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Alternative Respiratory Pathway

ITS ROLE IN SEED RESPIRATION AND ITS INHIBITION BY PROPYL GALLATE[1]

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ABSTRACT

Oxygen uptake during the first hours of imbibition in intact soybean and mung bean seeds showed a marked sensitivity to potassium cyanide but was unaffected by addition of either salicylhydroxamic acid or propyl gallate. However O_2 uptake by finely ground seed particles was very sensitive to the addition of either compound. The results indicated that O_2 uptake in intact, imbibing seeds was associated with a cyanide-sensitive process, most probably mitochondrial mediated respiration, and not the result of the cyanide-insensitive lipoxygenase activity which was readily detectable in ground seed particles.

The antioxidant propyl gallate was found to inhibit specifically alternative pathway electron transfer in isolated mung bean mitochondria. Half-maximal inhibition occurred with 2 to 5 micromolar propyl gallate. Kinetic analysis indicated that propyl gallate inhibition of the alternative pathway occurred at, or very near, the site of inhibition of the alternative pathway by salicylhydroxamic acid.

A high level of lipoxygenase activity was found to be associated with washed mitochondria isolated from a variety of etiolated plant tissues. Most of this lipoxygenase activity could be eliminated from mung bean mitochondria if the mitochondria were purified on a discontinuous sucrose gradient. This indicated that the mitochondrial-associated activity was probably the result of nonspecific adsorption of lipoxygenase onto the mitochondrial membranes during isolation.

Studies of respiration in imbibing seeds, although few in number, have nonetheless given a variety of results with regard to the nature of the respiratory pathways operating during imbibition and the earliest stages of germination. Wilson and Bonner (27) observed that mitochondria isolated from peanut embryos were essentially deficient in Cyt *c* for up to 16 h following imbibition. However even in the apparent absence of Cyt *c*, O_2 uptake in these mitochondria was greater than 70% inhibited by 0.1 mM KCN suggesting that most of the respiratory flux was via the main, Cyt pathway. Yentur and Leopold (28) however reported that O_2 uptake by imbibing soybean seeds was predominantly cyanide-insensitive during the first 4 h of imbibition and shifted to a fully cyanide-sensitive process between 4 and 8 h following the beginning of imbibition. O_2 uptake during the first 4 h was found to be sensitive to SHAM[2] suggesting that it was associated with mitochondrial electron transfer through the "alternative" respiratory pathway (21, 8). Burguillo and Nicolas (5) found a different pattern in germinating chick pea where the respiration was observed to be predominantly cyanide-sensitive for the first 12 h of germination with a cyanide-insensitive pathway appearing afterwards; reaching a maximal level between 72 and 96 h of germination.

In further studies of O_2 uptake by imbibing soybean, Parrish and Leopold (16) observed that ground soybean particles showed a burst of cyanide-insensitive O_2 uptake immediately following imbibition. They later showed that this O_2 uptake was also sensitive to SHAM but was not due to mitochondrial stimulated electron transfer (17). Instead, they attributed this SHAM-sensitive, cyanide-insensitive O_2 uptake to activity of the enzyme lipoxygenase. This was supported by the observations that addition of linoleic acid markedly stimulated O_2 uptake by the soybean particle suspension and O_2 uptake by imbibing seed particles was itself inhibited by propyl gallate, a known inhibitor of lipoxygenase (26). These results led Parrish and Leopold (16, 17) to conclude that in whole tissue, SHAM sensitivity alone was not a sufficient criterion to classify a given O_2 uptake as being due to the mitochondrial alternative pathway. They also suggested that the two inhibitors, SHAM and propyl gallate, could be used to distinguish between alternative pathway and lipoxygenase mediated O_2 uptake.

Here, we have examined O_2 uptake during the earliest stages of germination (1–5 h imbibition) in intact soybean and mung bean seeds to determine if sensitivities to inhibitors of O_2 uptake in intact seeds mirror those found using ground seed particles. In addition, we have characterized the effects of propyl gallate on the cyanide-sensitive and alternative electron transfer pathways in isolated mung bean mitochondria. Finally, we have observed a marked lipoxygenase activity in preparations of washed mitochondria. However this lipoxygenase activity was found not to be specifically located in the mitochondria.

MATERIALS AND METHODS

Plant Material and Mitochondrial Isolation. Ransom soybeans (*Glycine max* [L.] Merr.) and mung beans (*Phaseolus aureus* Roxb.) were both obtained from local commercial sources. Washed mitochondria were isolated from 3- to 5-day-old etiolated mung bean hypocotyls according to the procedures of either Bonner (3) or Moreland and Boots (14). Purification of the above mitochondrial preparations was accomplished by centrifugation in a discontinuous sucrose density gradient as described by Douce *et al.* (8).

