Plant Physiol. (1992) 100, 1263–1268
0032-0889/92/100/1263/06/$01.00/0

Received for publication March 26, 1992
Accepted June 2, 1992

Oxygen Radical Generation by Isolated Microsomes from Soybean Seedlings[1]

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ABSTRACT

The generation of active oxygen species by microsomes isolated from soybean seedlings was studied. NADPH-dependent superoxide anion production was 5.0 ± 0.4 nmol·min^{-1} mg^{-1} of microsomal protein. Hydrogen peroxide generation by microsomes was 1.40 ± 0.05 nmol·min^{-1} mg^{-1} of protein. Hydroxyl radical production, in the presence of ferric EDTA, evaluated through the generation of formaldehyde from dimethyl sulfoxide or *tert*-butyl alcohol was 0.50 ± 0.04 and 0.44 ± 0.03 nmol·min^{-1} mg^{-1}, respectively. NADH proved to be suitable as cofactor for oxygen radical generation by microsomes from soybean seedlings. Because transition metals are implicated in radical generation by biological systems, the ability of microsomal membranes to reduce iron complexes was studied. Ferric ATP, ferric citrate, ferric ADP, ferric diethylenetriamine pentaacetic acid, and ferric EDTA were efficiently reduced in the presence of either NADPH or NADH as cofactor. The pattern of effectiveness of the different ferric complexes, on superoxide anion, hydrogen peroxide, and hydroxyl radical production, was similar to that found with animal microsomes. The data presented here indicate that microsomal ability to catalyze oxygen radical generation must be considered as an important contribution to cellular radical steady-state concentrations in cells from soybean seedlings.

Hydroperoxide metabolism in plants, particularly during seed germination, has received little attention. However, information about hydroperoxide production and utilization could be relevant to understanding important processes such as lipid peroxidation and redox changes. Previous work from our laboratory has indicated that superoxide anion and H_2O_2 are normal metabolites of plant cells (36, 37). Both oxidants are kept at low steady-state levels by the action of superoxide dismutase, catalase, and GSH and ascorbate peroxidases, presumably located at the cytosol of the soybean embryonic axes (10, 37). Mitochondrial H_2O_2 is generated through the primary production of O_2^- and its consequent dismutation (15). Mitochondria seem to be the most important cellular source of H_2O_2 (36), but other sources such as ER and peroxisomes should not be excluded.

In plants, Cyt P-450-dependent mono-oxygenases have been described in several species (2). They are involved in important metabolic pathways leading to lignins (19, 33), phytoalexins (24), pigments (20), steroids (32, 38), alkaloids (26), cutins (42), and hydroxy-fatty acids (3, 40). Plant Cyt P-450s are also implicated in the metabolism of exogenous compounds (14, 44, 45) and in the detoxification of herbicides (29). Most of these oxygenases occur in the microsomal fraction, and all are dependent on molecular O_2 and reducing equivalents provided by NADPH or NADH. Microsomal electron transport leads to the generation of active O_2 species, such as H_2O_2, O_2^-, and $^{\cdot}OH$, in animal tissues (34, 35). Reduction of O_2 to superoxide radical in plants has been demonstrated previously by microsomal membranes from carnation flowers, by a plasma membrane-bound NAD(P)H oxidase upon infection of potato tubers with incompatible races of *Phytophtora infestans* (27), and by radish plasmalemma vesicles (43).

In the present study, we provide evidence that microsomal membranes from soybean seedlings produce superoxide anion and H_2O_2 at detectable rates in the presence of either NADPH or NADH as cofactor. Because transition metals are absolutely required to catalyze the generation of active O_2 species, the effect of exogenous iron on the generation of O_2 radicals by microsomes isolated from soybean seedlings was evaluated.

MATERIALS AND METHODS

Plant Material

Soybean seeds (*Glycine max* var Hood) were grown for 7 d in the dark at 26°C over water-saturated filter paper. The final weight of soybean seedlings was 1.17 ± 0.07 g/seedling.

