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Responses of Superoxide Dismutase and Glutathione Reductase Activities in Cotton Leaf Tissue Exposed to an Atmosphere Enriched in Oxygen[1]

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ABSTRACT

Responses of superoxide dismutase (EC 1.15.1.1) and glutathione reductase (EC 1.6.4.2) activities were evaluated in leaf tissue from intact cotton plants (Cotton Branch 1697) which were exposed to 75% O_2, 350 microliters per liter CO_2 for 48 hours. Soluble protein was extracted from O_2-treated and control tissue, and enzyme levels were determined. Superoxide dismutase activity in cotton leaf tissue was high (26 units per milligram protein) under normal conditions of 21% O_2, saturating light, and limiting CO_2, and neither qualitative nor quantitative differences in the cyanide-sensitive or -insensitive forms of the enzyme occurred in response to hyperoxic conditions. Glutathione reductase activity, however, was 2- to 3-fold higher in extracts from tissue exposed to 75% O_2. No increase in activity was observed for the peroxisomal enzymes, glycolate oxidase (EC 1.1.3.1) and catalase (EC 1.11.1.6). Results are consistent with an integrated pathway involving superoxide dismutase and glutathione reductase for protection of sensitive leaf components against detrimental effects of intermediate reduction products of O_2.

In higher plants, O_2 is a product of photosynthesis, a necessary reactant in physiological processes, and the terminal electron acceptor in light-independent mitochondrial respiration. Many plants, particularly those which fix CO_2 exclusively via the reductive pentose phosphate pathway also exhibit light-dependent photorespiratory O_2 uptake. Further uptake of molecular O_2 and its reduction to H_2O_2 in isolated chloroplasts, the Mehler reaction, has been considered to be a component of pseudocyclic electron transport in algae and higher plants. This process, however, occurs to a significant extent in higher plants only if conditions are not optimal for normal CO_2 fixation (19). Metabolism of O_2 is initiated by a univalent reduction to superoxide free radical; dismutation of superoxide anions catalyzed by superoxide dismutase (EC 1.15.1.1) results in formation of H_2O_2 and O_2 (3, 20) and concludes with the ultimate reduction of H_2O_2 (4, 13, 16). This cycling of O_2 leads to no net evolution or uptake but results in a transport of electrons or O_2 intermediates to locations removed from the immediate site of potential oxidative damage. Intermediate reduction states of O_2 are associated with respiratory chain activity and glycolate metabolism and may impair cellular metabolism through inactivation of enzymes by oxidation of critical thiol groups, production of other reactive O_2 species which may react with cellular components, or diversion of normal metabolic pathways. The ability of photosynthetic cells to tolerate these potentially toxic effects of oxidative damage has been proposed to depend upon the properties of various chemicals which are found in these organisms, including glutathione, ascorbate, pigments, and enzymes (4, 13, 16, 18, 26).

Inasmuch as enzymes in higher plants can be induced or activated in response to changes in specific environmental factors, it was expected that levels of enzymes, associated with O_2 metabolism, would be regulated to accommodate elevated O_2 concentration. O_2-dependent induction of superoxide dismutase with parallel increases in O_2 tolerance has been observed in bacteria (14), animals (10), yeast (15), blue-green algae (1, 5), and green algae (3, 22). Similar responses for superoxide dismutase in higher plants have not been reported, although chloroplasts are damaged by a 10-fold increase in superoxide concentration (4).

Moreover, in the absence of effective catalase in the chloroplast (16), H_2O_2 production by superoxide dismutase requires operation of an alternative mechanism for the removal of that oxidant. Millimolar concentrations of glutathione and ascorbate in chloroplasts (4, 13) are consistent with a protective function for these antioxidants. NADPH-glutathione reductase (EC 1.6.4.2) occurs in the chloroplast and functions to maintain a high GSH/GSSG ratio (2, 13, 24).

We have evaluated the effect of increased atmospheric O_2 concentration upon levels of certain enzyme activities, particularly superoxide dismutase and glutathione reductase, in leaf tissue of cotton, a C_3 species.