Seed Imbibition. Intact seeds (10 g) were surface-sterilized by soaking for 10 min in 0.5% NaOCl, washed extensively with distilled H_2O, and transferred to a respirometer chamber (see below) for O_2 uptake measurements. When seeds were imbibed for 1 or more h prior to carrying out O_2 uptake measurements, the seeds were placed in a 250-ml Erlenmeyer flask containing 150 ml distilled H_2O. Air was continuously bubbled through this solution to prevent its going anaerobic during the course of imbibition. Mung bean and soybean particles were obtained from seed tissue which had been ground in a hammer mill and sieved to a size of 2 mm or less.

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[2] Abbreviations: SHAM: salicylhydroxamic acid; 1799: bis(hexafluoroacetonyl) acetone.

O_2 Uptake Measurements. Mitochondrial electron transfer was measured polarographically using a Clark-type O_2 electrode (Yellow Springs Instruments) fitted to a 2-ml glass cell (Gilson Medical Electronics) thermostatted at 25 C. The reaction medium contained 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM K-phosphate buffer (pH 7.2), and approximately 0.4 mg/ml mitochondrial protein. O_2 uptake by intact seeds and ground seed particles was also measured polarographically in a reaction chamber fashioned from a 100-ml round bottom flask as described by Laties (12). Total chamber volume was 130 ml and all reactions were carried out in distilled H_2O at 25 C.

Lipoxygenase Measurements. Lipoxygenase activity in the mitochondrial preparations was assayed polarographically in the standard mitochondrial reaction buffer (pH 7.2). Linoleic acid was solubilized in Tween 80 according to Surrey (25). The reaction was initiated by addition of linoleic acid (3.6 mM final concentration) to a 2-ml reaction cell containing the appropriate mitochondrial fraction (0.1–0.2 mg protein/ml).

Reagents and Solutions. Protein was measured according to the method of Lowry *et al.* (13). Propyl gallate, SHAM and linoleic acid (grade II) were obtained from Sigma. Stock solutions of 0.1 M propyl gallate and 0.2 M SHAM in absolute ethanol were made fresh daily. 1799[2] was a gift of Dr. P. G. Heytler (Du Pont de Nemours, Wilmington, Del.). All other reagents were of the highest purity commercially available.

RESULTS

Oxygen Uptake by Intact Seeds. Figure 1 shows the time course of the onset of O_2 uptake observed during the initial stages of imbibition by intact mung bean seeds. The previously dry seeds had been imbibing for approximately 10 min as a result of the surface sterilization treatment prior to addition to the O_2 electrode chamber. A lag of 20–30 min was observed before any appreciable O_2 uptake was measured. Following this lag, the rate of O_2 uptake began to increase in an autocatalytic fashion, reaching a rate of about 50 nmol O_2 consumed/g fr wt·min after 5 h of imbibition. Over the next 19 h, this rate increased another 2-fold.

The extent of the observed lag was shortened by 5–10 min if distilled H_2O was vacuum infiltrated into the seeds prior to placing them in the electrode chamber. Removal of the seed coat after the surface sterilization treatment reduced the observed lag to less than 5 min and resulted in significantly higher rates of O_2 uptake by the imbibing seeds. However this O_2 uptake was mostly cyanide-insensitive indicating that mechanical removal of the seed coat had probably damaged the cotyledon tissue, releasing an otherwise latent lipoxygenase activity. Identical results were obtained if soybeans replaced mung beans in these experiments.

Table I shows the effects of several inhibitors on the rates of O_2 uptake observed in both ground soybean seed particles and intact, imbibing seeds. Five-h imbibition was chosen for the intact seeds because it represented a time sufficient to give both reasonably high and relatively stable rates of O_2 uptake. The results obtained with soybean seed particles were similar to those reported by Parrish and Leopold (17). A high rate of O_2 uptake was observed which was insensitive to KCN (7% inhibition) but was inhibited by either SHAM (26%) or propyl gallate (63%). In intact seeds, the rate of O_2 uptake was less than 20% of that observed with the seed particles and 0.25 mM KCN completely inhibited this rate. Maximal inhibition did not appear immediately after addition of KCN but appeared gradually over the course of a 25- to 30-min period. Alternatively, neither 5 mM SHAM nor 0.5 mM propyl gallate had any appreciable effect (<10% inhibition) upon the rate of O_2 uptake by intact seeds even when present during the entire 5-h imbibition period.

