EVALUATION OF OXIDATIVE ENZYMES IN LEAF TISSUE FROM INTACT COTTON PLANTS EXPOSED TO DIFFERENT OXYGEN CONCENTRATIONS

by

Joyce Geraldine Foster

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APPROVED:

J. L. Hess, Chairman

L. B. Barnett

R. D. Brown, Jr.

E. M. Gregory

L. D. Moore

R. R. Schmidt

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Blacksburg, Virginia

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LIST OF ABBREVIATIONS

C.B.:	cotton branch
EDTA:	ethylenediaminetetraacetic acid
EGTA:	ethylenebis (oxyethylenenitrilo) tetraacetic acid
GSH:	reduced glutathione
GSSG:	oxidized glutathione
NBT:	nitroblue tetrazolium
RuBP:	ribulose-1,5-bisphosphate
PGA:	3-phosphoglyceric acid
PVP:	polyvinylpyrrolidone
TEMED:	N,N,N',N'-tetramethylenediamine

INTRODUCTION

Like all aerobic organisms, higher plants metabolize atmospheric oxygen. Most photosynthetic organisms have the capacity to produce oxygen from the oxidation of water as well as the ability to utilize oxygen as a terminal electron acceptor in respiration. Further, in plants which photorespire, there exists a light-dependent utilization of oxygen in addition to typical light-independent mitochondrial respiration.

It is proposed that, in plants maintained in an oxygen-enriched atmosphere, levels of enzymes associated with oxygen metabolism would be regulated to accomodate the altered oxygen concentration. In order to examine this hypothesis, Gossypium herbaceum L. C.B. 1697 was cultured in controlled environment growth chambers and treated with ambient concentrations of carbon dioxide in atmospheres elevated in oxygen concentration. Soluble protein from leaf tissue was isolated, and activities of the following enzymes were quantified in these extracts: catalase, peroxidase, glycolate oxidase, glyoxylate reductase, glutathione reductase, superoxide dismutase, malate dehydrogenase, and acid phosphatase. In this way responses of cytosolic, chloroplastic,

and peroxisomal enzymes to an elevated concentration of atmospheric oxygen could be compared.

To insure that comparisons between treated and control tissue involved as few variables as possible, effort was directed toward the successful propagation and growth of genetically uniform, diploid cotton plants. Further, the Old World cotton, selected as a representative diploid variety, contained unusually large quantities of phenolics so that it was necessary to optimize extraction procedures for isolation of soluble protein and active enzymes from leaf tissue.

LITERATURE REVIEW

Organismic Importance of Oxygen

Requirements for oxygen during various stages of growth and development are indicative of multiple functions for oxygen in higher plant metabolism. Seed germination, particularly for seeds like cotton which have a high content of oil and protein (1), is favored by high oxygen tensions. Apparently a high demand for oxygen during the early period in the germination process is associated with a high level of cyanide-resistant, cytochrome-independent respiration attributed to an alternate oxidase functioning at that time (2). Once aerial plant parts are established, downward movement of oxygen entering those tissues occurs by a gaseous diffusion process through continuous intracellular spaces (3,4). This internal transport of oxygen appears to supply respiratory needs (4), but exposure of mesophytic plants to low-oxygen conditions, such as water-logged soil, for only a short time results in irreparable damage to the roots (5) and reduces crop yield (6). Apparently for plants with small, developing root systems, internal oxygen supply is adequate for respiratory demands, but with accumulation

of a large respiratory sink in extensive root systems, this source of oxygen is inadequate to supply remote parts of the system (7), and growth of plants depends upon roots obtaining oxygen from the soil (5).

At constant, ambient carbon dioxide concentrations, net photosynthesis increases when the concentration of oxygen surrounding the leaf is reduced from 21% (8), and growth of soybean plants in low oxygen concentrations increases dry matter production both in the aerial portion and in the roots (9). While atmospheric concentrations of oxygen less than 21% give rise to increases in vegetative growth, oxygen concentrations greater than 15% are required for normal pod and seed development in soybeans (10). Because elevated oxygen atmospheres containing ambient or elevated carbon dioxide inhibited total growth of soybeans without affecting the balance of vegetative to reproductive growth, Quebedeaux and Hardy (9,10) concluded that some unknown reaction requiring at least atmospheric levels of oxygen, but independent of carbon dioxide, is necessary for optimization of all phases of reproductive growth and suggest a unique chemical reaction or physical process asso-

ciated with translocation and accumulation of assimilated materials.

Oxygen participates in the biosynthesis of the plant senescence hormone ethylene (11). Moreover, the rate of fruit ripening, reflected in intensity of the respiratory climacteric, is oxygen-dependent and probably due to the contribution of the alternate, cyanide-insensitive oxidase (12). Thus, throughout the life cycle of the higher plant, oxygen as a reactant contributes to germination, root growth, senescence, flowering and ripening, and, as a product, is released during periods of photosyntheis.

Impact of Oxygen on Higher Plant Metabolism

Photosynthesis and the Warburg Effect - Oxygen's importance in the higher plant is apparent from the number and variety of biochemical processes in which it participates. Green plant photosynthesis, described by light-dependent oxygen evolution, is associated with fixation of carbon dioxide by ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) and reduction of the carbon utilizing intermediates of the reductive pentose phosphate pathway. Although oxygen may enhance photosynthesis under saturating irradiances and high concentrations of

carbon dioxide, an effect attributed to oxygen-dependent regeneration of the carbon dioxide acceptor, ribulose bisphosphate (13), oxygen inhibits carbon dioxide fixation in plants photosynthesizing in ambient carbon dioxide, a phenomenon first described by Warburg (14) and usually ascribed to increased glycolate synthesis that occurs with increased concentrations of oxygen (15,16). This reversible inhibition of photosynthesis apparently arises from dual activity of the carboxylase/oxygenase enzyme (17) and competition between oxygen and carbon dioxide for the active center of the enzyme (17,18,19). At high oxygen/carbon dioxide ratios, oxygen not only impedes access of carbon dioxide to the active center, but also competes effectively for that binding site (20); kinetic analyses reveal that apparent K_m values for carbon dioxide and oxygen approximate apparent K_i values for oxygen or carbon dioxide (18, 21), TABLE I. Ku and Edwards (22) have reported that, under atmospheric conditions of oxygen and carbon dioxide, the increased inhibition of photosynthesis one observes with increasing temperatures correlates well with concurrent increases in the solubility ratio of oxygen/carbon dioxide in the leaf. Maintenance of a constant solubility ratio

TABLE I

K_m and K_i values of ribulose bisphosphate carboxylase/oxygenase at pH 8.3-8.4

Activity	K_m or K_i for CO_2	K_m or K_i for O_2	Reference
Carboxylase	18 μM	350 μM	(18)
Oxygenase	19 μM	196 μM	(18)
Carboxylase	34 μM	370 μM	(21)
Oxygenase	38 μM	390 μM	(21)

by controlling external concentrations of the two gases resulted in a constant percentage inhibition over a temperature range of 25-35°. Steiger and Beck (23) suggest that, in vivo, chloroplastic oxygen concentration, which may depend on photosynthetic oxygen production as well as external oxygen tension, is equal to that in the cytoplasm and that the velocity of oxygen exchange between the cell and its environment determines the intracellular concentration of oxygen. Changes in oxygen concentration in leaf tissue, simultaneous with changes in oxygen levels in the external environment, attest to the responsiveness of this tissue to the environment (24) and correlate with observations of increased oxygenation rates of ribulose bisphosphate with increasing concentrations of atmospheric oxygen (15,16,25).

Photorespiration - Higher plants which utilize only the reductive pentose phosphate cycle for fixation of carbon dioxide possess a photorespiratory pathway which, in contrast to photosynthesis, is characterized by oxygen consumption and carbon dioxide evolution. The primary event in photorespiration is the oxidative cleavage of ribulose bisphosphate catalyzed by ribulose bisphosphate carboxylase/oxygenase (15,16,25,26). While regeneration of the oxygen

acceptor is light-dependent, subsequent oxidation of the photorespiratory substrate, glycolate, and release of carbon dioxide proceed by light-independent processes (8). Like the Warburg effect, photorespiration is stimulated by oxygen and inhibited by carbon dioxide (27,28), and can continue for prolonged periods in carbon dioxide-free air (27). Because it releases previously fixed carbon dioxide, photorespiration expends photosynthetic energy and has been regarded as an energy-wasteful process whose net effect is a decrease in plant productivity (29). On the other hand, reactions of the glycolate pathway may prevent accumulation of glycolate to toxic levels (30) and provide a mechanism whereby a portion of the carbon in glycolate can be converted to carbohydrate as efficiently as possible (8). It has also been proposed from the observed correlation between chlorophyll accumulation and glycolate oxidase activity that glycolate can function as the starting substrate for synthesis of 5-aminolevulinic acid, a metabolite of chlorophyll biosynthesis (31).

Photoinhibition - In intact spinach chloroplasts and photosynthetic cells incubated in visible light in the

absence of carbon dioxide, capacity for subsequent carbon dioxide-dependent photosynthetic oxygen evolution is substantially reduced (32). This oxygen-dependent effect termed photoinhibition apparently results from irreversible damage to light-harvesting components of the photosynthetic apparatus caused by light energy trapped in the thylakoid system and consequential electron flux. Under conditions of limited supply of exogenous carbon dioxide during illumination, photorespiratory carbon dioxide production may prevent depletion of carbon dioxide in cells and thereby protect photosynthetic capacity by providing the substrate necessary for reactions utilizing photochemical energy. Osmond and Björkman (33) suggest that photorespiration prevents photodestruction of the photosynthetic apparatus during periods of water stress and high irradiance by generating carbon dioxide which can be refixed when stomata are closed.