MATERIALS AND METHODS

Plant Culture. Diploid cotton plants. *Gossypium herbaceum* L. Cotton Branch 1697, were generated by vegetative propagation from a single parent plant. Plants were grown in a Perlite-Vermiculite (1:1) potting medium in controlled environment chambers at 30 C and 70 to 80% RH. A 16-h day/8-h night cycle was provided with irradiances of 200 to 300 $\mu E\ m^{-2}\ s^{-1}$ supplied by a mixture of cool-white fluorescent and incandescent bulbs. Plants were watered with 20% Hoagland nutrient.

Elevated O_2 Studies. For studies of effects of altered concentrations of atmospheric O_2, 60- × 60- × 120-cm vinyl chambers were erected inside a controlled environment chamber in order to maintain light and temperature at levels available during plant culture. Filtered air from a compressed air line, alone or supple-

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mented with specified quantities of CO_2 and O_2 from compressed gas cylinders, was introduced at a rate of 10 l/min to provide about 1.5 volume changes/h. The level of CO_2, 350 ± 50 μl/l, was monitored using an IR gas analyzer; atmospheric O_2 concentrations were monitored using a Beckman model 1008 Fieldlab O_2 analyzer. Humidity was maintained at 70 to 80% RH by bubbling gas through distilled H_2O as necessary.

Twelve plants, pruned to a single stem of approximately 25 cm 1 month prior to use, were introduced into the vinyl chambers at 30 C and acclimated in air for 1 to 3 days. O_2 concentration in one chamber then was elevated to 75 ± 5%.

Tissue Harvest. Time-dependent responses to atmospheric conditions were determined by harvesting fully expanded, 2- to 4-week-old leaves from two plants after 24 and 72 h of acclimation to the vinyl chamber, immediately before the elevation in atmospheric O_2 and after 24-, 48-, and 72-h exposure to elevated O_2. Samples contained 3 g tissue (four to six leaves) from only one plant, and all harvests were made at the midpoint of the light period to prevent variability in data arising from possible diurnal rhythms.

Protein Preparation. Leaf samples were crushed in liquid N_2 and then homogenized at 0 to 5 C with a Polytron tissue homogenizer in 25 ml 0.1 M Tris-Cl, (pH 6.9) containing 10 mM isoascorbate, 2% soluble PVP (PVP10, Sigma), and 0.5 g/g fresh tissue insoluble PVP (Polyclar AT supplied by General Aniline and Film Co., New York, N.Y.). When included in the homogenization medium, Triton X-100 was present at a concentration of 0.2%. After centrifugation (5 C) of the homogenate at 24,000*g* for 10 min, gel filtration chromatography of the supernatant fraction on Sephadex G-50-300 at room temperature yielded a soluble protein fraction suitable for protein determination and enzyme assays.

Protein Analysis. Protein was determined using the Coomassie G-250 dye-binding assay (7) with crystalline BSA as the standard reference protein.

Enzyme Assays. Unless noted otherwise, 1 unit of enzyme activity was that amount necessary to catalyze the decomposition of 1 μmol substrate/min at 25 C. Samples of 10 to 200 μl were used so that all assays depended on the amount of protein added.

Glutathione reductase activity was assayed by monitoring glutathione-dependent oxidation of NADPH at 340 nm in 0.15 mM NADPH, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione, and 50 mM Tris-Cl (pH 7.5) (24); units were expressed as nmol/min.

Superoxide dismutase was assayed spectrophotometrically as the inhibition of xanthine oxidase-dependent reduction of 10 μM ferricytochrome *c* monitored at 550 nm in 50 mM K-phosphate, 100 μM EDTA, and 50 μM xanthine (pH 7.8). One unit of superoxide dismutase inhibited by 50% a control rate, established by sufficient xanthine oxidase, of ferricytochrome *c* reduction of 0.025 A_{550}/min at 25 C (20). Cyanide-insensitive superoxide dismutase activity was quantified by the inclusion of 1 mM cyanide in the assay mixture; cyanide-sensitive activity was calculated by difference. Following electrophoresis using the standard 7.0% polyacrylamide gel system at pH 8.9, electrophoretically distinct forms of superoxide dismutase were detected as achromatic bands after incubating gels first in 25 mM nitroblue tetrazolium, then in 28 μM riboflavin, 36 mM K-phosphate (pH 7.8), and illuminating to initiate dye reduction (6). Inhibition of cyanide-sensitive forms of the enzyme, with a resulting loss of associated achromatic bands on gels, was accomplished by inclusion of 1 mM cyanide in staining solutions.