Table II presents the results obtained using mung bean seeds. The rate of O_2 uptake by mung bean seed particles was much lower than that observed with soybean particles, but still was significantly higher than the rate found with intact seeds. The one major difference between the mung bean and soybean results was the finding that cyanide, as well as SHAM and propyl gallate, inhibited O_2 uptake by mung bean seed particles. Again however, with intact seeds, the O_2 uptake rate was markedly cyanide-sensitive and insensitive to the addition of either SHAM or propyl gallate.

Effect of Propyl Gallate on Electron Transfer in Isolated Mung Bean Mitochondria. Figure 2 shows the effect of the addition of propyl gallate on succinate-mediated electron transfer through the cyanide-sensitive and alternative pathways in isolated mung bean mitochondria. In the presence of 0.25 mM KCN, electron flow was exclusively via the alternative pathway and was found to be markedly inhibited by increasing concentrations of propyl gallate. Half-maximal inhibition appeared with 3–4 μM propyl gallate and complete inhibition was obtained with 50 μM propyl gallate. The cyanide-sensitive pathway, obtained in the presence of 1.0 mM SHAM, was unaffected by added propyl gallate at concentrations up to 200 μM. Figure 3 shows that identical results were obtained if malate replaced succinate as the electron donor.

Inhibition of the alternative pathway by propyl gallate was independent of mitochondrial protein concentration and could be completely reversed by washing propyl gallate-treated mitochondria with propyl gallate-free reaction medium. In addition, propyl gallate did not interfere with the mitochondrial energy conserving apparatus as evidenced by the constancy of both the respiratory control and P/O ratios in the presence and absence of 50 μM propyl gallate.

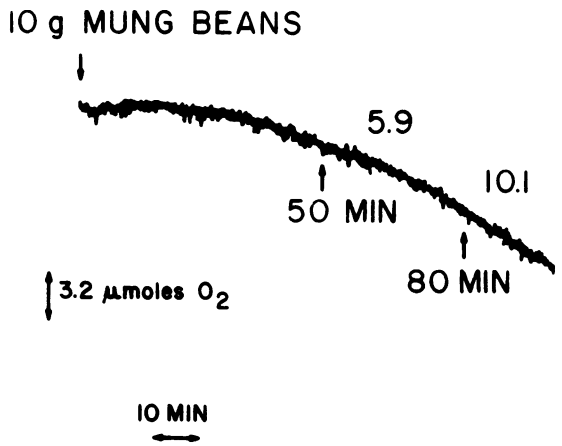

FIG. 1. O_2 uptake during the initial stages of imbibition by intact mung bean seeds. Seeds (10 g fresh weight) were treated as described. Rates, given above the electrode trace, are expressed as nmol O_2 consumed/g fresh weight·min.

Table I. *Inhibitor Sensitivities of O_2 Consumption in Imbibing Soybean Seeds and Seed Particles*

Seed particles were prepared and rates determined immediately after addition to the O_2 electrode chamber which contained 130 ml distilled H_2O and the appropriate inhibitor. Intact seeds were imbibed for 5 h in distilled H_2O prior to measuring O_2 consumption.

Inhibitor	Concentration	O_2 Consumption	
		Seed Particles	Imbibing Seeds
	mM	nmol O_2/g fresh wt·min	
Control		263	50
KCN	0.25	244	0
SHAM	5.0	195	46
Propyl gallate	0.5	97	47

Table II. *Inhibitor Sensitivities of O_2 Consumption in Imbibing Mung Bean Seeds and Seed Particles*

Conditions were as described in Table I.

Inhibitor	Concentration	O_2 Consumption	
		Seed Particles	Imbibing Seeds
	mM	nmol O_2/g fresh wt·min	
Control		78	46
KCN	0.25	17	15
SHAM	5.0	31	45
Propyl gallate	0.5	54	55