Microsomal Membrane Preparation

Hypocotyls were homogenized for 30 s in a blender with a buffer containing 0.1 M Tris-HCl (pH 7.0), 0.3 M mannitol, 5 mM EDTA, 0.1% BSA, 0.05% cysteine. The homogenate was filtered through several layers of gauze and then centrifuged at 10,000*g* for 30 min. The resulting supernatant was centrifuged at 100,000*g* for 60 min. The pellet (microsomal fraction) was resuspended in buffer containing 0.1 M Tris-HCl (pH 7.0), 20% (v/v) glycerol.

Cyt P-450 Content

Cyt P-450 was estimated according to the procedure of Omura and Sato (31).

[1] This study was supported by the International Foundation for Science (Sweden). S.P. is Career Investigator from Consejo Nacional de Investigaciones Científicas y Técnicas.

Enzyme Assays

NADH and NADPH Cyt *c* reductase activities were determined, recording changes in A_{550} (E = 21 mM^{-1} cm^{-1}). The reaction mixture contained 0.3 M potassium phosphate buffer (pH 7.7), 0.4 mM Cyt *c*, 100 μM NADPH or NADH, and 0.2 to 0.5 mg of microsomal protein. Superoxide dismutase activity was assayed spectrophotometrically at 550 nm by the method of McCord and Fridovich (28), in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 15 μM Cyt *c*, 50 μM xantine, and xantine-oxidase. The amount of homogenate inhibiting by 50% the reduction of Cyt *c* was taken as 1 unit of superoxide dismutase activity. Under the conditions of the assay, this amounts to 0.12 μg of superoxide dismutase/mL. Peroxidase activity was measured spectrophotometrically at 430 nm (E = 2.47 mM^{-1} cm^{-1}) in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0), 5 mM pyrogallol, and 1 mM H_2O_2 (41). Catalase was assayed spectrophotometrically at 240 nm (E = 40 M^{-1} cm^{-1}) in a reaction medium consisting of 50 mM potassium phosphate buffer (pH 7.0) (10).

O_2^- Generation

The rate of production of O_2^- was determined as the superoxide dismutase-sensitive rate of adrenochrome formation, measured at 485 to 575 nm (E = 2.97 mM^{-1} cm^{-1}) in a Perkin-Elmer dual-wavelength spectrophotometer. The reaction medium consisted of 40 mM potassium phosphate buffer (pH 7.4), 120 mM KCl, 1 mM EDTA, and 1 mM epinephrine (5).

NADH and NADPH Consumption

Cofactor consumption rates were determined spectrophotometrically at 340 nm (E = 6.2 mM^{-1} cm^{-1}). The reaction mixture contained 100 mM phosphate buffer (pH 7.4), 0.1 mM NAD(P)H, and about 1 mg of microsomal protein in a final volume of 3 mL.

H_2O_2 Generation

The production of H_2O_2 was determined by the generation of formaldehyde from the oxidation of methanol by the catalase-H_2O_2 compound I complex (22). Reactions were carried out in a buffer consisting of 40 mM Tris-HCl (pH 7.4), 120 mM KCl, 100 mM methanol, and about 1 mg of microsomal protein in a final volume of 1 mL, in the presence of either catalase or azide. Formaldehyde was determined fluorometrically (excitation = 415 nm, emission = 505 nm).

·OH Generation

The production of ·OH or compounds with the oxidizing power of ·OH was assayed by the generation of formaldehyde from DMSO or *tert*-butyl alcohol (9). The basic reaction system consisted of 40 mM potassium phosphate buffer (pH 7.4), 120 mM KCl, 0.4 mM NAD(P)H, either 30 mM DMSO or *tert*-butyl alcohol, and about 1 mg of microsomal protein in a final volume of 1 mL. The production of formaldehyde was determined by the Nash reaction (30).