Peroxidase was assayed by monitoring H_2O_2-dependent oxidation of reduced trichloroindophenol at 675 nm (21); a mM extinction coefficient of 273 was used to determine activity. The reaction mixtures contained 0.3 mM leuco-2,3',6-trichloroindophenol, 17 mM $Na_2S_2O_3$, 120 mM H_2O_2, and 40 mM Na-phosphate (pH 6.0).

Glycolate oxidase activity was detected by a modification of a general protocol proposed by Cohen (9). H_2O_2, formed as a product of glycolate oxidation, was coupled to diaminobenzidine oxidation. One unit was defined as a change of 0.01 A_{352}/min. Relatively insoluble diaminobenzidine was prepared as a stock solution by suspending 0.5 mg/ml with a glass homogenizer; the 3-ml reaction mixture contained 0.1 ml diaminobenzidine stock solution, 60 μg horseradish peroxidase, 10 mM glycolate, and 0.3 M K-phosphate (pH 8.3).

Catalase activity was measured as a function of H_2O_2 utilization at 240 nm (17). Glyoxylate reductase was quantified as nmol/min by monitoring glyoxylate-dependent oxidation of NADPH at 340 nm (25). Malate dehydrogenase activity was determined by monitoring oxaloacetate-dependent oxidation of NADH (23) at 340 nm. Hydrolysis at 40 C of *p*-nitrophenyl phosphate at pH 5 was used to estimate acid phosphatase activity (11); appearance of phenolate was measured at 400 nm.

RESULTS

Typical yields of soluble protein and levels of enzymic activities in extracts from 4-week-old cotton leaf tissue are presented in Table I. Data are expressed as averages ± 1 SD for eight samples of tissue harvested from separate, equivalently treated plants and demonstrate the reproducibility with which extracts can be prepared. With the exception of glutathione reductase and glyoxylate reductase, determinations for soluble protein and the enzyme activities were reproducible within a range of 10 to 20%; slightly greater variabilities for the pyridine nucleotide-dependent enzymes reflect variable quantities of NADPH oxidase activity in extracts. Data of this quality were obtained consistently with the extraction procedure developed for this tissue, and fluctuations of the magnitude predicted for enzymes subject to regulation by O_2 could be readily detected. It was further determined that position of plants in the growth chamber had no effect on enzyme levels in leaf tissue.

Figure 1 depicts representative data for oxidative enzyme levels in cotton leaf tissue as a function of length of exposure to an O_2-enriched atmosphere. Averages of enzyme activities/g fresh tissue for four samples harvested during two independent time-course evaluations are plotted as a function of time for tissue maintained in air or exposed to 75 ± 5% O_2 (Fig. 1). Although activities of most of the enzymes which were examined remained essentially constant over the 3-day treatment period as illustrated for super-

Table I. *Protein Yields and Enzyme Levels Observed in Extracts from G. herbaceum Cotton Branch 1697 Leaf Tissue Prepared by the Optimized Extraction Procedure*

Initial homogenization was performed in 25 ml 100 mM Tris-Cl (pH 6.9) containing 10 mM isoascorbate, 0.5 g Polyclar AT/g tissue, and 1% (w/v) PVP-10. Two rehomogenizations of the residue remaining after centrifugation were each performed in buffered isoascorbate containing 0.5% PVP-10. Supernatant fractions were combined and chromatographed on Sephadex G-50-300 to obtain the soluble protein fraction. Data are expressed as the average ± 1 SD for eight samples, each prepared from mature 4-week-old leaf tissue from separate, equivalently treated plants.