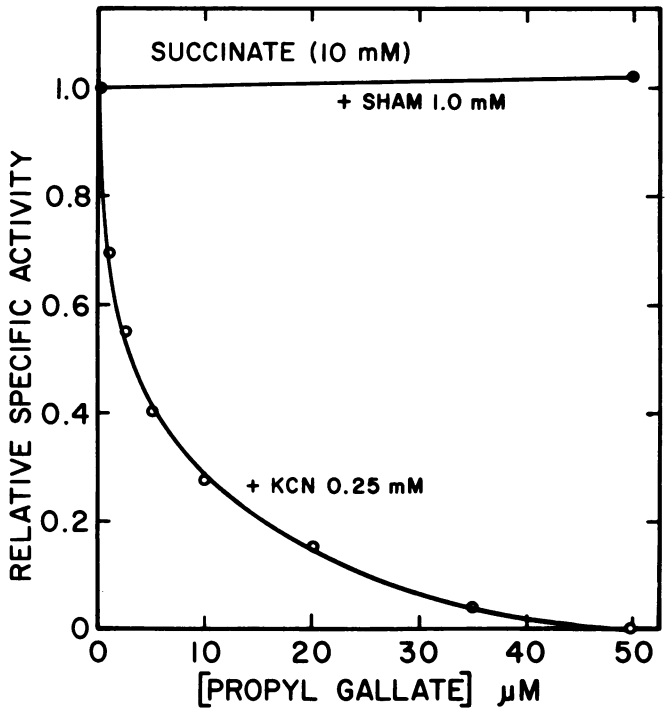

FIG. 2. Effect of propyl gallate on succinate stimulated electron transfer in isolated mung bean mitochondria. Mitochondria were incubated with 0.15 mM ATP, 2.5 μM 1799, and the appropriate inhibitor prior to initiating the reaction with 10 mM succinate. Propyl gallate (PG) was added, as indicated, after obtaining an initial steady-state rate. The uninhibited specific activities were: alternative pathway (+ 0.25 mM KCN), 28 nmol O_2 consumed/mg protein·min; main pathway (+ 1.0 mM SHAM), 193 nmol O_2 consumed/mg protein·min.

Figure 4 represents a kinetic analysis of the inhibition of both succinate- and malate-mediated alternative pathway electron transfer using the method of Dixon (7). Both substrates gave linear plots indicating that propyl gallate inhibition of the alternative pathway could be explained in terms of a simple single site hyperbolic-type binding system. Both inhibitions were noncompetitive with respect to reducing substrate so that the x-intercept in Figure 4 can be taken as the value of the inhibition constant (K_i) for propyl gallate (7). This value varied between mitochondrial preparations, but always fell within the range of 2 to 5 μM. Because the malate and succinate values shown in Figure 4 were obtained using different mitochondrial preparations, the similarity of the two lines suggested that the site of inhibition of the alternative electron transfer pathway by propyl gallate was the same whether malate or succinate was used as the substrate.

In Figure 5, an attempt was made to compare the sites of inhibition of the alternative pathway by SHAM and propyl gallate. A Dixon plot varying SHAM concentration in the absence and presence of different, constant concentrations of propyl gallate resulted in a series of parallel lines (Fig. 5). Parallel lines in such a plot can only be obtained if the two inhibitors are acting in a mutually exclusive fashion to inhibit a given activity (22). Such results are generally taken to indicate that the two inhibitors are acting at the same site of action. As a control, similar studies were carried out using either SHAM or propyl gallate and the inhibitor disulfiram which has been reported to give nonparallel lines with SHAM in the corresponding Dixon plot (11). Nonparallel lines

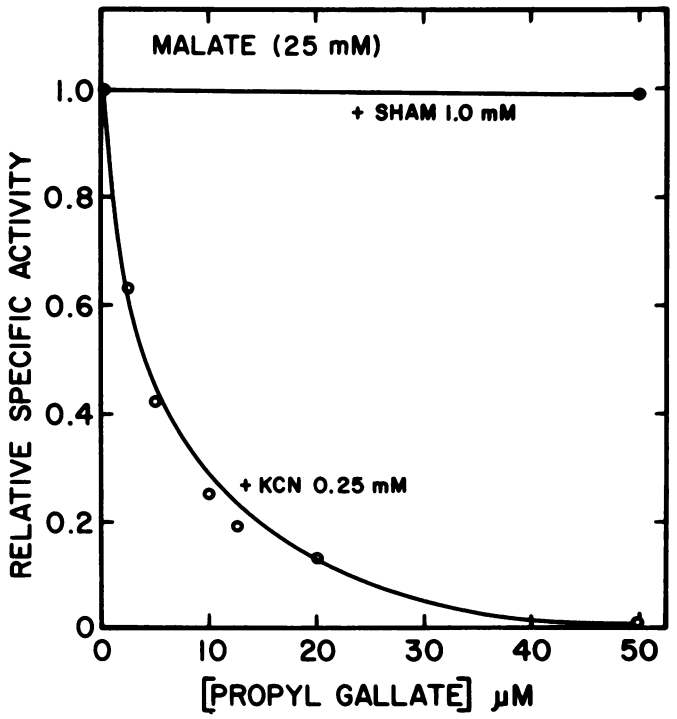

FIG. 3. Effect of propyl gallate on malate-stimulated electron transfer in isolated mung bean mitochondria. Conditions were as described in Figure 2 except that the reaction was initiated with 25 mM malate. The uninhibited specific activities were: alternative pathway, 35 nmol O_2 consumed/mg protein·min; main pathway, 185 nmol O_2 consumed/mg protein·min.