Rate of Reduction of Iron

The reduction of iron was monitored in a reaction system containing 0.1 M phosphate buffer (pH 7.4), about 0.10 to 0.35 mg of microsomal protein, either 0.5 mM NADPH or 0.5 mM NADH, and 5 mM α,α'-bipyridyl in a final volume of 3 mL. The formation of the stable ferrous-(bipyridyl)$_3$ complex was measured at 520 nm (E = 14 μM^{-1} cm^{-1}) (7).

The ferric complexes were prepared by dissolving ferric ammonium sulfate in 0.1 N HCl and then diluting with the respective chelator to the appropriate stock concentration. Ferric ATP was utilized as a 1:20 complex, whereas the other ferric complexes were all utilized as 1:2 complexes.

RESULTS

Mixed-function oxidase activity in microsomes from hypocotyls of soybean seedlings was characterized. Carbon monoxide was passed through a dithionite-reduced sample of soybean seedlings microsomes, and the detected peak at 450 nm was ascribed to Cyt P-450 content (32 ± 3 pmol/mg of protein). When reduction was carried out with NADH under aerobic conditions, the difference spectrum of soybean microsomes was similar to that of animal tissues, exhibiting a maximum at 425 nm and representing Cyt b_5 content (40 ± 3 pmol/mg of protein). Table I shows the activities of NADPH- and NADH-Cyt *c* reductases. Higher activity of NADH-Cyt *c* reductase than NADPH-dependent reductase is consistent with a higher consumption of NADH by the microsomes as compared with NADPH (Table I).

Figure 1 shows O_2^- production by microsomal membranes from soybean seedlings in the presence of NADPH. The rate of O_2^- generation was calculated from the superoxide dismutase-sensitive rate of adrenochrome formation. A linear response of the rate of O_2^- generation to the microsomal protein addition, in the presence of NADPH, was observed (Fig. 1). Similar patterns were obtained in the presence of NADH. The detected rates of O_2^- production were 5.0 ± 0.4 and 5.5 ± 0.4 nmol·min^{-1} mg^{-1} of protein in the presence of NADPH and NADH, respectively.

The production of H_2O_2 was assayed by determining the peroxidatic activity of catalase toward methanol (30). The rates of H_2O_2 generation by microsomes were 1.40 ± 0.05 and 0.87 ± 0.05 nmol·min^{-1} mg^{-1} of protein in the presence of NADPH and NADH, respectively.

The production of formaldehyde from DMSO or *tert*-butyl alcohol is a rapid and sensitive technique that has been

Table I. *Mixed Function Oxidase Activities and Cofactor Consumption by Microsomes Isolated from Hypocotyls of Soybean Seedlings*

Values are means ± SE.

	Content or Activity
Cyt P-450	32 ± 3 pmol·mg^{-1} of protein
Cyt b_5	40 ± 3 pmol·mg^{-1} of protein
NADPH-Cyt c reductase	4.6 ± 0.5 nmol·$min^{-1}mg^{-1}$ of protein
NADH-Cyt c reductase	13.2 ± 1.1 nmol·$min^{-1}mg^{-1}$ of protein
NADPH consumption	4.1 ± 0.3 nmol·$min^{-1}mg^{-1}$ of protein
NADH consumption	7.4 ± 0.7 nmol·$min^{-1}mg^{-1}$ of protein

