## Short Communication

# Confounding of Alternate Respiration by Lipoxygenase Activity[1]

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## ABSTRACT

The initial burst of respiratory activity ($Q_{O_2}$) of imbibing soybean (*Glycine max* [L.] Merr. var. Wayne) seed tissue is cyanide-insensitive, and sensitive to salicylhydroxamate: presumptive evidence for the presence of alternate respiration. The initial $O_2$ consumption is also highly sensitive to propyl gallate. Soybean lipoxygenase exhibits similar characteristics of insensitivity to cyanide and sensitivity to salicylhydroxamate and to propyl gallate. The initial burst of respiration is enhanced by the addition of linoleic acid, a lipoxygenase substrate. These results indicate that the conventional tests for alternate respiration in plant tissues can be confounded by lipoxygenase; they also suggest that propyl gallate can be used to assess the possible participation of lipoxygenase in the $O_2$ uptake by plant tissues.

Neither the biochemical nature nor the physiological significance of the cyanide-insensitive, alternate respiration is known, but its activity has now been demonstrated in a number of plant systems (2, 9). Alternate respiration is commonly defined or diagnosed as that portion of respiration which is insensitive to cyanide but inhibited by hydroxamic acids (2, 8). This activity is thought to be due to an electron flow which does not involve Cyt oxidase (2, 10). We present data which show that initial *in vivo* $O_2$ uptake by soybeans is not inhibited by KCN and is inhibited by hydroxamate; and that this apparent respiration may be due in part to the activity of lipoxygenase, an enzyme which catalyzes the dioxygenation of certain unsaturated fatty acids (5).

## MATERIALS AND METHODS

Respiratory activity was measured with a Clark-type $O_2$ electrode (Yellow Springs Instrument, Yellow Springs, Ohio) operated at 0.7 v in a 2 ml magnetically stirred, water-jacketed reaction vessel (Gilson Medical Electronics, Middletown, Wis.) at 25 C. Particles of soybean (*Glycine max* [L.] Merr. var. Wayne) seed tissue which had been ground in a Wiley mill and sieved for 0.5- to 1-mm sizes were added to the $O_2$ electrode reaction vessel, 0.1 g/test.

Crystallized soybean LOX[4] (lipoxidase) and LA (99%) were obtained from ICN Pharmaceuticals, Cleveland, Ohio. SHAM (99%) and PG were gifts of Aldrich Chemical, Milwaukee, Wis. and Eastman Chemical Products, Kingsport, Tenn., respectively. Tween 20 was combined 1:1 (w/w) with the LA to obtain a stable

emulsion in 10 mM phosphate buffer (pH 7.0) (11). A 1 mg/ml (3.56 mM) LA solution was used as substrate for the LOX assays. LOX was dissolved in 10 mM phosphate buffer (pH 7.0) and injected into the $O_2$ electrode reaction vessel to give a reaction mixture which had 100 $\mu$g of LOX/ml. Assuming a mol wt of about 100,000 (1), LOX concentration was about 1 $\mu$M.

## RESULTS AND DISCUSSION

The respiration of soybean particles was characterized by a burst of $O_2$ consumption during the first few min of imbibition (6). This respiratory burst was not inhibited by KCN and was inhibited by SHAM (Fig. 1). The initial respiratory activity was also markedly inhibited by PG (Fig. 2), being about 10 times more sensitive to PG than to SHAM. If the normally alkaline KCN solution was strongly buffered (100 mM) to maintain the pH at neutrality, the small promotion of respiration seen in Figure 1 was no longer observed; and there was still no evidence of inhibition in the first few min. SHAM and PG sensitivities were not affected by buffering at pH 7.0.

Suspecting that the SHAM inhibition might be related to LOX activity, we tested the cell-free enzyme's sensitivity to the hydroxamate and found (Fig. 3) that indeed it was, being 90% inhibited by 5 mM SHAM. Autooxidation of LA (in the absence of LOX) was unaffected by SHAM. The LOX activity was not inhibited by KCN (data not shown). The enzyme was about 10 times more sensitive to PG than to SHAM, being 90% inhibited at about 0.5 mM PG (data not shown).