Mehler Reaction and Pseudocyclic Photophosphorylation - Uptake and reduction of molecular oxygen to H_2O_2 by illuminated chloroplasts was established by Mehler in 1951 (34). Radmer and Kok (35) have since shown that oxygen and carbon dioxide are in direct competition for light-generated

reducing power of Photosystem I and that oxygen is the principal electron acceptor under conditions in which oxygen evolution occurs more rapidly than carbon dioxide reduction. Generation of ATP coupled to the transfer of reducing equivalents to oxygen permits flexibility in the production of ATP and NADPH (36,37). Forti and Gerola (38) reported that H_2O_2 is not formed when $NADP^+$ is available and propose that electron flow from Photosystem I is diverted to oxygen when insufficient ATP limits the rate of reoxidation of NADPH. Translocation of protons and cations and production of ATP by oxygen-mediated electron flow following a period of darkness may prime the carbon dioxide reduction system (35). Apparently plants utilize this pseudocyclic photophosphorylation mechanism involving H_2O_2 generation to supplement ATP provided by cyclic and noncyclic photophosphorylations, insuring adequate quantities of ATP for assimilation of carbon dioxide and other cellular phosphorylations (36,37).

Respiration - Plants exhibit multiple respiratory processes which consume oxygen and evolve carbon dioxide. Unlike photorespiration which is not saturated at 100% oxygen (15,39), dark, mitochondrial respiration associated

with oxidation of organic substrates and generation of high energy compounds is saturated at 2% oxygen and occurs at a rate in light that is less than (39) or equal to (40) its rate in the dark. In higher plants, there exists an additional mitochondrial respiratory process which has a high affinity for oxygen and is resistant to inhibitors of cytochrome oxidase (12). Termed cyanide-resistant respiration, this alternate path branches from the cytochrome-dependent respiratory chain before cytochrome b. Phosphorylation may (41) or may not (42) be linked with activity of the cyanide-insensitive alternate oxidase and the extent to which a plant utilizes the alternate pathway depends upon the species, nature of the tissue, and the mitochondrial state, but the intensity of electron flux through the cytochrome path probably determines the partitioning of electrons between the two paths (12).

Oxygen Fixation - Mass spectrometric techniques for tracing incorporation of [^{18}0] oxygen into metabolites of the photorespiratory pathway have detected label only at the carboxyl carbon in glycolate, glycine, serine, and 3-phosphoglyceric acid (25,30,43). Enrichments at a single site, combined with minimal exchange with unlabeled oxygen,

supports the thesis that plants fix atmospheric oxygen into the photorespiratory substrate and that this oxygen is retained in metabolites of the pathway, eventually appearing in the carboxyl oxygen of 3-phosphoglyceric acid. These observations are in agreement with the proposed pathway for photorespiration and the relationship between photorespiration and photosynthesis.

Generation of Reactive Oxygen

Triplet or ground state molecular oxygen which contains two unpaired electrons with parallel spins is not a very reactive chemical species. However, unless electron spin of this paramagnetic species is stabilized by a metal ion, reduction occurs via one-electron steps (44) and leads to the production of the superoxide free radical, $O_2^{\cdot -}$, a highly reactive chemical species (45). Further reduction produces hydrogen peroxide which can react with superoxide in a Fenton's type reaction (46,47) represented stoichiometrically by the Haber-Weiss reaction (48)

$$H_2O_2 + O_2^{\cdot -} \rightarrow O_2 + OH^- + OH^{\cdot} \qquad (I)$$

to generate the extremely reactive hydroxyl radical $OH^{\cdot}$.

Electronically excited or singlet state oxygen, $^1O_2$, is produced by photodynamic action in which excitation energy of a photosensitized compound is transferred to molecular oxygen (45). Singlet oxygen has been characterized as a product of spontaneous disproportionations of $O_2^{\cdot -}$ (49,50, 51) and H_2O_2 (46) and of the Haber-Weiss reaction (46,52).

Production of reactive oxygen species has been observed during a variety of metabolic processes in higher plants. Reduction of molecular oxygen to hydrogen peroxide by illuminated chloroplasts was established by Mehler (34). More recent investigations (53,54,55,56,57) demonstrated the production of the superoxide radical by chloroplasts in the presence of light and oxygen, and subsequent studies by Asada, et al., (58) revealed the Mehler reaction to be composed of two partial reactions, the first of which generates the superoxide free radical through univalent reduction of oxygen; hydrogen peroxide is then produced by the dismutation of superoxide anions. That superoxide occurs as an intermediate in the reduction sequence of Photosystem I has been inferred from: ascorbate oxidation (54,57), ascorbate-mediated phosphorylation (56), sulfite oxidation (59), photoreduction of cytochrome c (58), photooxidation of

epinephrine (58), and the production of a superoxide adduct of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (60). Asada and coworkers (58) have proposed that the primary electron acceptor for Photosystem I functions as the electron donor for molecular oxygen, and all evidence supports the contention that the major process responsible for oxygen uptake by chloroplasts is the reduction of oxygen to free superoxide radicals by Photosystem I (61). On the other hand, oxidation of superoxide anion in illuminated chloroplasts by oxidized electron transfer components results in the generation of singlet oxygen (62) thought to be responsible for photoinhibition (63); formation of reactive oxygen, however, does not occur during photooxidation of water in Photosystem II (61). Apparently superoxide anion is not involved in oxygenation reactions catalyzed by ribulose bisphosphate carboxylase/oxygenase (25).

Metabolism of oxygen in its different oxidation states occurs in cellular locations other than the chloroplast. In higher plant mitochondria, Rich and Bonner (64) have experimentally demonstrated generation of superoxide associated with respiratory chain activity; further, hydrogen peroxide may be the immediate product of the cyanide-insen-

sitive alternate cytochrome b oxidase (65). Thus, there appear to be several alternate, oxygen-consuming systems in mitochondria, of which only the hydroxamic acid- and antimycin A-insensitive, cyanide-sensitive oxygen uptake yields superoxide as an intermediate (64,66). In peroxisomes, photorespiratory glycolate metabolism also involves hydrogen peroxide (67), but not superoxide production (68). Both superoxide and hydrogen peroxide generation by plant cell wall peroxidases have been observed (69,70,71). A soluble NADPH oxidase from leaf tissue may also be responsible for generation of H_2O_2 (72). Simultaneous presence of hydrogen peroxide and superoxide is conducive to formation of the hydroxyl radical as confirmed by ethylene evolution from methional (11), and intracellular peroxide concentration increases at the onset of fruit ripening in plants (73).

Oxygen Toxicity and Protective Mechanisms

Halliwell (74) has recently reviewed the literature regarding the toxicity of oxygen at the biochemical level. Among several possible mechanisms by which oxygen can exert deleterious effects are: (a) inactivation of enzymes by oxidation of critical thiol groups, (b) production of

hydrogen peroxide, free radicals, and singlet oxygen which may react with numerous cellular components (c) oxidation of lipids to lipid peroxidases which inhibit enzyme activity and disrupt membrane structure and function, and (d) diversion of normal metabolic pathways into undesirable routes. Sensitivity of A. nidulans to the presence of 100% oxygen was reported (75) and the effects of oxygen on higher plants was apparent from photooxidative death of leaf tissue demonstrated using paraquat (76). Application of this bipyridyl herbicide, known to autoxidize and produce superoxide, results in lipid peroxidation and breakdown of chloroplast membranes (63).

Photosynthetic cells of higher plant tissues contain chemical constituents, including pigments and enzymes which protect cellular components from oxidative damage by reactive oxygen species. In the chloroplast, which is most accessible to light-oxygen toxicity (61), scavenging of singlet oxygen produced by the photosensitizing action of chlorophylls (45,74) occurs at a rate which approximates the rate of diffusion (77) and appears to be a major carotenoid function. These polyenes also quench the triplet excited state of chlorophyll, preventing transfer of exci-

tation energy to oxygen (78). Singlet oxygen is scavenged by α-tocopherol as well (74). A list of antioxidants compiled by Asada (61) is presented in TABLE II: cellular concentrations of these biomolecules, reaction rates, and intracompartmental capacity for regeneration of reduced oxidants insure the integrity of the photosynthetic apparatus. Walker, et al. (79) reported that 2 mM $MnCl_2$ added to isolated spinach chloroplast suspensions not only lowered the concentration of superoxide, but also utilized H_2O_2 during the regenerative process as illustrated by equations II and III, but it was not clear that this dismutation was important in vivo.

$$2\ Mn^{2+} + 2\ O_2^{\cdot -} + 4\ H^+ \rightarrow 2\ H_2O_2 + 2\ Mn^{3+} \qquad \text{(II)}$$

$$2\ Mn^{3+} + H_2O_2 \rightarrow O_2 + 2\ Mn^{2+} + 2\ H^+ \qquad \text{(III)}$$

Reduced glutathione, GSH[1] (80), ascorbate (74,81), and glyoxylate (82) can also be oxidized by H_2O_2; ascorbate and sugars may play a role in scavenging the hydroxyl radical (61).

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[1] Abbreviations used in this text are defined in the List of Abbreviations, page ix.