Assay	Yield
	units/mg protein
Enzyme activity	
Superoxide dismutase	14 ± 2
Glutathione reductase	1.5 ± 0.3
Catalase	250 ± 17
Peroxidase	1.5 ± 0.3
Glycolate oxidase	19 ± 3
Glyoxylate reductase	5 ± 1.8
Malate dehydrogenase	5.2 ± 0.7
Acid phosphatase	0.12 ± 0.02
Soluble protein, mg/g tissue	15.4 ± 1.4

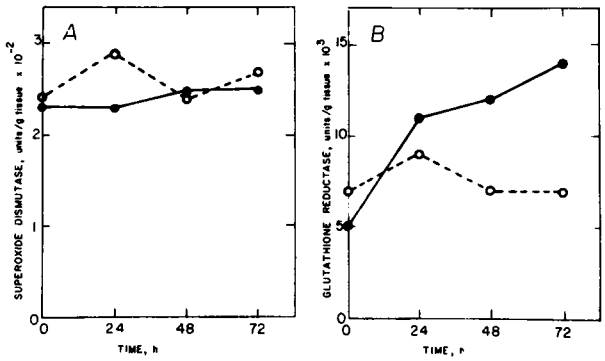

FIG. 1. Levels of enzyme activities in soluble extracts from mature cotton leaf tissue during exposure of the plant to 75 ± 5% O_2. A: superoxide dismutase assayed with the Cyt *c* procedure (20); B: NADPH-glutathione reductase assayed by following pyridine nucleotide oxidation (24). (O- - -O): Air controls; (●——●): 75 ± 5% O_2, 350 μl/l CO_2.

Table II. *Changes in Levels of Oxidative Enzymes in G. herbaceum Cotton Branch 1697 Leaf Tissue Exposed to 75 ± 5% O_2 for 48 h*

Plants were acclimated in air in vinyl chamber for 24 h prior to exposure to the high O_2 atmosphere. Data are expressed as the average ± 1 SD for eight samples, each prepared from mature, 2- to 4-week-old leaf tissue harvested at the midpoint of the photoperiod from separate, equivalently treated plants.

Atmosphere	Air[a]	75 ± 5% O_2, 350 ± 50 μl/l CO_2
	units/mg protein	
Enzyme activity		
Superoxide dismutase	26 ± 3.2	26 ± 7.1
Cyanide-sensitive	18 ± 3.1	18 ± 5.7
Cyanide-insensitive	7 ± 2.0	9 ± 2.0
Glutathione reductase	1.3 ± 0.4	4.6 ± 0.86**
Peroxidase	0.7 ± 0.10	1.1 ± 0.17**
Catalase	240 ± 44	210 ± 16
Glycolate oxidase	22 ± 2.6	28 ± 3.7
Glyoxylate reductase	5 ± 1.8	6 ± 1.1
Malate dehydrogenase	4 ± 0.4	4 ± 0.3
Acid phosphatase	0.1 ± 0.01	0.2 ± 0.02**
Soluble protein, mg/g tissue	13 ± 1.4*	11 ± 1.4

[a] Where data from the air and 75% O_2 atmospheres are significantly different, asterisks indicate the atmosphere producing the higher level of protein or enzymic activity and denote the P value as follows: * $0.005 < P < 0.01$; ** $P < 0.005$.

oxide dismutase in Figure 1A, changes in levels of enzymes sensitive to the elevated O_2, *e.g.* glutathione reductase, were quite apparent after 48 h (Fig. 1B). Visible symptoms of environmental stress were absent during the experimental period when ambient levels of CO_2 (350 ± 50 μl/l) were maintained during O_2 treatment.

Enzyme levels in tissue harvested from air controls or from plants exposed to 75 ± 5% O_2 for 48 h appear in Table II. Comparisons revealed protein levels to be 15% lower in O_2-treated tissue. In contrast, significantly higher specific activities for glutathione reductase (±254%), peroxidase (±57%), and acid phosphatase (+100%) were observed in extracts from tissue exposed to 75% O_2, whereas levels of superoxide dismutase, both cyanide-sensitive and -insensitive forms, glyoxylate reductase, malate dehydrogenase, catalase, and glycolate oxidase were independent of the atmosphere.