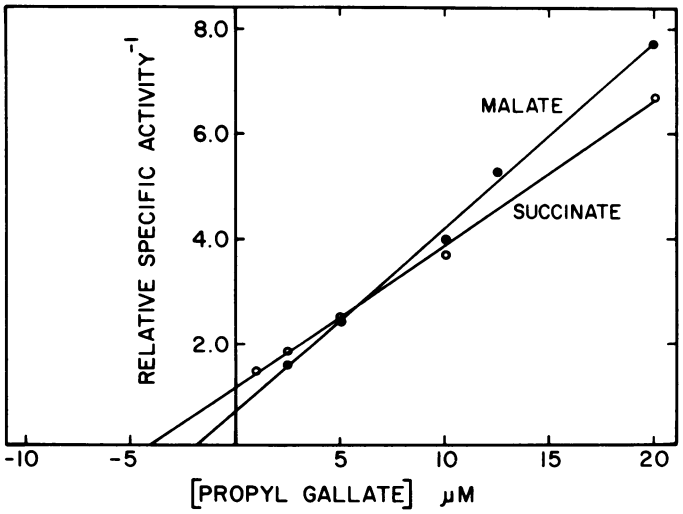

FIG. 4. Dixon plot of PG inhibition of alternative pathway electron transfer in isolated mung bean mitochondria. Conditions were as described for the alternative pathway in Figures 2 and 3.

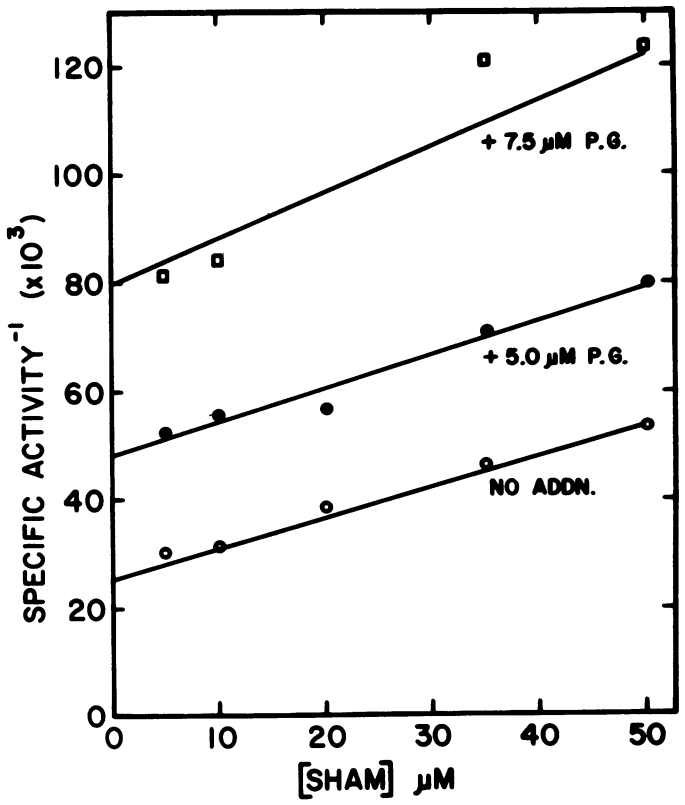

FIG. 5. Dixon plot of the inhibition of alternative pathway electron transfer by SHAM in the presence and absence of PG. Mung bean mitochondria were incubated with 0.15 mM ATP, 2.5 μM 1799, 0.25 mM KCN, SHAM (as indicated), and either 0 (○), 5.0 (●), or 7.5 (□) μM propyl gallate (PG). The reaction was initiated with 10 mM succinate.

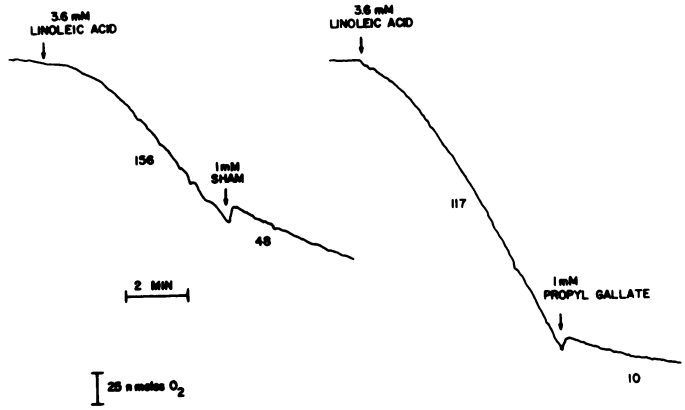

FIG. 6. Effect of linoleic acid on O_2 consumption by isolated mung bean mitochondria. The reactions were performed as described. Rates are expressed as nmol O_2 consumed/mg protein·min.

were obtained in both cases indicating that SHAM (or propyl gallate) and disulfiram act at separate sites.