TABLE II

Reactivity of chloroplast components with superoxide, their rate constants and concentrations (61)

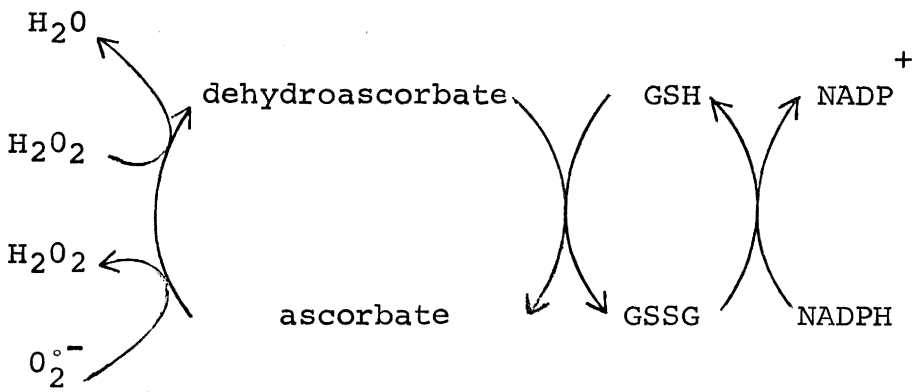
Reaction with $O_2^{\cdot -}$	Reaction Rate Constants (M^{-1} sec^{-1})	Concentration in Chloroplasts[a] (M)
cytochrome f (Fe^{3+}) → cytochrome f (Fe^{2+})	6.1×10^6 (pH 7.8)	6.2×10^{-5} [b,c]
plastocyanin (Cu^{2+}) → plastocyanin (Cu^{+})	1.1×10^6 (pH 7.8)	6.2×10^{-5} [b,c]
Mn^{2+} → Mn^{3+}	6.0×10^6 (pH 7.8)	4.0×10^{-4} [c]
ascorbate → dehydroascorbate	2.7×10^5 (pH 7.3)	2.5×10^{-3}
GSH → GSSG	6.7×10^5 (pH 7.8)	3.5×10^{-3}

[a] Values are average concentrations in intact chloroplasts.

[b] Value estimated assuming that chloroplast content is 1 molecule/400 molecules.

[c] Value estimated assuming that the concentration of chlorophyll in intact chloroplasts is 25 mM.

Protective mechanisms involving glutathione and ascorbate appear to be intimately related and involve several chloroplastic enzymes. The metabolic sequence (IV)



(IV)

proposed to function in the removal of superoxide and hydrogen peroxide generated in the chloroplast (81) also prevents formation of these activated forms of oxygen by using reducing equivalents from NADPH. In a paper given at the Fourth International Congress on Photosynthesis[2], Groden and Beck presented evidence for a chloroplastic ascorbate peroxidase. With the absence of catalase (EC 1.11.1.6) (59,73) and glutathione peroxidase (EC 1.11.1.9) (83,84)

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[2] Groden, D. and Beck, E. Characterisation of a membrane bound, ascorbate specific peroxidase from spinach chloroplasts. Fourth International Congress on Photosynthesis, 4-9 September, 1977, Reading, U.K. Details were obtained from a manuscript provided by the authors.

from chloroplasts, ascorbate peroxidase may well be the much sought intrachloroplastic enzyme for degradation of hydrogen peroxide generated within that organelle (23,61, 74). At the concentrations of reduced glutathione present in the chloroplast and the alkaline pH of the stroma during illumination, glutathione-dependent reduction of dehydroascorbate proceeds rapidly in the absence of an enzyme (80, 81); in plants, dehydroascorbate reductase (EC 1.8.5.1) required for ascorbate regeneration below pH 7.5 is localized in the cytosol (81). Reduction of glutathione, catalyzed by glutathione reductase (EC 1.6.4.2) found only in the chloroplast stroma in plant cells (81), occurs at the expense of NADPH generated photosynthetically during illumination and by glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activated in the dark (80). Equilibrium favors reduced glutathione which insures availability of this form for reduction of ascorbate and H_2O_2 as well as for protection of thiol groups of reductive pentose phosphate pathway enzymes against oxidative chemical species (81).

Degradation of extrachloroplastic H_2O_2 is accomplished by catalase in the peroxisome (65,73,85) and peroxidase (EC 1.11.1.7), distributed widely throughout plant cells

(74,86,87), according to the following reactions:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2 \qquad \text{(V)}$$

$$H_2O_2 + RH_2 \xrightarrow{\text{peroxidase}} R + 2\ H_2O \qquad \text{(VI)}$$

where RH_2 represents any one of a variety of possible reductants. Apparently catalase and other peroxisomal reactions are capable of destroying as much as 90% of the H_2O_2 produced by glycolate oxidase, maintaining H_2O_2 at nontoxic levels (65). Peroxidase-catalyzed utilization of H_2O_2 during lignification also results in lower intracellular levels of this compound (69).

Superoxide dismutases (EC 1.15.1.1), catalyze the reaction

$$O_2^{\cdot -} + O_2^{\cdot -} + 2\ H^+ \xrightarrow{\text{superoxide dismutase}} O_2 + H_2O_2\ . \qquad \text{(VII)}$$

When saturated with substrate [$K_m = (3.55 \pm 0.08) \times 10^{-4}$ M], these enzymes exhibit a rate constant [$V_{max} = (1.9 \pm 0.3) \times 10^9\ M^{-1}\ sec^{-1}$; pH 7.8, 8.5] which is a factor of 10^4 greater than the spontaneous rate of disproportionation at physiological pH values [$k = 2 \times 10^5\ M^{-1}\ sec^{-1}$, pH 7.4] (88-91). Proposed to produce only triplet state oxygen (32,74,92), superoxide dismutase restricts singlet oxygen formation

associated with spontaneous disproportionation of $O_2^{\cdot -}$ (49, 50); weak peroxidatic activity associated with the copper-zinc-containing superoxide dismutase (93) may also help limit production of $OH^{\cdot}$ Reaction I (74). Among higher plants superoxide dismutase activity, associated with at least two molecular forms of the enzyme (94), occurs in several subcellular locations, and multiple electrophoretic forms of both cyanide-sensitive and insensitive superoxide dismutases have been observed (95-98). The cyanide-sensitive copper-zinc enzyme, characteristic of higher eukaryotes, has been found in plants (61) in the cytosol (95,99), in etioplasts (100), and both in the stroma (99-102) and lamellae of chloroplasts (100,102,103); evidence for its existence in the intermembrane space of plant mitochondria has been presented (95). A cyanide-resistant, probably manganese-containing superoxide dismutase (94), occurs in the matrix of mitochondria (95,102) and has been reported to occur in spinach chloroplasts (99,102,104-106), but its presence on the outside of the chloroplast may only be due to association during isolation (102). Superoxide dismutase activity has not been observed in peroxisomes (107) or in the microsomal fraction (102).

A chloroplastic NADPH-glyoxylate reductase has been localized in chloroplasts (108) and may function in the dissipation of excess reducing equivalents via a glycolate-glyoxylate shuttle. In such a mechanism glyoxylate produced in the peroxisome during photorespiration would be transported to the chloroplast where excess NADPH formed there could be consumed in the reduction process. Glycolate produced would move back into the peroxisome where it could be oxidized for recycling. This mechanism would function as a Mehler-type reaction and thereby compete with, or eliminate, reduction of molecular oxygen by NADPH, minimizing production of reactive oxygen species (108). The proximity of chloroplasts and peroxisomes and elevated biosynthesis of glycolate under conditions of low carbon dioxide and high light intensity lend support for such a proposed glycolate-glyoxylate cycle (28).

Regulation of Oxidative Enzymes

Enzymes in higher plants can be induced or activated in response to changes in a specific environmental factor (109). While alterations in superoxide dismutase in higher plants in response to exposure to high oxygen tensions have

not yet been published, induction is predicted (74) on the basis of responses in bacteria (110,111), animals (112-114), yeast (115), blue-green algae (75), and green algae (104,116). In Streptococcus faecalis and Escherichia coli B, 16- and 25-fold increases in superoxide dismutase activity, respectively, occurred in rapid response to growth in hyperbaric oxygen (111) and cells grown under 100% oxygen contained elevated levels of superoxide dismutase which provided resistance to toxic effects of hyperbaric oxygen. In E. coli B this increase in superoxide dismutase activity was associated only with the manganese-containing enzyme (117), but high partial pressures of oxygen resulted in preferential synthesis of the iron-containing superoxide dismutase with some stimulation of the copper-zinc enzyme in Photobacterium leiognathi (110). Gregory, et al. (115), observed a 6.5-fold increase in the level of superoxide dismutase in Saccharomyces cerevisae grown under 100% oxygen compared to that present in anaerobically-grown cells. In this case both the cyanide-sensitive and cyanide-insensitive forms of superoxide dismutase were induced, and prior growth of S. cerevisiae at 1 atm of oxygen provided resistance to the lethal effects of 20 atm of oxygen. Superoxide dismutase activity is higher in lungs from

control mice exposed to ambient oxygen than in lungs from hypoxic mice (113), and Crapo and Tierney have shown that the enzyme is induced in lungs of 250-350 g rats during exposure to 85% oxygen, enabling rats previously acclimated to this level of oxygen to survive in 100% oxygen (112). The rate of oxygen tolerance paralleled the time course for the increase in superoxide dismutase activity in the lung, and the progressive loss of tolerance paralleled the decrease in pulmonary superoxide dismutase activity upon return of the animals to air. Crapo and coworkers (118) found that, on a whole lung basis, activity of both the copper-zinc- and manganese-containing superoxide dismutases was elevated by five days exposure to 85% oxygen. It has since been reported (114) that only mitochondrial superoxide dismutase in pulmonary macrophages is induced by exposure of ten-day old rats to 100% oxygen for 24 hours. Moreover, protein synthesis inhibitors revealed that higher levels of superoxide dismutase activity were due to enhancement of specific protein synthesis (113).

The first photosynthetic plant system for which correlation between superoxide dismutase and response to high oxygen tensions has been documented is Chlorella sorokiniana (116). Wild type cells exposed to high partial

pressures of oxygen for 48 hours exhibited twice as much superoxide dismutase activity on the basis of cell number as air grown cells; the magnitude of increase was less, but still significant, when expressed as a function of protein. An oxygen-resistant strain, which contained a 3.5-fold higher level of superoxide dismutase in air than the wild type strain and did not exhibit an increase in superoxide dismutase activity when grown under high oxygen tensions, tolerated higher light intensities before undergoing photooxidative damage. Induction of superoxide dismutase in Anacystis nidulans (75) required a high concentration of oxygen and occurred in response to growth of the blue-green alga in air. Absent from cells grown in nitrogen supplemented with carbon dioxide, superoxide dismutase protected the alga against photooxidative effects in pure oxygen. In Euglena, highest levels of superoxide dismutase in cells grown under photoautotrophic conditions implicate light as an additional causative agent in the adaptive formation of superoxide dismutase (104). Similarly, levels of superoxide dismutase were higher in light-grown cotton and maize leaves than in corresponding etiolated tissues (98).