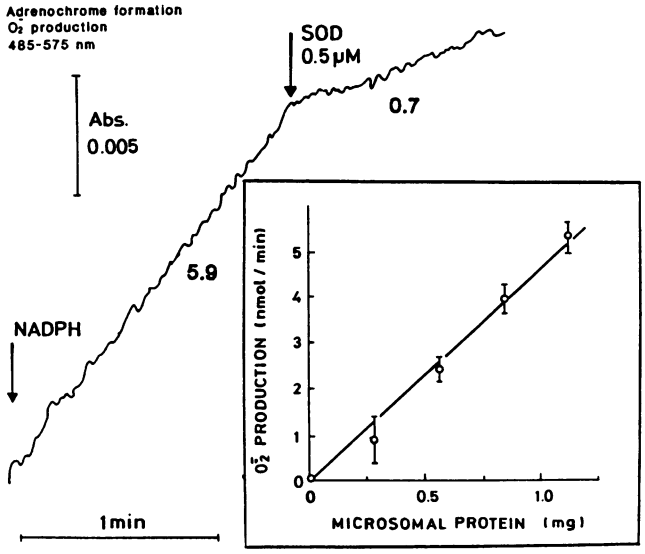

Figure 1. Rate of NADPH-dependent production of O_2^- by microsomes isolated from hypocotyls of soybean seedlings. The numbers near the traces indicate the rate of O_2^- production in nanomoles per minute per milligram of protein. Inset, Rate of NADPH-dependent generation of O_2^- as a function of the microsomal protein added. Experimental conditions are indicated in "Materials and Methods." SOD, Superoxide dismutase.

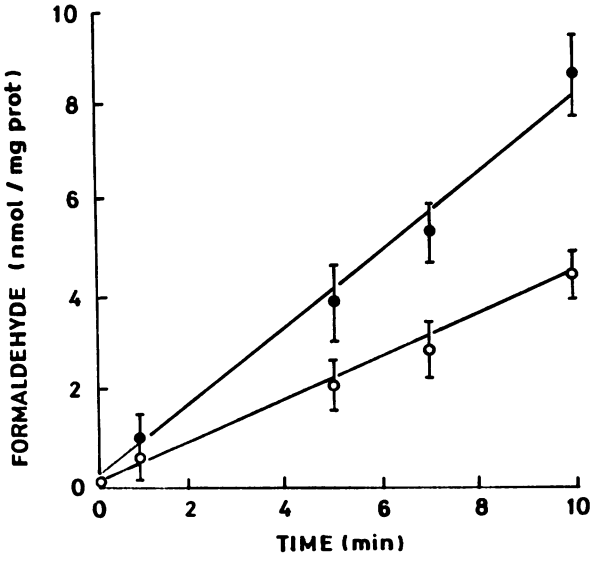

Figure 2. Production of formaldehyde from the oxidation of 30 mM DMSO by microsomes from soybean seedlings, in the presence of NADPH (O) or NADH (●). Experiments were carried out as described in "Materials and Methods," using 50 μM ferric EDTA as the iron catalyst. Values refer to means ± SE. prot, Protein.

Table II. *Rate of Reduction of Ferric Complexes by Soybean Seedling Microsomes*

Values are means ± SE. Ferric complexes were prepared as indicated in "Materials and Methods."

Ferric Complexes	Rate of Ferric Reduction	
	NADPH	NADH
	nmol·min^{-1}mg^{-1} of protein	
Ferric ATP	6.7 ± 0.5	11.7 ± 1.1
Ferric citrate	4.5 ± 0.3	7.3 ± 0.6
Ferric ADP	4.9 ± 0.5	9.9 ± 0.9
Ferric DTPA	4.6 ± 0.4	12.6 ± 1.0
Ferric EDTA	3.8 ± 0.1	6.2 ± 0.5

utilized to evaluate the generation of ·OH or ·OH-like compounds during animal microsomal electron transfer (34, 35). A time course for the oxidation of DMSO is shown in Figure 2 using either NADPH or NADH as cofactor in the presence of ferric EDTA. Essentially similar results were obtained when *tert*-butyl alcohol was utilized as the ·OH scavenger. Oxidation of either *tert*-butyl alcohol or DMSO was not detected in the absence of added iron.

Because transition metals, mainly iron, are implicated in radical generation by biological systems (6, 13), the ability of microsomal membranes to reduce iron complexes was studied. Table II shows that, in the presence of either NADPH or NADH, all the tested iron complexes were reduced. Ferric reduction rates were higher with NADH than with NADPH, with all of the ferric complexes utilized.