Lipoxygenase in Mitochondrial Preparations. Figure 6 shows the effect of the addition of 3.6 mM linoleic acid to a preparation of washed mung bean mitochondria. A marked O_2 uptake was observed which approached a linear rate only after an initial lag phase. This O_2 uptake was inhibited by either 1.0 mM SHAM or 1.0 mM propyl gallate. The above results suggested the presence of the enzyme lipoxygenase (17). Total activity, per mitochondrial preparation, varied between 1 and 5 units (μmol O_2 taken up/min) of lipoxygenase. In addition, mitochondria prepared by the methods of either Bonner (3) or Moreland and Boots (14) both showed comparable levels of lipoxygenase activity. High levels of lipoxygenase activity were also observed in washed mitochondria prepared from either fresh or "aged" potato tubers, soybean hypocotyls, and, to a much lesser extent, Jerusalem artichoke tubers. The lipoxygenase had a sharp pH optimum centered around pH 7.0 making it most similar to the lipoxygenase-2 isozyme of soybean (6).

Of more importance however was the question of whether this lipoxygenase activity was truly mitochondrial or simply an artifact of the mitochondrial preparation procedure. Table III shows the distribution of lipoxygenase activity during the course of isolation of mung bean mitochondria. The amount of lipoxygenase which finally appeared in the washed mitochondrial fraction represented less than 1% of the total. Total lipoxygenase activity was taken to be the sum of those activities found in the first supernatant and the unwashed mitochondria (Table III). Further washing of the mitochondrial fraction released little additional lipoxygenase activity. No differences were found between the properties of the lipoxygenase in the first supernatant and that associated with the final (washed) mitochondrial fraction.

The washed mitochondria were further purified on a discontinuous sucrose gradient according to the procedure described by Douce *et al.* (8). As shown in Figure 7, 85% of the mitochondrial activity (Cyt *c* oxidase) appeared in band 4 at the interface of the 1.2-M and 1.45-M sucrose layers. Lipoxygenase activity was spread throughout the entire gradient with peaks of activity appearing at each interface between successive sucrose concentrations. As a result, only 15% of the total lipoxygenase activity in the sucrose gradient was localized in band 4, the mitochondrial fraction. If the three fractions making up band 4 were pooled, diluted to obtain a sucrose concentration of 0.3 M and centrifuged, 50% of the remaining lipoxygenase activity appeared in the supernatant fraction and not in the mitochondrial pellet. The resulting mitochondria were >90% intact as measured by the succinate Cyt *c* reductase assay (8). It was therefore possible to remove up to 95% of the total lipoxygenase originally associated with the washed mitochondria preparation without appreciably disrupting the integrity of the mitochondrial membranes. This indicated that the lipoxygenase in the initial mitochondrial preparation was not itself located within the membrane system of the mitochondrion.

DISCUSSION

The results of Parrish and Leopold (16, 17) showed there was a burst of cyanide-insensitive O_2 uptake in imbibing soybean seed particles. This activity was sensitive to SHAM, a classic inhibitor of the alternative respiratory pathway (21). As demonstrated by Parrish and Leopold, the O_2 uptake in imbibing seed particles was mostly, if not entirely, due to the action of the enzyme lipoxygenase which was itself SHAM-sensitive (17). They went on to suggest that propyl gallate, a known lipoxygenase inhibitor (26), could be

Table III. *Distribution of Lipoxygenase Activity During the Isolation of Washed Mung Bean Mitochondria*

Mitochondria were isolated according to the procedure of Moreland and Boots (14) and lipoxygenase was assayed. Total lipoxygenase activity was taken as the sum of the activities in the first supernatant and the unwashed mitochondria fractions.

Fraction	Lipoxygenase Activity	
	Total Activity	Total
	units	%
Crude homogenate	473	91
First supernatant	492	94
Unwashed mitochondria	29	5.6
Second supernatant	20	3.8
Washed mitochondria	3.4	0.6