Induction of catalase and peroxidase by hyperoxia and

hyperbaric oxygen have also been observed. As compared to enzyme levels in anaerobically-grown cells, a 2-fold increase in catalase activity following exposure of cells to 100% oxygen occurred in Bacillus subtilis (119), E. coli K-12 his^- (115), and S. cerevisiae (115). Of two electrophoretic forms of catalase observed in E. coli K-12, one was constitutive and not induced, the other appeared only in cells grown in air (120). Catalase activity evaluated on a whole lung basis was significantly higher in 250-350 g rats exposed to 85% oxygen (118) relative to air, and a 3-fold elevation in catalase level in pulmonary macrophages from ten day-old rats occurred in vivo and in vitro upon exposure to 100% oxygen due to specific protein synthesis (114). Catalase levels in fungi and cress grown in air have been compared with those observed after exposure of plants to hyperbaric oxygen (121). High pressure oxygen treatment of fungi resulted in a 2-5-fold increase in catalase activity during the first 1-3 days followed by a slow decrease which became more rapid after 15 days. Cress subjected to the same treatment exhibited an immediate decrease in catalase activity, and no activity remained after 15 hours. The change in level of catalase activity appeared

to be important as high levels of catalase in cress did not correlate with low susceptibility to the effects of hyperbaric oxygen. A 2-fold increase in peroxidase activity over anaerobic levels was observed in *E. coli* K-12 his^- grown in 100% oxygen (115). Further studies with *E. coli* K-12 grown in air revealed that catalase and peroxidase were co-induced with components of the respiratory chain (120). Increase in peroxidase activity which appeared before the increase in catalase activity was transitory while catalase reached a stable plateau.

Because of its proposed role in protecting cells against effects of oxygen, glutathione reductase activity has been predicted to increase rapidly upon exposure of organisms to atmospheres enriched in oxygen, but activity of this enzyme decreased in erythrocytes and remained unaltered in plasma during exposure to high oxygen tensions (122). In winter-hardy plants increases in glutathione reductase activity during cold weather are proposed to protect proteins during freeze dehydration by scavenging oxidizing agents or by reducing disulfide bonds (123).

Agricultural Significance of Cotton

Agricultural importance of the cotton plant arises from its salt tolerance and ability to grow in regions where limited water supply prohibits culture of many other crop species (124-127). Additionally, insecticidal activity of sesquiterpenoid aldehyde quinones in pigment glands of cotton tissues provides endogenous defenses against many insects (128). Moreover, the plant produces fiber which is both adaptable and durable and seed components suitable for human and livestock consumption and for the manufacture of various industrial commodities.

EXPERIMENTAL PROCEDURES

Materials

Cotton seeds, Gossypium herbaceum L., Cotton Branch 1697, were obtained from P. A. Fryxell, United States Department of Agriculture, College Station, Texas; G. hirsutum L., cv Coker 310 seeds were supplied by Coker Pedigree Seed Company, Hartsville, South Carolina. Rootone F ® used for propagation of cuttings was a product of Amchem Products, Incorporated (Freemont, California); Terra-lite ® commercial medium grade vermiculite and perlite used for the potting mixture were purchased from Wetzel Seed Company (Harrisonburg, Virginia).

For studies involving altered levels of atmospheric components, compressed oxygen, grade 2.6 (99.6% minimum purity) and carbon dioxide, grade 2.8 (99.8% minimum purity), both contaminated only with air, were obtained from Airco, Air Reduction Company, (New York, New York). Filters for compressed air lines were obtained from Deltech Engineering, Incorporated (New Castle, Delaware). Analyzed, nitrogen-balanced carbon dioxide mixed gases used as standards for infrared gas analysis were products of Matheson (East

Rutherford, New Jersey).

Liquid nitrogen for rapid freezing of cotton leaf tissue was obtained from Chemtron (Richmond, Virginia). For extraction of soluble protein, insoluble polyvinylpyrrolidone (PVP), Polyclar AT, was generously provided by General Aniline and Film Corporation (Linden, New Jersey); soluble polyvinylpyrrolidone, PVP-10 (average molecular weight 10,000), Tris (Sigma 7 - 9 and Trizma Base), D-isoascorbic acid, aerosol silicone antifoam, and Sephadex G-50-300 (100-300 μ mesh) for gel filtration chromatography were purchased from Sigma Chemical Company (St. Louis, Missouri). Sephadex G-10 (40-120 μ mesh) and G-50 (20-80 μ mesh) were products of Pharmacia Fine Chemicals (Piscataway, New Jersey).

Xanthine, sodium salt, grade III; xanthine oxidase, grade I or III; cytochrome *c*, type III; nitro blue tetrazolium, grade III; horseradish peroxidase; oxidized glutathione, grade II; *p*-nitrophenyl phosphate, disodium salt (Sigma 104 phosphatase substrate); NADH, disodium salt, grade III; NADPH, tetrasodium salt, type I; glyoxylic acid, sodium salt; and bovine serum albumin, fatty acid-free, components of protein and enzyme assay mixtures, were

purchased from Sigma Chemical Company (St. Louis, Missouri). Glycolic acid, 99 + %, and 3,3'-diaminobenzidine were obtained from Aldrich Chemical Company, (Milwaukee, Wisconsin); Coomassie brilliant blue G-250 and 2,3',6-trichloroindophenol, sodium salt were purchased from Eastman Kodak Company (Rochester, New York); oxalacetic acid was a product of Calbiochem (San Diego, California). Scintillation grade Triton X-100 was obtained from Amersham/Searle Corporation (Arlington Heights, Illinois).

For analytical gel electrophoresis, high purity acrylamide and N,N'-methylenebisacrylamide were obtained from Ames Company (Elkhart, Indiana); electrophoresis-grade ammonium persulfate from Bio-Rad Laboratories (Richmond, California); N,N,N',N'-tetramethylenediamine (TEMED) from Canalco (Rockville, Maryland); riboflavin from Mann Research Laboratories (New York, New York); and ammonia-free glycine from Nutritional Biochemical Corporation (Cleveland, Ohio).

Other chemicals used were reagent grade.

METHODS

Culture of Plants

Diploid plants, Gossypium herbaceum L. C.B. 1697, were generated by propagation of cuttings such that all plants were the product of a single seed, thus minimizing genetic variation. Cuttings 12-15 cm in length were treated with a commercial auxin-containing fungicide and planted in nutrient-moistened vermiculite, 10-15 cuttings per 25 cm plastic pots. After 4-5 weeks under conditions used for maintenance of growing plants, well-rooted cuttings were transplanted individually into 15 cm plastic pots containing a 1:1 perlite:vermiculite mixture.

Plants were maintained under conditions favorable for rapid leaf development in controlled environment growth chambers manufactured for the Virginia Agricultural Experiment Station at Virginia Polytechnic Institute by the Sherer Gillett Company, Marshall, Michigan. A photoperiod consisting of 15 hours per day fluorescent lighting and 16 hours per day incandescent lighting, extending 0.5 hour before and after the fluorescent exposure period, was used; plants were illuminated with photosynthetically active

radiation of 200-300 $\mu E\ m^{-2}\ sec^{-1}$ as measured with a Lambda Instrument Corporation (Lincoln, Nebraska) model LI-185 Quantum/Radiometer/Photometer equipped with a Quantum flux sensor. Temperature was maintained at 30 ± 1°; relative humidity within chambers was 70-80%. Plants were watered on alternate days with 20% Hoagland's nutrient solution #1 (129), TABLE III, and nutritional status of plants was evaluated on the basis on mineral composition of leaf tissue and the potting medium. Analyses of the potting medium were performed by the Virginia Polytechnic Institute and State University Soils Testing Laboratory according to their soil testing procedures manual (130). After extracting the potting medium, pH was determined directly using a pH meter, soluble salts by conductivity, and nitrate by an ion-specific electrode. Potassium was assayed by flame photometry, phosphorous by absorption spectroscopy, and calcium, magnesium, and manganese were quantified by atmoic absorption spectrophotometry. Nutrient composition of the potting medium prior to use is reported in TABLE IV. Following acid digestion of dried leaf tissue, calcium, magnesium, manganese, and iron were quantified by atomic absorption spectrophotometry according to procedures detailed in the

TABLE III

Composition of Hoagland's nutrient solution #1 (129)

Component[a]	Concentration
Macronutrients[b]	mM
KH_2PO_4	1
KNO_3	5
$Ca(NO_3)_2$	5
$MgSO_4$	2
$FeSO_4$[c]	0.1
Fe-citrate	0.1
Micronutrients[d]	ppm
H_3BO_3	0.5
$MnCl_2$	0.5
$ZnSO_4$	0.05
$CuSO_4$	0.02
H_2MoO_4	0.01

[a] Nutrient was prepared with tap distilled water and adjusted to pH 6.8 with 1 N KOH.

[b] Macronutrients were added as concentrated stock solutions.

[c] Stock solution was prepared immediately before use.

[d] Micronutrients were added as a single, concentrated stock solution.