The effect of the iron complexes on ·OH generation by soybean seedling microsomes was evaluated. Table III indicates that ferric EDTA and ferric DTPA[2] were very effective in catalyzing the oxidation of DMSO by the microsomes, whereas ferric ADP and ferric citrate were not. The pattern of effectiveness of the different ferric complexes was the same with either NADPH or NADH. Because H_2O_2 appears to serve as the precursor of ·OH through an iron-catalyzed reaction, the effect of iron on H_2O_2 generation was assayed. In the presence of NADPH or NADH, either ferric EDTA or ferric DTPA increased significantly the rate of production of H_2O_2, whereas ferric ADP and ferric citrate were not effective (Table III).

The activities of enzymic antioxidants, such as catalase, peroxidases, and superoxide dismutase, were measured in total homogenate from soybean seedlings (Table IV). Vitamin E content in microsomal membranes, evaluated by HPLC, was 1.2 ± 0.1 pmol/mg of protein, suggesting that both enzymic and nonenzymic antioxidants play an active role in the cellular defense system against O_2 toxicity.

DISCUSSION

Previous data from our laboratory indicate that the rate of mitochondrial O_2^- production and H_2O_2 steady-state levels in soybean embryonic axes at the onset of germination were 0.8 to 1.3 nmol·min^{-1} mg^{-1} of mitochondrial protein (37)

[2] Abbreviation: DTPA, diethylenetriamine pentaacetic acid.

Table III. *Comparison of the Effect of Different Ferric Complexes on the Oxidation of DMSO and tert-Butyl Alcohol and on the Generation of H_2O_2 by Microsomes Isolated from Soybean Seedlings*

Values are means ± SE.

	·OH Production		H_2O_2 Production
	DMSO	*tert*-Butyl alcohol	
	nmol min^{-1} mg^{-1} of protein		
NADPH	nd[a]	nd	1.40 ± 0.05
+Ferric EDTA	0.50 ± 0.04	0.44 ± 0.03	2.30 ± 0.02
+Ferric DTPA	0.44 ± 0.03	0.54 ± 0.04	2.40 ± 0.02
+Ferric ADP	0.21 ± 0.01	0.15 ± 0.02	1.10 ± 0.05
+Ferric citrate	0.09 ± 0.01	0.12 ± 0.01	1.15 ± 0.05
NADH	nd	nd	0.87 ± 0.05
+Ferric EDTA	0.90 ± 0.08	1.11 ± 0.09	2.70 ± 0.06
+Ferric DTPA	1.06 ± 0.14	0.70 ± 0.06	2.17 ± 0.07
+Ferric ADP	0.15 ± 0.01	0.15 ± 0.02	1.30 ± 0.03
+Ferric citrate	0.18 ± 0.02	0.12 ± 0.01	1.25 ± 0.05

[a] nd, Nondetectable, indicates that the values are under the limit of detection in the assay conditions.

and 0.3 μM (36), respectively. Extramitochondrial O_2^- production was not considered, because at early stages of germination (2 h of incubation) the microsomal contribution seems less important. As soon as an active metabolism was established in the soybean seedling, production of active O_2 species by microsomal membranes became significant. The enzymic activities responsible for radical generation include Cyt P-450, Cyt *c* reductase, and Cyt b_5. Several studies have identified these activities in different plant tissues (4, 23, 39). In the current report, mixed-function oxidase activities were determined in microsomes isolated from soybean hypocotyls, and O_2^-, H_2O_2, and ·OH production were measured in the presence of either NADPH or NADH as cofactor.

Microsomal O_2^- generation was 5.0 ± 0.4 and 5.5 ± 0.4 nmol·min^{-1} mg^{-1} of microsomal protein, in the presence of either NADPH or NADH as cofactor, respectively. These values are within the same range of production by peroxisomal membranes from *Pisum sativum* L. leaves (11), reported as 10 nmol·min^{-1} mg^{-1} of protein, and of cotton roots extracts (2.2 ± 0.9 nmol·min^{-1} mg^{-1} of protein) (8). By comparison with other O_2 radicals, O_2^- was considered rather unreactive (21), even though it was recently reported that O_2^- can either be deleterious to [4Fe-4S]-containing dehydratase (17, 18) or react with NO to produce peroxinitrite (1). However, the main danger of O_2^- seems to lie in its ability to dismutate to H_2O_2 and to form ·OH (12).