With the exception of catalase, which exhibited a significant O_2-dependent decrease (−32%) in activity/g fresh tissue (Table III), the comparison of other enzyme activities was similar whether activity was expressed on the basis of soluble protein or fresh tissue weight.

Table III. *Oxygen-dependent Changes in Enzyme Levels in G. herbaceum Cotton Branch 1697 Leaf Tissue Exposed to 75 ± 5% O_2, 350 ± 50 μl/l CO_2 for 48 h*

Changes were calculated using enzyme activities in extracts from 2- to 4-week-old fresh tissue obtained from control tissue and tissue exposed to 75 ± 5% O_2 for 48 h; yield of soluble protein/g tissue was 15% lower in the O_2-treated tissue.

Enzyme	Change[a]	
	units/mg protein	units/g tissue
	%	
Superoxide dismutase	Unchanged	Unchanged
Glutathione reductase	+ 254**	+ 183**
Peroxidase	+ 57**	+ 22*
Catalase	Unchanged	− 32**
Glycolate oxidase	Unchanged	Unchanged
Glyoxylate reductase	Unchanged	Unchanged
Malate dehydrogenase	Unchanged	Unchanged
Acid phosphatase	+ 100**	+ 85*

[a] Asterisks define those enzymes for which statistically significant changes in activity occurred as a result of the high-O_2 treatment: * $0.005 < P < 0.01$; ** $P < 0.005$.

Since O_2 treatment appeared not to alter total levels of superoxide dismutase activity, electrophoresis of extracts was performed to examine possible existence of O_2-related differences in electrophoretic forms of this enzyme. Polyacrylamide disc gels stained for superoxide dismutase activity indicated five distinct electrophoretic forms of superoxide dismutase having relative mobilities of 0.31, 0.46, 0.53, 0.61, and 0.86 (Fig. 2); of these, only the form having an R_F value of 0.46 was insensitive to 1 mM cyanide. Extracts from air controls and tissue exposed to 75 ± 5% O_2 produced staining patterns which were qualitatively and quantitatively identical.

Further extraction of the particulate fraction from tissue homogenates was performed to determine whether differential association of superoxide dismutase with the insoluble residue was responsible for the absence of an O_2-dependent effect upon levels of this enzyme. Analysis of protein solubilized from this fraction with 0.2% Triton X-100 indicated similar yields from controls and O_2-treated tissues (Table IV). Superoxide dismutase activity in these homogenates could be completely eliminated by boiling the extracts. Addition of 1 mM cyanide during assay of these samples by the Cyt *c*-dependent spectrophotometric assay resulted in a level of activity which was greater than that observed in the absence of cyanide. Apparently, in the absence of cyanide, proteins such as Cyt *c* oxidase solubilized during the Triton X-100 procedure interfere with the rate of Cyt *c* reduction. Analysis of superoxide dismutase activity on disc gels revealed similar electrophoretic patterns for control and treated tissue samples, the predominant cyanide-sensitive form having a relative mobility of 0.86.

DISCUSSION

Conceptually, O_2 toxicity in photosynthetic organisms seems a paradox since O_2 is a primary product of photosynthesis. However, organisms, including plants, which survive an aerobic environment contain enzymes, such as superoxide dismutase, catalase, and peroxidase, which function to maintain low concentrations of reactive O_2 intermediates, such as superoxide and H_2O_2 (4, 18). The apparent viability of higher plants exposed to an O_2-enriched atmosphere and the observed increases in superoxide dismutase activity in blue-green and green algae exposed to O_2 (1, 22) directed us to examine the response of oxidative enzymes in cotton leaves.