TABLE IV

Analysis of mineral composition of potting medium

Nutrient	Mineral, ppm		
	Perlite[a]	Vermiculite[a]	1:1 Perlite: Vermiculite[b]
pH	7.0±0.1	6.4±0.1	6.8±0.1
Calcium	160±27	310±33	560±60
Magnesium	16±10	480[c]	480[c]
Phosphorus	5±3	37±9	38±9
Potassium	40±18	330±58	510±51
Soluble salts	<10	<10	42±60
Nitrate	<5	<5	7±2
Manganese	3±1.3	0.2±0.07	8±2

[a] Values represent an average ± 1 standard deviation for 3 replicates.

[b] Values represent an average ± 1 standard deviation for 4 replicates.

[c] Under standard assay conditions, concentration was greater than upper limit of detection.

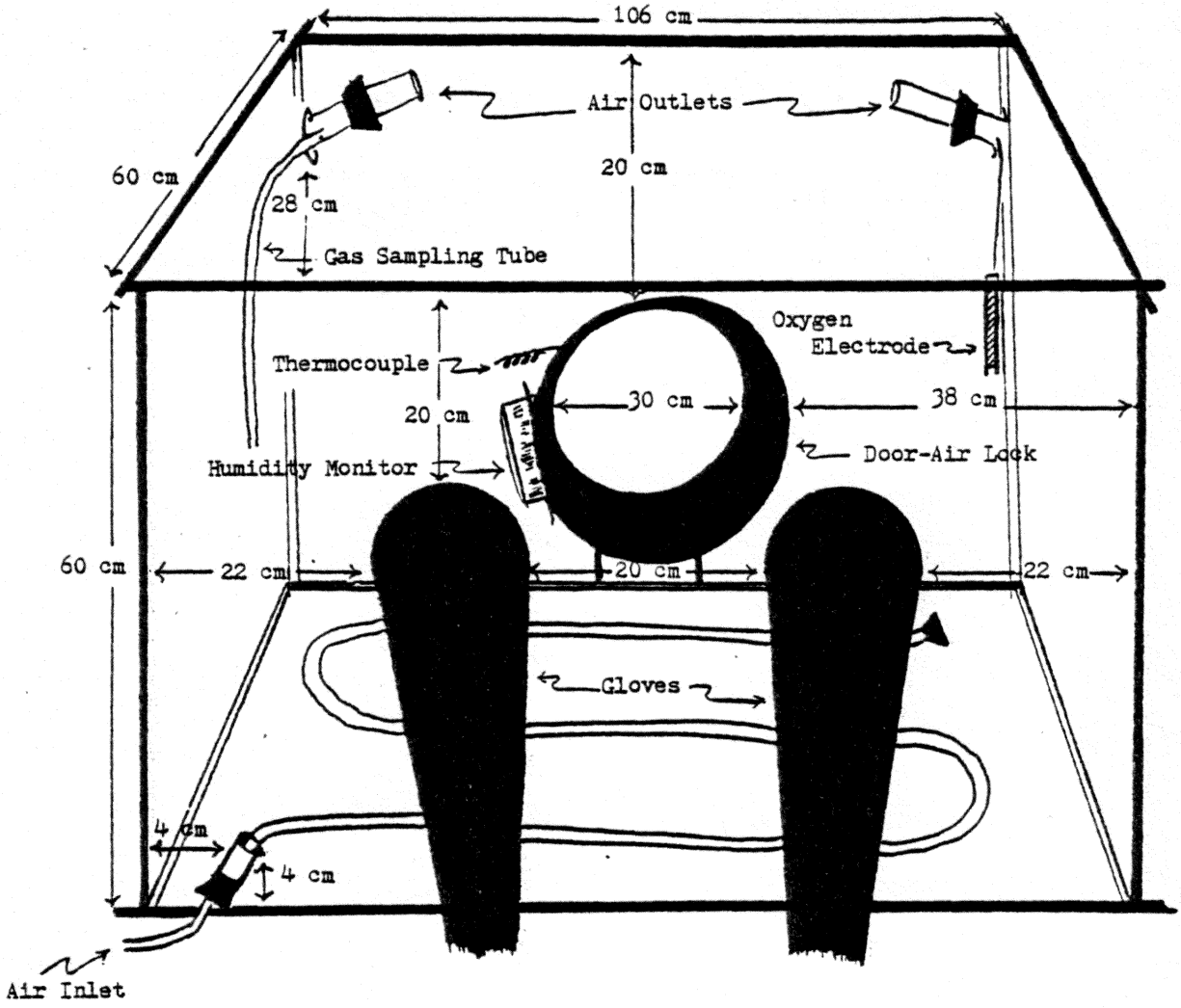
Perkin-Elmer atomic absorption handbook (131).

Elevated Oxygen Studies

Growth Chambers - For studies of effects of elevated concentrations of atmospheric oxygen on levels of enzyme in cotton leaf tissue, two 12 mil 60 x 60 x 106 cm, vinyl chambers designed to be erected inside Sherer chambers described for growth and maintenance of plants were constructed by the G-F Supply Division, Standard Safety Equipment Company (Palatine, Illinois) according to FIGURE 1. A 3.3 m length of tygon tubing (10 mm i.d.), sealed at one end, was connected to the entry port at the base of the chamber and looped across the floor, terminating diagonally across from the entry port as indicated in FIGURE 1. Holes were punched in the tubing parallel to the floor in the following manner to provide even dispersion of incoming gas and uniform circulation of gases within the chamber: from chamber inlet for a distance of 0.3 m, no holes; from 0.3 m to 0.9 m, 3 mm diameter holes on alternating sides of the tubing spaced 15 cm apart; from 0.9 to 2.1 m, 4 mm holes through both walls of the tubing spaced 15 cm apart; from 2.1 m to the end of the tubing, 5 mm holes through both walls spaced

FIGURE 1

Design of vinyl growth chambers for environmental oxygen studies.


106 cm
Air Outlets
20 cm
60 cm
28 cm
Gas Sampling Tube
Oxygen
Electrode
Thermocouple
20 cm
30 cm
38 cm
Humidity Monitor
Door-Air Lock
60 cm
22 cm
20 cm
22 cm
Gloves
4 cm
4 cm
Air Inlet

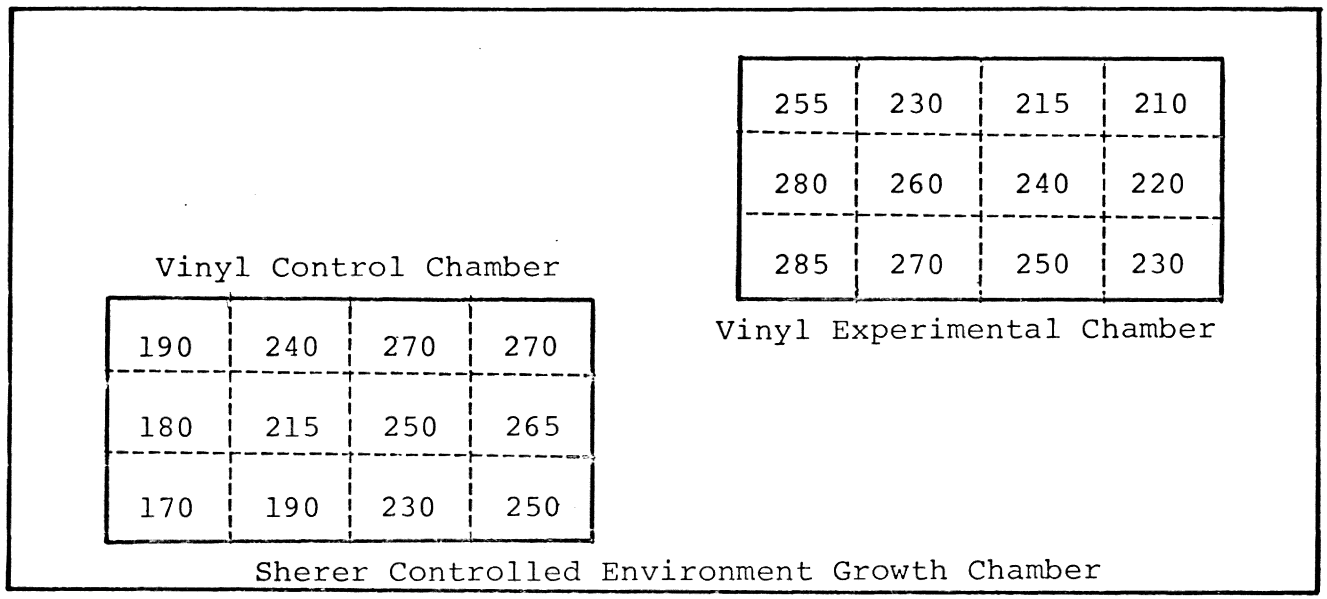
15 cm apart. Gases exited the chamber through outlet ports located at each end of the top of the chamber. For studies involving greater than ambient concentrations of atmospheric oxygen, exiting gases were funnelled out of the Sherer chamber via tygon tubing which did not restrict the normal flow of gas passing through outlet ports. Each vinyl chamber was equipped with an Abbeon Cal (Santa Barbara, California) model HTAB 169B relative humidity and temperature indicator mounted on the door frame. Gases introduced into these chambers were bubbled through water as necessary to maintain the desired relative humidity. Day/night temperatures in the Sherer chamber were established to maintain a temperature of 30 $\pm$ 1° within the vinyl chambers as monitored with a copper/constantan thermocouple. Photosynthetically active radiation at mid-chamber height is given in FIGURE 2 for centers of positions indicated.

Chamber Atmosphere - Filtered air from a compressed air line, alone or mixed with specified quantities of carbon dioxide and oxygen from compressed gas cylinders, was flushed through chambers at a rate of 9.5 $\pm$ 0.5 l/min to give about 1.5 volume changes per hour. Air and oxygen

FIGURE 2

Photosynthetically active radiation at mid-chamber height in vinyl growth chambers.

Light intensities determined at centers of sections are reported as $\mu E/m^2$ sec.


