggesting that PG does, in fact, inhibit the alternative oxidase (Table I; Fig. 3) is likely due to the disparity in concentrations of PG utilized—between 0 and 0.01 mM in Figure 1, but ranging to 0.5 mM in Table I and Fig. 3. Hence, it is likely that the concentrations of PG used in the experiments reported in Figure 1 were too low to detect interference with SHAM binding.

Table III presents a summary of $K_I$ values for PG and SHAM in slices, isolated mitochondria, and isolated soluble lipoxygenase. Clearly, the $K_I$ for SHAM is almost identical in slices and mitochondria, and is about 10-fold lower than the $K_I$ for SHAM inhibition of lipoxygenase. The $K_I$ for PG inhibition of slice $O_2$ consumption is about 40 times higher than the $K_I$ for inhibition of $O_2$ consumption by isolated mitochondria, but is only slightly higher (1.5 times) than the $K_I$ of PG for lipoxygenase.

If PG were acting at two sites in the mitochondria, with one identical to the site of SHAM inhibition, $\alpha$ would be large but would not approach $\infty$. However, experimental conditions may not have been favorable for observation of both inhibition sites due to the low concentrations of PG used. For example, the $K_I$ for PG in mitochondria (alternative oxidase) was 3% of that for lipoxygenase (Table III), which may also be bound to the washed mitochondrial fraction. The concentrations of PG used in the experiments reported in Figure 2 were much lower than those necessary to observe significant lipoxygenase activity. Also, if the second site of PG inhibition was mitochondrial in nature, it may be associated with complex I. All experiments reported herein used succinate as the substrate for mitochondrial respiration and, therefore, complex I would be bypassed.

We have previously demonstrated that ethylene-induction of CN-resistant respiration is not associated with increased glucose metabolism (9). In this regard, Table IV presents evidence for an increase in the affinity of mitochondrial membranes for lipoxygenase after the development of CN resistance. An alteration in the mitochondrial membrane after ethylene treatment, nonspecifically increasing the affinity of the membrane for soluble proteins would result in an increase in total lipoxygenase activity contaminating our mitochondrial preparations. However, such a nonspecific increase in affinity could not result in a specific activity of lipoxygenase higher than that observed in the soluble fraction. Since the observed specific activity of lipoxygenase in the mitochondrial fraction of ethylene-treated tubers was about double that observed in the corresponding soluble fraction, any 'contamination' would have to be specific (at least in part) for lipoxygenase. Regardless of whether the increased specific activity of mitochondrially associated lipoxygenase is the result of contam-

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Table II. Kinetic Parameters of State 3 Respiration (Succinate as Substrate) of a Washed Mitochondrial Fraction from Ethylene-Treated Potatoes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$V_{act}$</th>
<th>$V_{ext}$</th>
<th>$\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>28.1</td>
<td>39.5</td>
<td>0.9</td>
</tr>
<tr>
<td>SHAM</td>
<td>25.7</td>
<td>38.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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FIG. 2. Yonetani-Theorell plot of the state 3 rate of $O_2$ consumption by a washed mitochondrial fraction isolated from ethylene-treated tubers. Respiration was measured in the presence of 400 $\mu$M KCN and the concentration of SHAM and PG noted in the figure. Succinate at 10 mM was used as substrate.

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The results of a multiple inhibition experiment using both PG and SHAM on mitochondria preparations are presented as a Yonetani-Theorell plot in Figure 2. Most (94%) of the observed variation of the data can be explained by regressing $1/\alpha$ against the first two variables in equation 1 ($I_1$ and $I_2$) and excluding the last term ($I_3$). No further reduction in deviation sums of squares occurred when the third term was included in the regression analysis. The third term will become insignificant when $\alpha \to \infty$, i.e. $1/\alpha \to 0$. The data are sufficiently accounted for by the equation:

$$1/\alpha = 0.055 + 0.434 I_1 + 246 I_2$$
The difference between 'control' and 'ethylene-treated' results in Table IV must be the result of an increased mitochondrial affinity specific for lipoxygenase. While this observed shift in lipoxygenase affinity may reflect increased membrane lipid metabolism, which may be associated with the development/maintenance of CN resistance (14, 15), this is at present an area of speculation.