H_2O_2 generation was detected in the presence of either NADPH or NADH as cofactor. The ratio of NADPH-dependent H_2O_2 to NADH-dependent H_2O_2 was 1.7 for soybean seedling microsomes and 5 for rat liver microsomes (12). This difference could be ascribed to a different relative activity of the enzymes involved in plant, as compared to animal, microsomes.

Isolated microsomes from soybean seedlings in the presence of either NADPH or NADH and iron can catalyze the production of ·OH-like species as reflected by the production of formaldehyde from DMSO and *tert*-butyl alcohol. Iron catalytic activity is required for the production of ·OH. Ferric EDTA and ferric DTPA are effective catalysts for plant microsomal generation of ·OH, whereas ferric ATP and ferric citrate are poor catalysts. This pattern of effectiveness could be ascribed to the higher redox potential of iron when complexed to EDTA or DTPA (25) or could be dependent on iron availability, which is related to the dissociation constant of the complex.

It would appear that the production of H_2O_2 is a better candidate than reduction of the ferric complexes as the limiting step for ·OH generation. For example, with ferric EDTA as the iron catalyst, rates of NADPH-dependent ·OH production, H_2O_2 generation, and reduction of iron from the ferric to the ferrous state are about 0.50 ± 0.04, 1.40 ± 0.05, and 3.8 ± 0.1 nmol·min^{-1} mg^{-1} of protein, respectively. Regarding the nature of the electron donor, iron reduction is significantly higher in the presence of NADH as compared with NADPH. This increased rate of reduction could reflect the higher activity of NADH-Cyt *c* reductase as compared with NADPH-Cyt *c* reductase activity (Table I).

At the cellular level, O_2 radical damage is controlled through enzymic and nonenzymic antioxidants. The activities of catalase, peroxidases, and superoxide dismutase measured in the soybean seedling homogenates were higher, as compared with previous data obtained in embryonic axes allowed to imbibe for 2 h (37). The rearrangement of energy-yielding processes during seed development causes an increase in O_2 uptake, leading to a more active production of O_2 radicals, which would determine the need for an increase in the activity of the enzymic antioxidant system. Regarding the nonenzymic antioxidants, it has been reported that tocopherols and tocotrienols are accumulated in organs and tissues with high sensitivity to oxidation, mainly the seeds (16). It is feasible that during germination and growth vitamin E content could be affected, but our data indicate that microsomal membranes contain detectable amounts of vitamin E. Further study is needed to explore the role of vitamin E in protection during growth.

Research on the microsomal contribution to free radical generation will provide a sound basis for an integrative

Table IV. *Enzymic Antioxidants in Soybean Seedling Homogenates*

Values are means ± SE.

Antioxidant	Activity
Catalase	14.4 ± 1.3 μmol of H_2O_2·min^{-1}mg^{-1} of protein
Peroxidases	1.1 ± 0.1 mmol·min^{-1}mg^{-1} of protein
Superoxide dismutase	11.5 ± 1.6 units·mg^{-1} of protein

evaluation of cellular total production rates that will contribute to the understanding of the complex role of these species in plant metabolism. The measurement of rates of production of O_2 radicals by microsomes, mitochondria, and peroxisomes will allow the calculation of cellular steady-state concentrations in physiological conditions after stress or in aged soybean. After steady-state concentrations are compared, the involvement of radicals in cellular damage could be evaluated. On the other hand, a detailed study of antioxidant status will contribute to the finding of experimental strategies for improving seed germination and plant growth.