To investigate further the possible role of LOX in the apparent initial respiration, soybean seed particles were placed in buffered solution with or without SHAM and $O_2$ uptake was measured with and without the addition of 50 $\mu$g/ml (0.18 mM) LA substrate (Fig. 4). In the absence of SHAM, when LA was added, there was an immediate 9- to 10-fold increase in respiratory rate and all of the $O_2$ remaining in the solution was quickly consumed. When 5 mM SHAM was present, the initial $O_2$ consumption was inhibited approximately 50%, and the addition of LA caused only a transient 2- to 3-fold increase in $O_2$ consumption. (Added Tween 20 was without effect on the respiration rates in the presence of absence of hydroxamate.) The maximal rate observed after addition of the LA was almost 90% less in the presence of the 5 mM SHAM.

The initial respiration ($O_2$ uptake) by ground soybean tissue is cyanide-insensitive and hydroxamate-inhibited: presumptive evidence for alternate respiration. However, $O_2$ consumption by cell-free soybean LOX responds in the same way to the two poisons, and both the tissue and purified LOX are sensitive to PG. These results show clearly that the criteria often used as evidence for alternate oxidase activity can be misleading in preparations which have LOX activity.

The insensitivity of LOX to cyanide and its potent, specific inhibition by hydroquinones (such as PG) are well documented (1, 3). We believe that this is a first report of the inhibition of LOX by SHAM. It has been suggested that the hydroxamic acids may inhibit some enzymes by inactivating a nonheme iron (8, 12) although this mechanism is now in dispute (7). LOX has been

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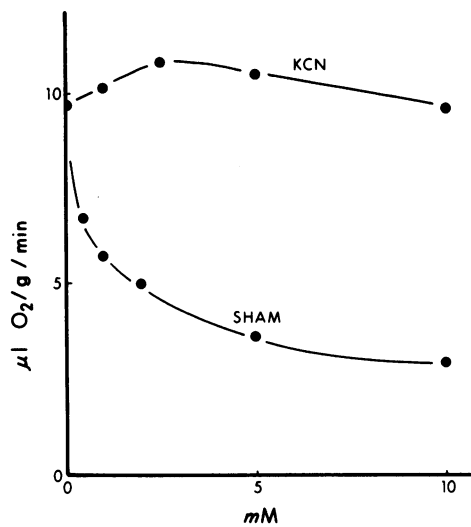[4] Abbreviations: LOX: lipoxygenase; LA: linoleic acid; SHAM: salicylhydroxamic acid; PG: propyl gallate.

FIG. 1. Effect of various concentrations of KCN and SHAM on the initial burst (2 min) of $O_2$ uptake by soybean seed tissue.
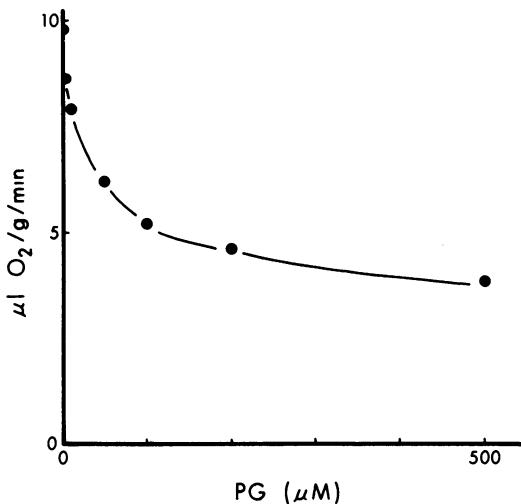


FIG. 2. Inhibition of initial burst of $O_2$ uptake by soybean seed tissue by various concentrations of propyl gallate.
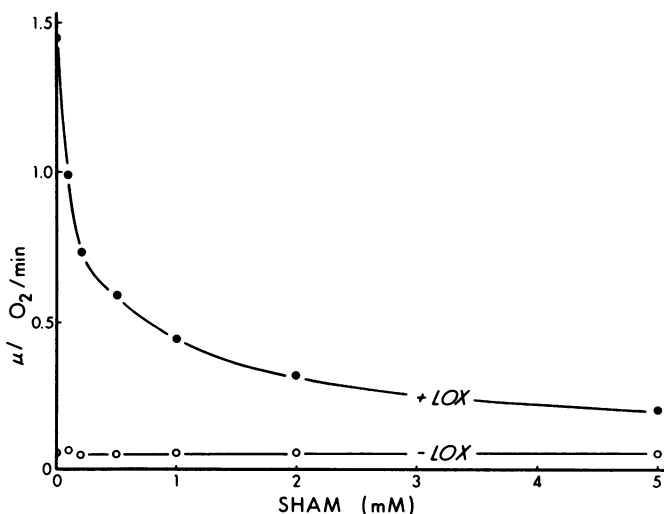