Figure 4 demonstrates that PG was capable of preventing the aging-induced development of the alternative pathway. The apparent $K_I$ for this inhibition was 0.08 mM, which is similar to the $K_I$ of the low-affinity site for slice $O_2$ consumption of 0.13 mM (Fig. 3). Although this similarity may be fortuitous, it is alternatively explainable if the action of PG in preventing cyanide-resistant respiratory development reflects the general antioxidant properties of PG rather than its inhibition of the alternative oxidase. The presence of 1 mM SHAM in the aging solution had no effect on the development of the alternative pathway, demonstrating that inhibition of the alternative oxidase cannot effect development of the alternative oxidase. Since PG inhibits alternative pathway activity, it is necessary that slices aged in PG be thoroughly washed prior to respiratory determinations. By treating slices known to be cyanide resistant (i.e. ethylene-treated) with PG and then washing the slices as described, virtually all of the inhibition of the alternative pathway caused by PG was removed. It was also noted that aging slices in the presence of 1 mM PG...
resulted in a 60% inhibition of the wound-induced respiration as well as preventing alternative pathway development. The relationship between this wound-induced respiration and the alternative pathway is unclear, especially since the alternative pathway is inoperative in the absence of applied cyanide. These results do, however, support the suggestion made earlier that PG may be acting on different sites in slices and isolated mitochondria.

It has been reported by Goldstein et al. (6) that lipoxygenase activity (linoleic acid as substrate) is associated with a washed mitochondrial fraction from wheat, which also apparently contains an operational alternative pathway. Of the two mitochondrial fractions separable by Percoll density gradient centrifugation, one fraction contained a linoleic acid oxidizing component while the other did not. Also, the apparent loss of cyanide resistance in response to applied Krebs cycle intermediates accompanied this purification step and only returned in the fraction with the linoleic acid oxidizing ability following the addition of linoleic acid. The authors correctly point out the inherent difficulties this presents. A controversy arises concerning present, past, and future use of a nonpurified mitochondrial fraction for alternative pathway studies. Also, the possibility presented by Goldstein et al. (6), that in a washed mitochondrial fraction lipoxygenase activity may be contributing to O\textsubscript{2} consumption in the absence of added fatty acid, is intriguing. However, in potato we feel that the difference in the K\textsubscript{f} of PG for lipoxygenase and mitochondrial alternative oxidase is indicative of the fact that the same enzyme is not responsible for both types of O\textsubscript{2} uptake. Also, along these same lines, Dizen-gremel and Lance (4) reported that a sucrose density gradient purification of mitochondria from aged potato slices did not eliminate the alternative pathway activity as was the case with the wheat mitochondria.

**CONCLUSIONS**

The existence of at least three major sources of O\textsubscript{2} consumption by potato slices, Cyt oxidase, the alternative oxidase, and lipoxygenase, has given rise to apparently confusing results in the literature. This paper demonstrates a kinetic procedure by which the relative anaplerotic contributions of these enzymes can be discerned. Using multiple inhibitor experiments, low concentrations of PG had no apparent influence on the K\textsubscript{f} for SHAM and, thus, was apparently acting at a site distinct from that influenced by SHAM in freshly cut slices from ethylene-treated tubers. However, a nonlinear Dixon plot of PG inhibition was derived at a wider range of PG concentrations, suggesting that PG is capable of inhibiting O\textsubscript{2} consumption at two or more sites or via two or more mechanisms in slices. On the other hand, PG and SHAM appeared to act at or near identical sites in isolated mitochondria, although mitochondria appear to have a much higher affinity for PG than for SHAM.

Ethylene treatment of tubers resulted in the induction of CN-insensitivity of slice respiration, and also increased both the total and specific activity of lipoxygenase associated with mitochondria. The presence of PG during slice aging apparently prevented the aging-dependent induction of CN-insensitivity, with about the same K\textsubscript{f} as for one component of slice O\textsubscript{2} consumption.

**LITERATURE CITED**

15. WARING AJ, GG LATIES 1977 Inhibition of the development of induced respiration and cyanide-insensitive respiration in potato tuber slices by cerulenin and dimethylaminoethanol. Plant Physiol 60: 11–16