Vinyl Control Chamber
190 240 270 270
180 215 250 265
170 190 230 250
255 230 215 210
280 260 240 220
285 270 250 230
Vinyl Experimental Chamber
Sherer Controlled Environment Growth Chamber

were passed individually through Gilmont (great Neck, New York) #13 or Matheson (East Rutherford, New Jersey) #604 flowmeters and into 4 l heavy walled Erlenmeyer flasks which served as mixing vessels; flow rates for carbon dioxide were monitored with a Gilmont #11 flowmeter. Gas mixtures from the mixing flasks were channelled into the closed vinyl chambers.

Atmospheric composition was analyzed periodically during the course of an experiment and appropriate adjustments were made in flow rates of individual gases to maintain concentrations of oxygen and carbon dioxide within specified ranges. Carbon dioxide was monitored by sampling the chamber atmosphere with a Cole Parmer (Chicago, Illinois) model 2 Dyna-vac pressure and suction pump, collecting withdrawn gases in Markson (New City, New York) 100% saran 5 l gas-collecting bags. Specific analysis for carbon dioxide was performed using a Beckman (Fullerton, California) model 865 infrared gas analyzer standardized with Matheson (East Rutherford, New Jersey) carbon dioxide-analyzed, nitrogen balanced mixed gases. All measurements were made using a flow rate of 3 l/min. Atmospheric oxygen concentrations were monitored continuously using a Beckman (Fullerton,

California) model 1008 Fieldlab oxygen analyzer standardized against carbon dioxide and air.

Tissue Acclimation and Exposure to Altered Atmospheres- Plants were introduced randomly into vinyl chambers and acclimated for 1-3 days at ambient levels of oxygen and carbon dioxide. Following acclimation, oxygen, supplemented with carbon dioxide, was introduced into the experimental chamber at a rate of 20 l/min to elevate the oxygen concentration to 75 ± 5% within 10-15 min. The flow rate was then reduced to that used during acclimation and the ratio of individual gases was regulated to maintain this level of oxygen in the presence of ambient concentrations of carbon dioxide (350 ± 50 ppm). Acclimation conditions were maintained for the air control. Transpiration rates were measured using a Lambda Instruments Corporation (Lincoln, Nebraska) model LI-60 diffusive resistance meter.

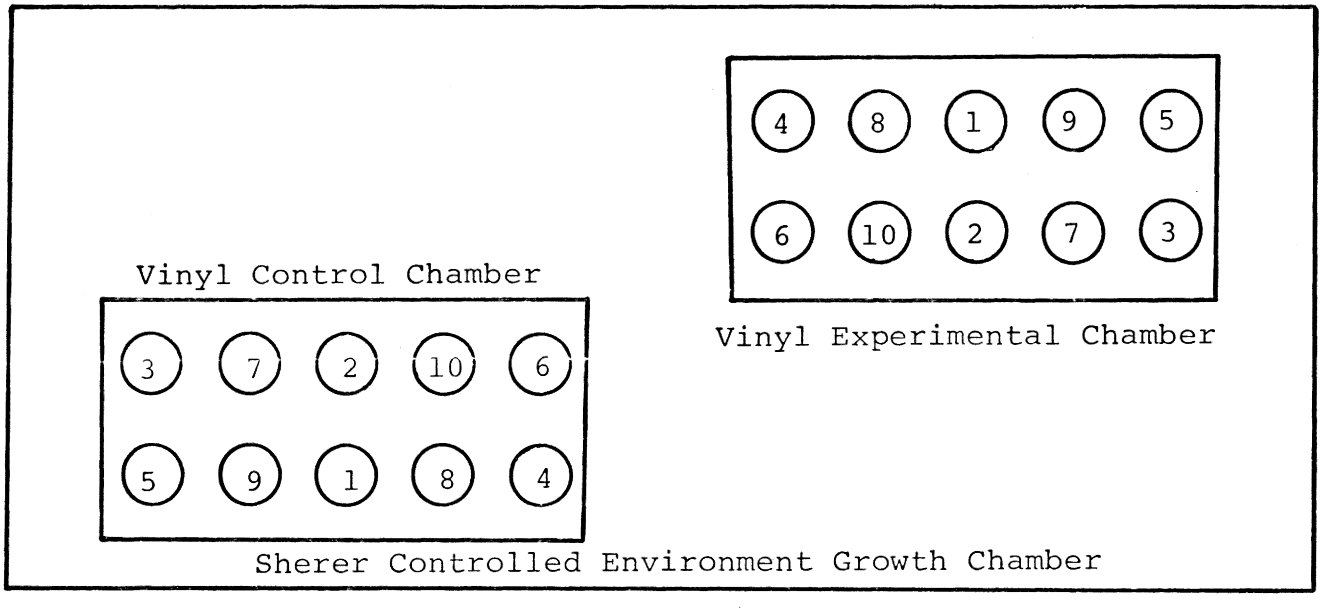
Tissue Harvest - Positions in chambers were numbered as indicated in FIGURE 3 so that possible position or chamber effects could be evaluated. A similar designation was used when fewer plants were present. To monitor time-related responses to environmental conditions, plants were harvested in numerical sequence, two plants per chamber

FIGURE 3

Plant locations and harvest sequence

for evaluation of position effects.

For time-course studies plants were harvested in numerical order, 2 plants per harvest time. For end-point studies all plants in a chamber were harvested at the same time.


Vinyl Control Chamber
3 7 2 10 6
5 9 1 8 4
4 8 1 9 5
6 10 2 7 3
Vinyl Experimental Chamber
Sherer Controlled Environment Growth Chamber

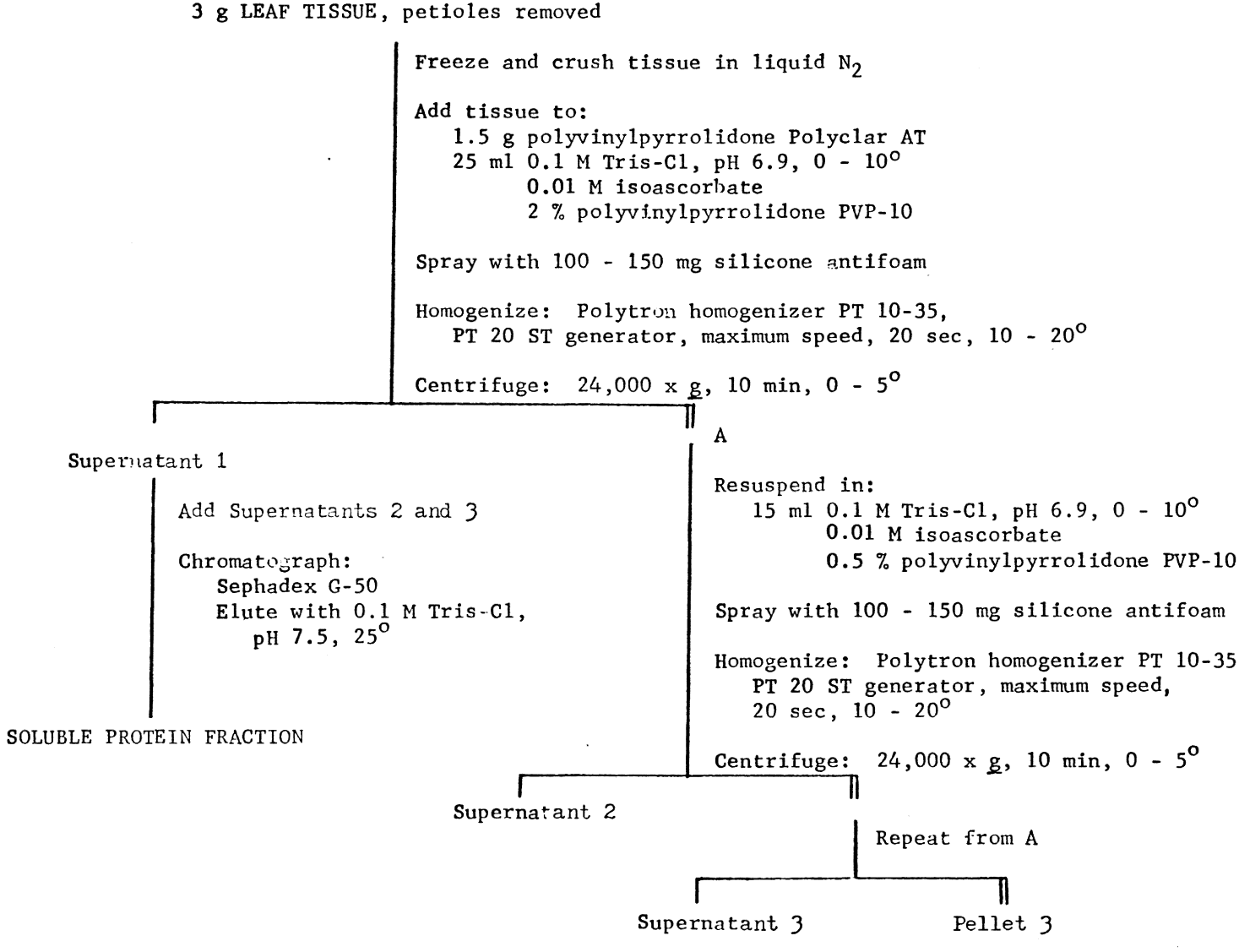
per harvest time. For such studies harvests were made after 24 hours of acclimation, after 72 hours of acclimation immediately prior to exposure to altered atmospheric levels of oxygen, and after 24, 48, and 72 hours exposure to elevated oxygen; four plants were harvested prior to the acclimation period. Alternatively, high oxygen and air control experiments were staggered by 48 hours, and after a specified period of treatment, all plants in the chamber were harvested. At harvest time fully expanded leaves, 15-30 days of age were selected. Each sample was composed of tissue from only one plant; all harvests were made at the midpoint of the light period to prevent variations in data arising from possible diurnal rhythms.