Utilization of O_2 as an alternative electron acceptor during

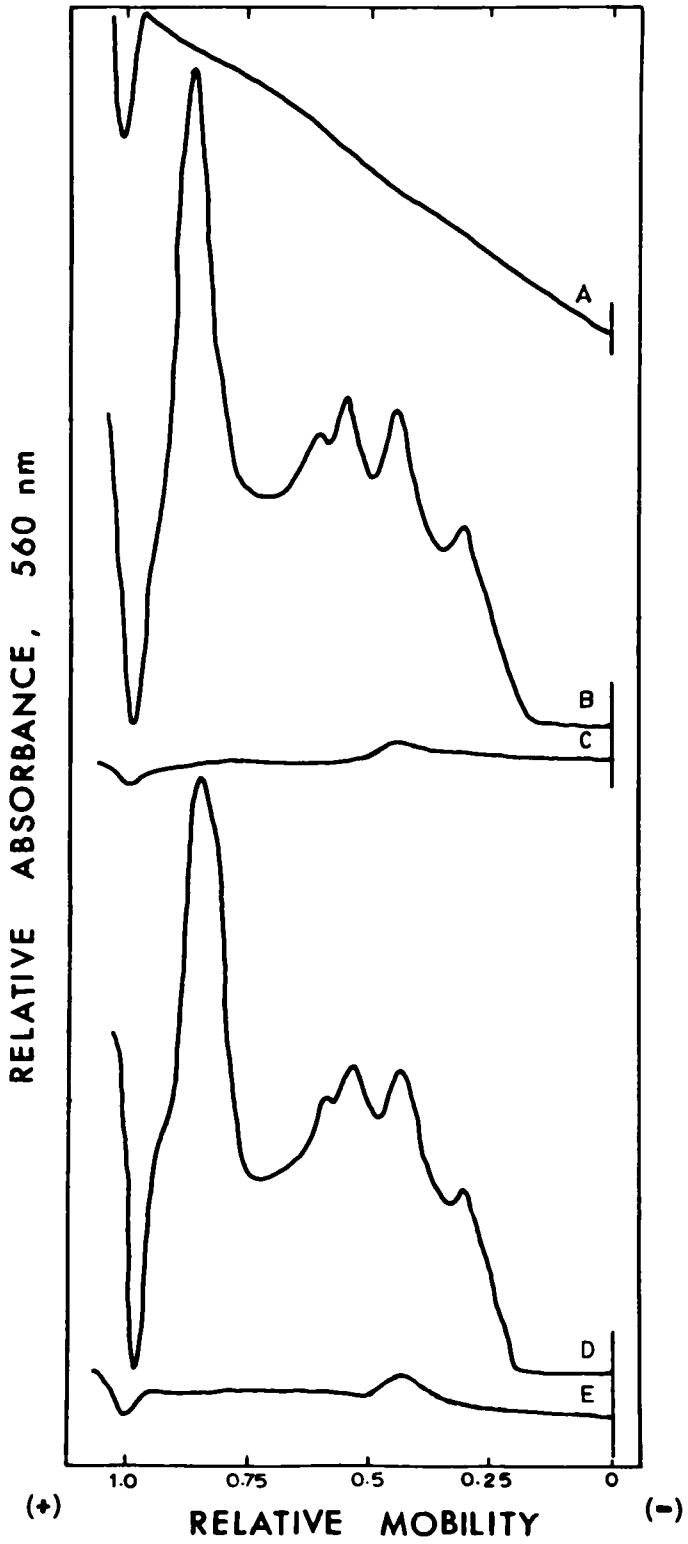

FIG. 2. Electrophoretic forms of superoxide dismutase from cotton leaf tissue at normal (21%), and elevated O_2 (75%) concentrations. Soluble protein (50 μg) from extracts of *G. herbaceum* Cotton Branch 1697 in 5% sucrose was layered on 7.0% polyacrylamide gels. Superoxide dismutase was located on the gels following electrophoresis using the activity stain (6) in the presence and absence of 1 mM KCN as indicated. A: blank gel; B: air control; C: air control + cyanide; D: 75 ± 5% O_2, 48 h; E: 75 ± 5% O_2, 48 h + cyanide. Full-scale sensitivity was 0.4 A for scans A, B, and D, and 1.0 A for scans C and E.