LITERATURE CITED

1. **Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA** (1990) Apparent hydroxyl radical production by peroxynitrite4: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA **87:** 1620–1624
2. **Benveniste I, Lesot A, Hasenfratz MP, Durst F** (1989) Immunochemical characterization of NADPH-cytochrome P-450 reductase from Jerusalem artichoke and other higher plants. Biochem J **259:** 847–853
3. **Benveniste I, Salaun JP, Simon A, Reinchhart D, Durst F** (1982) Cytochrome P-450-dependent ω-hydroxylation of lauric acid by microsomes from pea seedlings. Plant Physiol **70:** 122–126
4. **Borlakoglu JT, John P** (1989) Cytochrome P-450-dependent metabolism of xenobiotics. A comparative study of rat hepatic and plant microsomal metabolism. Comp Biochem Physiol **94C:** 613–617
5. **Boveris A** (1984) Oxygen radicals in biological systems. Methods Enzymol **105:** 429–435
6. **Braughler JM, Duncan LA, Chase RL** (1986) The involvement of iron in lipid peroxidation. Importance of ferric to ferrous ratios in initiation. J Biol Chem **261:** 10282–10289
7. **Buege JA, Aust SD** (1978) Microsomal lipid peroxidation. Methods Enzymol **52:** 302–310
8. **Cakmak I, Marschner H** (1988) Enhanced superoxide radical production in roots of zinc-deficient plants. J Exp Bot **39:** 1449–1460
9. **Cederbaum AI, Cohen G** (1984) Microsomal oxidant radical production and ethanol oxidation. Methods Enzymol **105:** 516–522
10. **Chance B, Sies H, Boveris A** (1979) Hydroperoxide metabolism in mammalian organs. Physiol Rev **59:** 527–605
11. **Del Rio LA, Fernandez VM, Rupérez RL, Sandalio LM, Palma JM** (1989) NADH induces the generation of superoxide radicals in leaf peroxisomes. Plant Physiol **89:** 728–731
12. **Dicker E, Cederbaum AI** (1991) NADH-dependent generation of reactive oxygen especies by microsomes in the presence of iron and redox cycling agents. Biochem Pharmacol **42:** 529–535
13. **Farber JL, Kyle ME, Coleman JB** (1990) Biology of disease: mechanisms of cell injury by activated oxygen species. Lab Invest **62:** 670–679
14. **Fonne-Pfister R, Simon A, Salaun JP, Durst F** (1988) Xenobiotic metabolism in higher plants. Involvement of microsomal cytochrome P-450 in aminopyrine N-demethylation. Plant Sci **55:** 9–20
15. **Forman HJ, Boveris A** (1982) Superoxide radical and hydrogen peroxide in mitochondria. In WA Pryor, ed, Free Radicals in Biology, Vol 5. Academic Press, New York, pp 65–87
16. **Franzen J, Haab MM** (1991) Vitamin E content during development of some seedlings. Phytochemistry **30:** 2911–2913
17. **Gardner PR, Fridovich I** (1991) Superoxide sensitivity of the *Escherichia coli* 6-phosphogluconate dehydratase. J Biol Chem **3:** 1478–1483
18. **Gardner RP, Fridovich I** (1991) Superoxide sensitivity of the *Escherichia coli* aconitase. J Biol Chem **29:** 19328–19333
19. **Grand C** (1984) Ferulic acid 5-hydroxylase: a new cytochrome P-450 dependent enzyme from higher plant microsomes involved in lignin synthesis. FEBS Lett **169:** 7–11
20. **Hagmann ML, Heller W, Grisebach H** (1983) Induction and characterization of a microsomal flavonoid 3′-hydroxylase from parsley cell cultures. Eur J Biochem **134:** 547–554
21. **Halliwell B** (1982) The toxic effects of oxygen on plant tissues. *In* LA Oberley, ed, Superoxide Dismutase, Vol I. CRC Press, Boca Raton, FL, pp 89–123
22. **Hildebrandt AG, Roots I, Tjoe M, Heinemeyer G** (1978) Hydrogen peroxide in hepatic microsomes. Methods Enzymol **52:** 342–350
23. **Jansson I, Shenkman JB** (1977) Studies on three microsomal electron transfer enzyme systems. Specificity of electron flow pathways. Arch Biochem Biophys **178:** 89–107
24. **Kochs G, Grisebach H** (1989) Phytoalexin synthesis in soybean: purification and reconstitution of cytochrome P-450 3,9-dihydroxypterocarpan 6a-hydroxylase and separation from cytochrome P-450 cinnamate 4-hydroxylase. Arch Biochem Biophys **273:** 543–553
25. **Koppenol WH** (1987) Thermodynamics of reactions involving oxyradicals and hydrogen peroxide. Bioelectrochem Bioenerg **18:** 3–11
26. **Madyastha KM, Meehan TD, Coscia CJ** (1976) Characterization of a cytochrome P-450 dependent monoterpene hydroxylase from the higher plant *Vinca rosea*. Biochemistry **15:** 1079–1102
27. **Mayak S, Legge RL, Thompson JE** (1983) Superoxide radical production by microsomal membranes from senescing carnation flowers: an effect on membrane fluidity. Phytochemistry **22:** 1375–1380
28. **McCord JM, Fridovich I** (1969) Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J Biol Chem **244:** 6049–6055
29. **Mougin C, Cabanne F, Canivec M, Scalla R** (1990) Hydroxylation and N-demethylation of chlorotoluron by wheat microsomal enzymes. Plant Sci **66:** 195–203
30. **Nash T** (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem J **55:** 416–422
31. **Omura T, Sato R** (1964) The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem **239:** 2370–2378
32. **Petersen M, Seitz HU** (1985) Cytochrome P-450 dependent digitoxin 12 α-hydroxylase from cell cultures of *Digitalis lanata*. FEBS Lett **188:** 11–14
33. **Potts JRM, Weklych R, Conn EE** (1974) The 4-hydroxylation of cinnamic acid by sorghum microsomes and the requirement for cytochrome P-450. J Biol Chem **249:** 5019–5026
34. **Puntarulo S, Cederbaum AI** (1988) Comparison of the ability of ferric complexes to catalyze microsomal chemiluminescence, lipid peroxidation, and hydroxyl radical generation. Arch Biochem Biophys **264:** 482–491
35. **Puntarulo S, Cederbaum AI** (1988) Increased NADPH-dependent chemiluminescence by microsomes after chronic ethanol consumption. Arch Biochem Biophys **266:** 435–445
36. **Puntarulo S, Galleano M, Sanchez RA, Boveris A** (1991) Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. Biochem Biophys Acta **1074:** 277–283
37. **Puntarulo S, Sanchez RA, Boveris A** (1988) Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol **86:** 626–630
38. **Rahier A, Taton M** (1986) The 14 a-demethylation of obtusifoliol by a cytochrome P-450 monooxygenase from higher plant microsomes. Biochem Biophys Res Commun **140:** 1064–1072