Protein Preparation

Soluble protein was extracted from leaf tissue according to the procedure outlined in FIGURE 4. Grinding media containing 0.01 M isoascorbate were prepared using 0.1 M potassium phosphate, pH 7.5 or 0.1 M Tris-Cl, pH 7.5. Following addition of isoascorbate and soluble polyvinylpyrrolidone, pH was adjusted to 6.9 with 1 N HCl to provide for optimal effectiveness of polyvinylpyrrolidone.

FIGURE 4

Optimized procedure for extraction of soluble protein from cotton leaf tissue.


3 g LEAF TISSUE, petioles removed
Freeze and crush tissue in liquid N_2
Add tissue to:
1.5 g polyvinylpyrrolidone Polyclar AT
25 ml 0.1 M Tris-Cl, pH 6.9, 0 - 10°
0.01 M isoascorbate
2 % polyvinylpyrrolidone PVP-10
Spray with 100 - 150 mg silicone antifoam
Homogenize: Polytron homogenizer PT 10-35,
PT 20 ST generator, maximum speed, 20 sec, 10 - 20°
Centrifuge: 24,000 x g, 10 min, 0 - 5°
Supernatant 1
Add Supernatants 2 and 3
Chromatograph:
Sephadex G-50
Elute with 0.1 M Tris-Cl,
pH 7.5, 25°
SOLUBLE PROTEIN FRACTION
A
Resuspend in:
15 ml 0.1 M Tris-Cl, pH 6.9, 0 - 10°
0.01 M isoascorbate
0.5 % polyvinylpyrrolidone PVP-10
Spray with 100 - 150 mg silicone antifoam
Homogenize: Polytron homogenizer PT 10-35,
PT 20 ST generator, maximum speed,
20 sec, 10 - 20°
Centrifuge: 24,000 x g, 10 min, 0 - 5°
Supernatant 2
Repeat from A
Supernatant 3
Pellet 3

Insoluble polyvinylpyrrolidone was premoistened in grinding medium containing 2% PVP-10; vacuum infiltration for 15-20 minutes removed trapped air which would have otherwise interfered with tissue homogenization and maximized potential for binding phenolic compounds. When liquid nitrogen was not used, tissue was chopped and infiltrated with isolation medium in the same manner prior to tissue disruption. Most efficient disruption of cotton leaf tissue added to the grinding medium in the polycarbonate tube was achieved by the combined impact and shearing forces produced by a Brinkmann Instruments (Westbury, New York) Polytron model PT 10-35 tissue homogenizer equipped with a PT 20 ST saw toothed generator. Chilling the generator on ice prior to each use and placing samples in an ice-salt water bath during homogenization was necessary to maintain sample temperature at 10-20°; addition of 100-150 mg silicone antifoam prevented excessive foaming. All centrifugations to sediment insoluble PVP and tissue fragments were performed at 0-5°. Gel filtration chromatography of combined supernatants (approximately 45 ml) on a 2.5 x 45 cm Sephadex G-50 column at 20-25° was necessary to separate soluble proteins from isoascorbate, soluble PVP, and

endogenous small molecular weight molecules which interfere with analytical procedures. Column eluate collected in 2 ml fractions was monitored at 280 nm; UV-absorbing fractions defining the initial peak were pooled, then analyzed for protein and enzymatic activity.

Proteins which remained associated with the particulate fraction, pellet 3, were extracted as outlined in FIGURE 5. Soluble protein released by the individual homogenizations indicated in FIGURES 4 and 5 was obtained by chromatographing each supernatant (approximately 15 ml) separately.

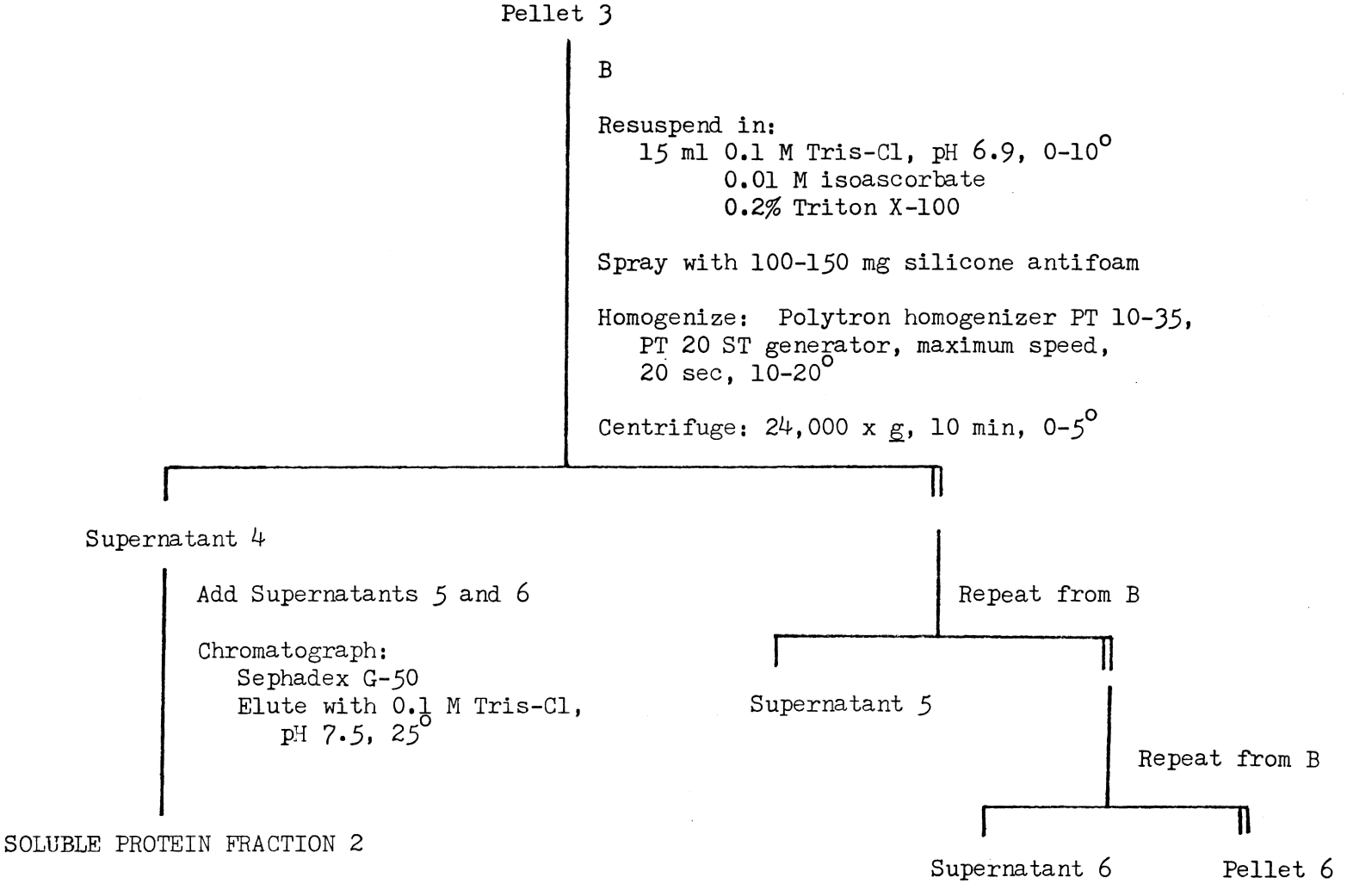
Analytical Procedures

Protein Analysis - Protein determinations were carried out using the Coomassie G-250 dye-binding assay (131,132) with crystalline bovine serum albumin as the standard reference protein. Analyses performed prior to the development of the Coomassie procedure utilized the technique of Lowry, et al., (134).

Superoxide Dismutase (EC 1.15.1.1) - Superoxide dismutase present in extracts was assayed by its ability to inhibit the reduction of ferricytochrome c according to

FIGURE 5

Extraction of protein associated with tissue fragments and insoluble PVP.


Pellet 3
B
Resuspend in:
15 ml 0.1 M Tris-Cl, pH 6.9, 0-10°
0.01 M isoascorbate
0.2% Triton X-100
Spray with 100-150 mg silicone antifoam
Homogenize: Polytron homogenizer PT 10-35,
PT 20 ST generator, maximum speed,
20 sec, 10-20°
Centrifuge: 24,000 x g, 10 min, 0-5°
Supernatant 4
Add Supernatants 5 and 6
Chromatograph:
Sephadex G-50
Elute with 0.1 M Tris-Cl,
pH 7.5, 25°
SOLUBLE PROTEIN FRACTION 2
Repeat from B
Supernatant 5
Repeat from B
Supernatant 6
Pellet 6

the method of McCord and Fridovich (135). The assay was carried out in 3.0 ml at 25° in a reaction mixture composed of 0.05 M potassium phosphate, pH 7.8, 0.1 M EDTA, 10^{-2} mM ferricytochrome c, 5 x 10^{-2} mM xanthine, sample, and sufficient xanthine oxidase to reduce ferricytochrome c at 550 nm at a rate of 0.025 $\pm$ 0.001 absorbance units per minute. Prior to use in assays, commercially prepared xanthine oxidase was dialyzed overnight at 0-5° against a buffer composed of 10 mM potassium phosphate, pH 7.8, 1 mM sodium salicylate, and 0.005% EDTA[3]. Aliquots were diluted with this buffer immediately before each use such that 10-12 µl produced the standard rate of ferricytochrome c reduction. One unit of superoxide dismutase activity was defined as that amount which would inhibit the standard rate of reduction of ferricytochrome c by 50%. A calibration curve based on the response produced by purified bovine erythrocyte superoxide dismutase was used in determining specific activities of superoxide dismutase in cotton leaf extracts. Assays were carried out in the absence of xanthine oxidase

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[3] Gregory, E. M., Personal communication.

to evaluate endogenous interference. Relative amounts of the cyanide-sensitive Cu-Zn superoxide dismutase and the cyanide-insensitive forms of the enzyme were quantified by the inclusion of 1 mM cyanide in the reaction mixture.