Table IV. *Effect of Triton X-100 upon Solubilization of Protein and Superoxide Dismutase Activity from Insoluble Material from Homogenates of Cotton Leaf Tissue Maintained in Air or Exposed to O_2 for 48 h*

Initial homogenization was performed in 100 mM Tris-Cl (pH 6.9) containing 10 mM isoascorbate, 2% PVP-10, and 0.5 g Polyclar AT/g tissue. Rehomogenization of the insoluble residue from a previous homogenization was performed in 100 mM Tris-Cl (pH 6.9) containing 10 mM isoascorbate and 0.5% PVP-10. Homogenizations 2 and 3 were performed in the presence of 0.2% Triton X-100.

Assay	Homogenate		
	1[a]	2[b]	3[b]
Soluble protein			
Air			
mg/g tissue	13 ± 1.4	3 ± 0.5	1 ± 0.05
% of total	76	18	6
75 ± 5% O_2, 350 ± 50 μl/l CO_2			
mg/g tissue	11 ± 1.4	3 ± 0.2	1 ± 0.02
% of total	73	20	7
Superoxide dismutase			
Air			
units/g tissue	350 ± 47	60 ± 1	20 ± 1
% of total	81	14	5
75 ± 5% O_2, 350 ± 50 μl/l CO_2			
units/g tissue	290 ± 79	50 ± 1	10 ± 2
% of total	83	14	3

[a] Data are averages of eight samples ± 1 SD.
[b] Data are averages of two samples ± 1 SD.

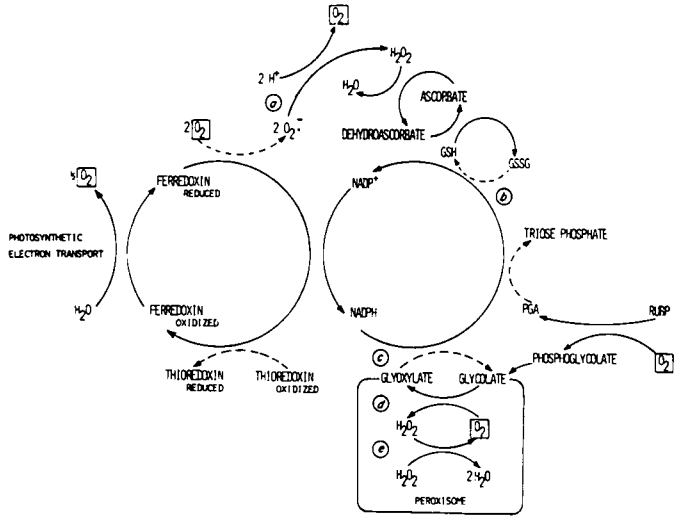

FIG. 3. Opportunities for O_2 metabolism in the chloroplast. Reactions designated by letters are catalyzed by enzymes which were analyzed in soluble extracts from cotton leaf tissue: a: superoxide dismutase; b: glutathione reductase; c: glyoxylate reductase; d: glycolate oxidase; e: catalase. Broken arrows indicate alternative electron acceptors which may function when normal electron flow to phosphoglyceric acid is limiting, although each reaction probably occurs in balance with normal electron flux.

photosynthesis (Fig. 3), especially when availability of $NADP^+$ is limiting, may impose upon higher plant leaf cells a high concentration of superoxide even under normal growth conditions, *i.e.* 21% O_2, saturating light intensity, and limiting CO_2. If O_2 concentrations surrounding the leaf are increased, the intracellular concentration of O_2 would also increase along with its probability of serving as an electron acceptor. For cotton leaves exposed to elevated O_2 for 48 h, we observed similar electrophoretic patterns for superoxide dismutase (Fig. 2) and constant levels of both the cyanide-sensitive and -insensitive superoxide dismutase activities

in comparison to control tissue (Tables II and IV). Apparently, the cotton leaf does not respond as do algae to elevated O_2 concentrations.