39. **Rich PR, Bendall DS** (1975) Cytochrome components of plant microsomes. Eur J Biochem **55:** 333–341
40. **Salaun JP, Benveniste I, Reichhart D, Durst F** (1978) Induction and specificity of a (cytochrome P-450)-dependent laurate in-chain-hydroxylase from higher plant microsomes. Eur J Biochem **90:** 155–159
41. **Saunders BC, Holmes-Siedle AG, Stark BP** (1964) The properties and uses of a versatile enzyme and of some related catalysts. *In* BC Saunders, ed, Peroxidase. Butterworths & Co, London, UK, pp 158–168
42. **Soliday CL, Kolattukudy PE** (1977) Biosynthesis of cutin. ω-Hydroxylation of fatty acids by a microsomal preparation from germinating *Vicia fava*. Plant Physiol **59:** 1116–1121
43. **Vianello A, Macri F** (1989) NAD(P)H oxidation elicits anion superoxide formation in radish plasmalemma vesicles. Biochem Biophys Acta **980:** 202–208
44. **Young O, Beevers H** (1976) Mixed function oxidases from germinating castor bean endosperm. Phytochemistry **15:** 379–385
45. **Zimmerlin A, Durst F** (1990) Xenobiotic metabolism in plants: aryl hydroxylation of diclofop by a cytochrome P-450 enzyme from wheat. Phytochemistry **29:** 1729–1732