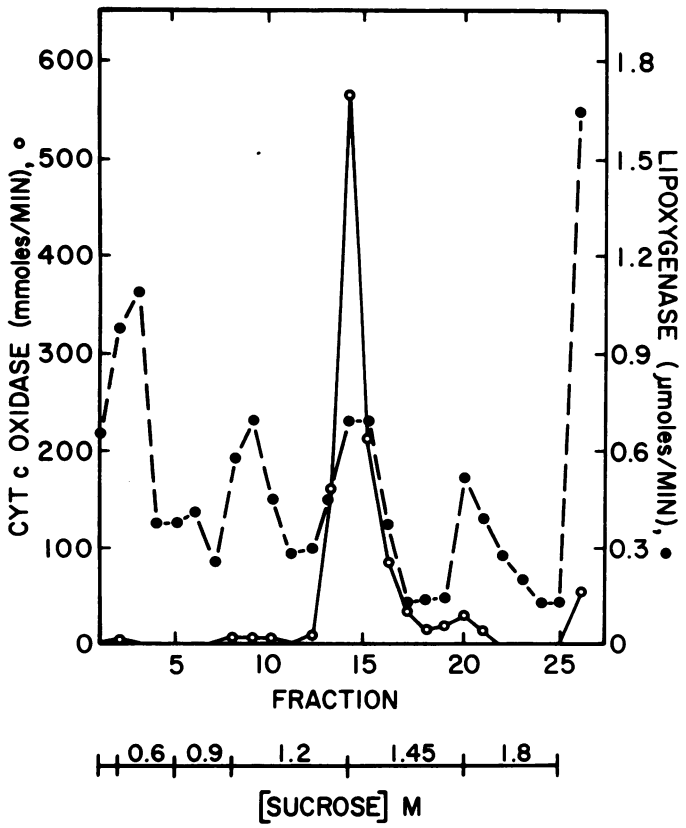

FIG. 7. Distribution of Cyt *c* oxidase and lipoxygenase activities in fractions from washed mung bean mitochondria separated on a discontinuous sucrose density gradient. The gradient, prepared and run according to Douce *et al.* (8), was fractionated and each fraction was assayed for lipoxygenase and Cyt *c* oxidase activities.

used to distinguish SHAM-sensitive lipoxygenase activity from SHAM-sensitive alternative oxidase activity. The present report grew out of our interest in two questions which we felt were left unanswered by Parrish and Leopold. What role does lipoxygenase play during the early stages of imbibition in intact seeds, and what is the effect of propyl gallate on the alternative pathway in isolated mitochondria?

As demonstrated in Tables I and II for both soybean and mung bean seeds, respectively, the inhibitor sensitivities associated with O_2 uptake in intact, imbibing seeds differed markedly from those seen with ground seed particles. O_2 uptake in intact seeds was very sensitive to KCN but virtually unaffected by either SHAM or propyl gallate. As noted earlier, no effects of SHAM or propyl gallate were observed even when these compounds were present during the entire 5-h imbibition period. These latter results ameliorate, but do not entirely rule out, problems that might arise due to the reduced ability of SHAM or propyl gallate to reach their respective sites of action in the intact seed.

Although many cyanide-sensitive oxidase reactions are known, it is not unreasonable to speculate that the cyanide-sensitive O_2 uptake which appears during the first 5 h of imbibition in intact seeds is due to the gradual "hydration activation" of mitochondria in the imbibing seed tissue. Grinding the seeds up prior to imbibition on the other hand releases both lipoxygenase and suitable unsaturated lipoxygenase substrates giving rise to a large O_2 uptake activity unrelated to that which appears in intact seeds. Cyanide sensitivity associated with initial rates of O_2 uptake in imbibing seeds was also observed by Burguillo and Nicolas (5) in chick-pea, Wilson and Bonner (27) in isolated peanut embryos and even Parrish and Leopold (16) when they used intact soybean cotyledons. We cannot explain why our results with soybean differ from those of Yentur and Leopold (28) who observed considerable inhibition of O_2 uptake by both SHAM and KCN in soybean cotyledons after 5 h imbibition. They also reported a significant drop in the rate of O_2 uptake between 3 and 9 h imbibition which we never observed. In our hands, any attempt to physically remove the cotyledons from the seed coat led to the appearance of some lipoxygenase activity and this could well account for the differences.

Figures 2 and 3 demonstrate that in isolated mung bean mitochondria, propyl gallate acted as a specific, reversible inhibitor of alternative pathway electron transfer. Similar results were obtained using mitochondria isolated from aged potato tubers. The concentration of propyl gallate required to obtain half-maximal inhibition of the alternative pathway ranged between 2 and 5 μM which is 50- to 10-fold lower than the concentration of hydroxamic acids required to inhibit this pathway (half-maximal) in mung bean mitochondria. The above conclusions, reached using isolated mitochondria, have been confirmed for intact potato tuber tissue where propyl gallate has been shown to specifically inhibit, at very low concentrations, O_2 uptake associated with the alternative respiratory pathway (Harry W. Janes, personal communication). Clearly, propyl gallate cannot be used to distinguish between lipoxygenase-mediated and alternative pathway-mediated O_2 uptake as was suggested by Parrish and Leopold (17). SHAM and propyl gallate seemed to act at the same site of action to inhibit the alternative pathway (Fig. 5). Given the significantly lower concentrations required to inhibit alternative pathway electron transfer, and the apparently common site of action, propyl gallate should serve as a useful alternative to SHAM in many cases, inasmuch as complete inhibition of the alternative pathway can be accomplished with 50–100 μM propyl gallate instead of 0.5–1.0 mM SHAM.