The specific activities of superoxide dismutase detected by the Cyt *c*-dependent assay in crude extracts from higher plants (20–25 units/mg protein) (3), including our measurements in cotton leaf tissue (Table II), are similar to, or greater than, those present in extracts from eukaryotic algae (15–24 units/mg protein) (3, 5, 18). For microorganisms, superoxide dismutase ranges from 0 to 30 units/mg protein, and susceptibility to O_2 toxicity correlates with low levels of this enzyme (14, 15, 22). Additionally, the 2- to 3-fold higher specific activity of the Cu-Zn superoxide dismutase purified from higher plant sources (18) and the higher content (50–75%) of this form of superoxide dismutase in land plants (3) may permit higher plants to tolerate large fluctuations in atmospheric O_2, even of the magnitude employed in experiments reported here. Further, absence of the Cu-Zn superoxide dismutase in eukaryotic algae (3) may account for the necessity to increase superoxide dismutase activity in *Chlorella* in response to exposure to elevated O_2 concentration.

The observed increase in glutathione reductase (Table III) may reflect increased glutathione turnover in tissue exposed to 75% O_2. Thus, the flux of H_2O_2, a product of superoxide dismutation, could be more readily accommodated in the stressed plant (Fig. 3). Although specific activity of glutathione reductase in extracts from air-grown leaf tissue is relatively low, it compares favorably with activity reported for the enzyme in crude protein extracts from other plant tissues (2, 13, 24), and its activity, expressed as a function of fresh tissue weight, is consistent with that found in evergreens (12). Thus, the 150 to 250% increase in catalytic activity (Table III) implicates glutathione reductase in accommodating oxidative stress in treated cotton leaves. A similar increase in leaf glutathione reductase was observed in leaf tissue harvested from plants during periods of low growth temperature (12) which could also correlate with increased O_2 concentration in cells. The increased activity in the treated leaves may be caused by a specific increase in chloroplast glutathione reductase, but the enzyme also occurs in cellular compartments other than the chloroplast (13).

Glutathione peroxidase has not been detected in plant tissues and catalase is located in peroxisomes (4, 16). Removal of H_2O_2, generated in the chloroplast, may be accomplished by an ascorbate-mediated, reduced glutathione-dependent reduction sequence (Fig. 3) which is catalyzed by ascorbate peroxidase (16) or occurs nonenzymically at pH 7.5, which exists in the chloroplast during photosynthesis (13). In this manner, glutathione reductase would not only provide protection of chloroplastic components against oxidation by H_2O_2, but would also minimize potential for H_2O_2 inactivation of the predominant Cu-Zn superoxide dismutase localized within the chloroplast (3, 4).

Glyoxylate reductase was not increased in tissue exposed to 75% O_2. Further, experimental conditions (high O_2/CO_2 ratio) were consistent with carbon metabolism through the photorespiratory pathway. Neither glycolate turnover nor peroxisomal metabolism was elevated beyond a point which can normally be accommodated in the plant since we observed no significant increase in levels of the peroxisomal enzymes glycolate oxidase or catalase (Tables II and III).

Significantly higher levels of peroxidase and acid phosphatase activities, but lower levels of catalase activity in extracts from O_2-treated cotton leaf tissue, may indicate general O_2-related tissue damage. Elevated H_2O_2 concentrations may release peroxidase from membrane structures with which it is normally associated, as in the case of the insoluble ascorbate peroxidase of spinach chloroplasts (16). The *in vitro* activation of acid phosphatase by oxidized glutathione has been observed (8), although the function for this effect is not understood.

In higher plants, apparently both superoxide dismutase and glutathione reductase are intimately related to O_2 metabolism in the chloroplast. These two enzymes function effectively at pH values which exist in this organelle during photosynthesis, and their interdependence is mediated by ascorbate turnover. Protection for the photosynthetic apparatus would result from superoxide dismutase catalysis of superoxide disproportionation and accessibility to reducing equivalents, via glutathione reductase, necessary for the ultimate reduction of H_2O_2. It needs to be determined to what extent ascorbate peroxidase in chloroplasts is affected by atmospheric O_2 levels.

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