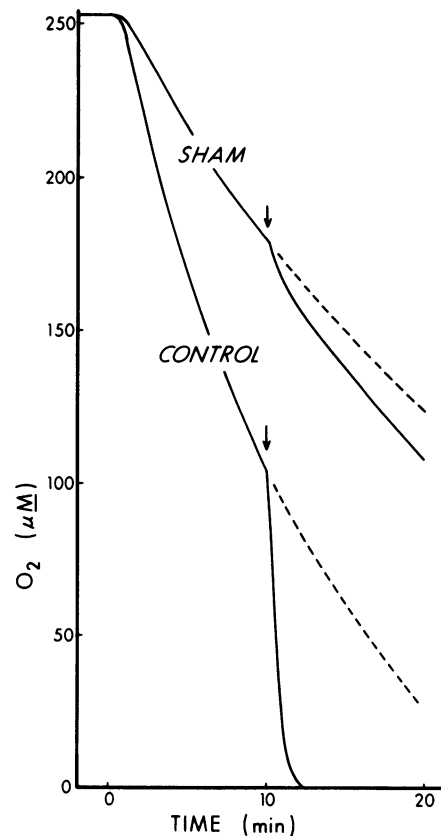FIG. 3. Effect of SHAM on oxidation of linoleic acid in the presence and absence of lipoxygenase.



FIG. 4. Promotion of $O_2$ uptake by soybean seed tissue with linoleic acid in presence or absence of 5 mM SHAM. Tissue was added at zero time. LA (50 μM final concentration) was added at arrows.

shown to possess 1 molecule of nonheme iron/molecule of enzyme (4). PG is thought to inhibit LOX by preventing or quenching free radical reactions (1). Because of its free radical scavenging ability, PG is also a good inhibitor of autooxidation. Significantly, 5 mM SHAM, which inhibits in vitro LOX activity by 90%, has no effect on autooxidation (Fig. 3).

The experiments reported here show that a part of the cyanide-insensitive $O_2$ consumption by imbibing soybean particles is due to LOX activity. We suggest, then, that the cyanide insensitivity in intact tissues is not itself convincing evidence for alternate respiration, and neither is the inhibition of $O_2$ consumption by hydroxamic acids such as SHAM (12–14). A distinction between alternate respiration and LOX utilization of $O_2$ can be made through the use of PG.

LITERATURE CITED

1. AXELROD B 1974 Lipoxygenases. In JR Whitaker, ed, Food Related Enzymes. American Chemical Society, Washington DC, pp 324–348
2. BAHR JT, WD BONNER 1973 Cyanide-insensitive respiration. I. The steady state of skunk cabbage spadix and bean hypocotyl mitochondria. J Biol Chem 248: 3441–3445
3. BONNER WD 1957 Soluble oxidases and their functions. Annu Rev Plant Physiol 8: 427–452
4. CHAN HWS 1973 Soybean lipoxygenase: an iron-containing dioxygenase. Biochim Biophys Acta 327: 32–35
5. CHRISTOPHER J, E PISTORIUS, B AXELROD 1970 Isolation of an isozyme of soybean lipoxygenase. Biochim Biophys Acta 198: 12–19
6. PARRISH DJ, AC LEOPOLD 1977 Transient changes during soybean imbibition. Plant Physiol 59: 1111–1115
7. RICH PR, WD BONNER 1977 EPR studies of higher plant mitochondria in relation to alternative respiratory oxidations. Plant Physiol 59: S-326
8. SCHONBAUM GR, WD BONNER, BT STOREY, JT BAHR 1971 Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Plant Physiol 47: 124–128
9. SOLOMOS T 1977 Cyanide resistant respiration in higher plants. Annu Rev Plant Physiol 28: 279–297

10. SOLOMOS T, GG LATIES 1975 The mechanism of ethylene and cyanide action in triggering the rise in respiration in potato tubers. Plant Physiol 55: 73–78

11. SURREY K 1964 Spectrophotometric method for determination of lipoxidase activity. Plant Physiol 39: 65–70

12. VAN DER PLAS LHW, GS SCHOENMAKER, SJ GERBANDY 1977 CN-resistant respiration in a Convolvulus arvense L. cell culture. Plant Sci Lett 8: 31–33

13. 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

14. YENTUR S, AC LEOPOLD 1976 The respiratory transition during seed germination. Plant Physiol 57: 274–276