The appearance of the substituted hydroxamic acids as specific inhibitors of the alternative pathway in plant mitochondria (21) has been a major boost to the study of this enigmatic oxidase, but has also led to some misconceptions concerning the exact nature of the alternative oxidase (or at least the site of action of hydroxamic acids). More specifically, the well characterized ability of hydroxamic acids to chelate ferric ions (1) has led some workers to invoke the involvement of an iron-sulfur protein on the alternative pathway (23). This concept is now generally felt not to be correct (4, 15). Rich *et al.* (20) recently reported that several soluble redox enzymes were inhibited by substituted hydroxamic acids. In the case of the two enzymes most extensively studied, tyrosinase and horseradish peroxidase, it was observed that the inhibition by hydroxamic acids was competitive with respect to the enzyme's phenolic substrate. By extrapolating the above results to considerations of the alternative oxidase, Rich *et al.* (20) suggested that inhibition of the alternative pathway by hydroxamic acids might be due to competition with the reducing substrate of the alternative oxidase, the most reasonable candidate being some reduced species of ubiquinone.

Comparison of the chemical structures of SHAM and propyl gallate (Fig. 8) appears to support the argument presented above. The hydroxamic acid moiety is not itself essential for specific inhibition of the alternative pathway, while the phenolic characteristics of propyl gallate are obvious. In addition, propyl gallate was found to serve as a substrate for both tyrosinase (mushroom) and horseradish peroxidase (unpublished results), a characteristic partially shared with SHAM. As a competitive inhibitor, SHAM is only able to bind at the active sites of these enzymes but not undergo subsequent reaction. We have seen nothing to indicate that propyl gallate can serve as an electron donor in isolated mitochondria.

Both SHAM and propyl gallate inhibit lipoxygenase (17) which has no phenolic substrate, however. The ability of propyl gallate to inhibit lipoxygenase has generally been associated with its

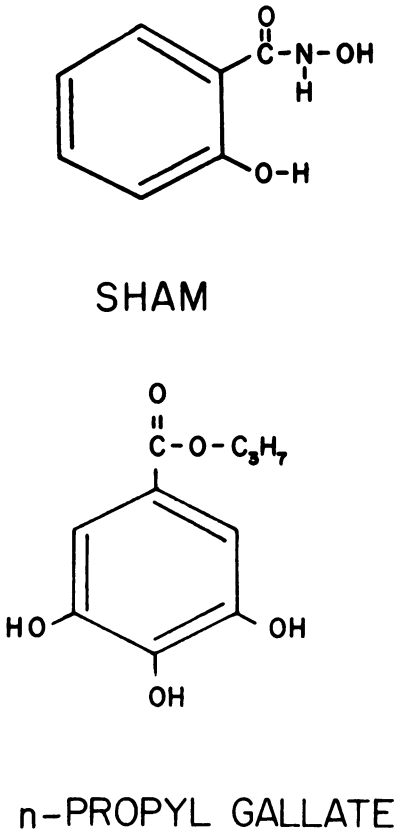

FIG. 8. Chemical structures of SHAM and *n*-propyl gallate.

ability as an antioxidant to remove free radicals formed during the course of the reaction (9). This aspect of its chemistry should be considered in hypothesizing its mechanism of inhibition of the alternative pathway. The radical form of ubiquinone, ubisemiquinone, has been postulated, in some schemes, to play a role at, or at least very near, the branch point of the alternative pathway (18, 19). Finally, in an effort not to confuse the issue, but merely to present all the facts, it should be noted that propyl gallate, like SHAM, has a marked ability to chelate ferric ions (24).

The appearance of high levels of lipoxygenase activity in the washed mung bean mitochondria preparation (Fig. 6) was initially somewhat surprising. The results shown in Table III and Figure 7 however indicate that this lipoxygenase activity is not mitochondrial. In sucrose gradient purified mitochondria less than 0.1% of the total lipoxygenase present in the initial crude homogenate appeared in the final mitochondrial fraction. The presence of lipoxygenase seems to be universal among etiolated plant tissues (2). Expectedly, we have observed lipoxygenase in washed mitochondria isolated from a wide variety of sources and Goldstein *et al.* (10) recently reported the presence of lipoxygenase in washed, but not purified, mitochondria isolated from wheat seedlings. The presence of this enzyme must therefore be taken into account in studies using simple washed plant mitochondria.

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