The assay of Beauchamp and Fridovich (136) for superoxide dismutase based on nitro blue tetrazolium reduction on polyacrylamide gels was used for the detection of electrophoretically distinct forms of the enzyme. Analytical polyacrylamide disc gel electrophoresis was performed using the standard pH 8.9, 7.0% gel system developed by Davis (137). Samples were layered on gels in 5% sucrose. Following electrophoresis at 2 mA per tube for 1.5-2 hours, gels were incubated in 2.5 mM nitro blue tetrazolium for 20 minutes, then in a solution containing 28 mM TEMED, 2.8 x 10^{-2} mM riboflavin, and 36 mM potassium phosphate, pH 7.8 for 15 minutes. Gels were removed from solution and illuminated to initiate dye reduction via superoxide. Inactivation of the cyanide-sensitive copper-containing form of the enzyme, with a resulting loss of the associated achromatic bands on gels, was accomplished by the inclusion of 1 mM cyanide in both solutions used in the gel-staining process.

Catalase (EC 1.11.1.6) - Determination of catalase activity was performed at 25° according to Beers and Sizer (138). Rates for endogenous, nonenzymatic decomposition of H_2O_2 were determined by assaying boiled fractions of the extracts.

Glycolate Oxidase (EC 1.1.3.1) - Glycolate oxidase was assayed using a modification[4] of the diaminobenzidine procedure proposed by Cohen (139). The relatively insoluble diaminobenzidine was prepared as a stock solution by homogenizing 0.5 mg diaminobenzidine/ml in a glass homogenizer. Analysis was carried out in 3.0 ml in an assay mixture which contained 0.1 ml solubilized diaminobenzidine, 60 µg horseradish peroxidase, 10 mM glycolate, and sample in 0.3 M potassium phosphate, pH 8.3, 25°. Hydrogen peroxide formed during glycolate oxidation, when coupled to the oxidation of diaminobenzidine by horseradish peroxidase, produces an oxidized chromophore which can be monitored at 352 nm. The reaction mixture was monitored prior to and following addition of glycolate to determine the level of glycolate-dependent oxidation of diaminobenzidine. One

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[4] Russ, P. N., Personal communication.

unit of glycolate oxidase activity was defined as that amount necessary to produce an absorbance change of 0.01 unit per minute.

Peroxidase (EC 1.11.1.7) - Extracts were assayed for peroxidase activity using the trichloroindophenol assay of Nickel and Cunningham (140). Calculations were perfomed using an apparent extinction coefficient of 273 mM^{-1} cm^{-1} for oxidized trichloroindophenol determined using a known amount of peroxidase quantified with the diaminobenzidine assay of Cohen (139)[5]. Nonenzymatic oxidation of reduced trichloroindophenol by components present in extracts was determined by assaying boiled samples.

Glutathione Reductase (EC 1.6.4.2) - The procedure of Schaedle and Bassham (141) was used to determine glutathione reductase activity in soluble protein extracts at 25°. Endogenous interference was evaluated by monitoring the reaction mixture in the absence of glutathione.

Glyoxylate Reductase (EC 1.1.1.26) - Glyoxylate reductase was quantified at 25° according to the method of Tolbert et al. (71), and endogenous interference was eval-

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[5] Gregory, E. M., Personal communication.

uated by monitoring the reaction mixture in the absence of glyoxylate.

Malate Dehydrogenase (EC 1.1.1.37) - The assay of NADH-specific malate dehydrogenase was carried out at 25° in the direction of malate production (142), and endogenous interference was evaluated in the absence of substrate.

Acid Phosphatases (EC 3.1.3.2) - Acid phosphatase activity was determined at 40° according to DeLeo and Sacher (143).

Statistical Analysis of Data

Significant differences between enzyme activities and protein levels in high-oxygen samples and air controls were evaluated statistically using a two sample, one tail t-test. When variances were not equal as determined by variance ratio tests, the t-statistic was approximated by the Behrens-Fisher test (144) for which

$$t_{obs} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \qquad \text{(VIII)}$$

and

$$df = \frac{\left(\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}\right)^2}{\frac{\left(\frac{S_1^2}{n_1}\right)^2}{n_1}\frac{\left(\frac{S_2^2}{n_2}\right)^2}{n_2}} \qquad \text{(IX)}$$

where t_{obs} is the approximated t-statistic, df is the number of degrees of freedom, and for the two sets of data, $\bar{x}_1$ and $\bar{x}_2$ are the averages, S_1^2 and S_2^2 are the variances, and n_1 and n_2 are the numbers of observations. Because the large numbers of tests performed on the data increases the liklihood of rejecting the hypothesis when it should not be rejected, only those differences having a P value less than 0.01 were considered to be significant.

RESULTS

Culture of Plants

G. herbaceum C.B. 1697 plants which were maintained on Huffman's nutrient (145), TABLE V, for 4-6 months developed aberrantly; leaf tissue became chlorotic; and variable biochemical information was obtained. To evaluate impact of nutrient composition on plant growth, a short-term experiment was designed to compare Hoagland's nutrient, which contains only nitrate as the nitrogen source (TABLE III), with Huffman's nutrient which contains both nitrate (60%) and ammonia (40%).

It was determined from the generally excellent appearance of leaf tissue, as well as mineral analyses of both the potting medium and leaf tissue, that a 1:1 perlite: vermiculite mixture, supplied with Hoagland's nutrient, supported plant growth, providing tissue of consistent quality for biochemical and physiological studies. The pH of potting media supplied with Huffman's nutrient became quite acid (pH 4.8) after short-term growth of newly propagated plants, although pH in the absence of plants remained constant. The pH of medium watered with Hoagland's nutrient

TABLE V

Composition of Huffman's nutrient (145)

COMPONENT[a]	CONCENTRATION
Macronutrients[b]	mM
$NH_4H_2PO_4$	2
NH_4NO_3	4
KNO_3	6
$CaCl_2$	4
$MgSO_4$	1
Fe-EDTA	0.05
Micronutrients[c]	ppm
H_3BO_3	0.3
$MnCl_2$	0.1
$ZnSO_4$	0.1
$CuSO_4$	0.03
H_2MO_4	0.05

[a] Nutrient prepared with tap distilled water had a pH of 6.8.

[b] Macronutrients were added as concentrated stock solutions.

[c] Micronutrients were added as a single concentrated stock solution.

remained constant, even in presence of plant growth. Nitrate and calcium concentrations in the potting medium correlated with concentrations of these components in nutrient solutions, and high accumulation of soluble salts during this time period occurred with both nutrient solutions.

While leaves of plants grown with full-strength Hoagland's nutrient remained turgid and uniformly pigmented for the entire 8-week experimental period, leaves of plants maintained on full strength Huffman's nutrient became chlorotic after 3-5 weeks, lost turgor after 6-7 weeks, and became dessicated and crumbled to the touch after 8 weeks. Appearance of brown specks along veins and margins of lower leaf surfaces, a marked decrease in the uptake of moisture from the potting medium, and ultimately death of plants indicated stress related to nutrient imbalances. Mineral analysis of this leaf tissue revealed high levels of magnesium, TABLE VI. Plants grown for short periods on full strength Hoagland's nutrient or maintained on 20% nutrient for 1-2 yr and repeatedly pruned and permitted to leaf out exhibited levels of magnesium which fell within the sufficiency range for cotton leaf tissue. Leaf concentrations

TABLE VI

Mineral content of cotton leaf tissue

Nutrient Treatment	Mineral, ppm			
	Ca	Mg	Fe	Mn
Short-term, Full-strength Huffman's Nutrient[a]	19,000	13,000	200	680
Short-term, Full-strength Hoagland's Nutrient[a]	23,000	9,000	250	580
Long-term, 20% Hoagland's Nutrient[b]	19,000	9,000	130	730
Sufficiency Range[c]	19,000-35,000	3,000-9,000	30-300	30-350

[a] Newly propagated cuttings transplanted into 1:1 perlite:vermiculite were supplied with full strength nutrient (500 ml/application) twice weekly for 8 weeks. Values represent an average of triplicates.

[b] Plants propagated by cuttings were maintained for 1-2 years on 20% Hoagland's nutrient supplied on alternate days at a rate of 300±100 ml/application. Values represent an average of 4 replicates.

[c] Sufficiency data for cotton were reported by Sabbe and MacKenzie (146).

of the micronutrient manganese were similar in both treatments, but greater than the sufficiency level, for all growth conditions. Further, levels of iron were adequate and clearly not implicated in abnormal leaf appearance.

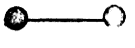
Extraction of Soluble Protein and Active Enzymes

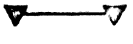
Extraction of soluble protein from leaf tissue of G. herbaceum C.B. 1697 in the presence of only buffered isoascorbate resulted in yields of less than 1 mg protein/g fresh tissue, FIGURE 6. A pink color, characteristic of these extracts, correlated with a low yield of protein, but actual levels varied with harvest time during the day/night cycle, and highest yields occurred at the end of the light period. Inadequate tissue disruption was not responsible for low protein yields as microscopic evaluation was necessary to discern nondisrupted tissue fragments. Increasing the concentration of the antioxidant, isoascorbate, and including the serine protease inhibitor, phenylmethylsulfonyl fluoride, metal chelators, EDTA and EGTA, or scavengers of the hydroxyl radical, benzoate and mannitol, during extraction failed to produce better protein yields. Addition of Dowex-1 (Cl^-) to scavenge phenolic

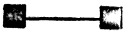
FIGURE 6

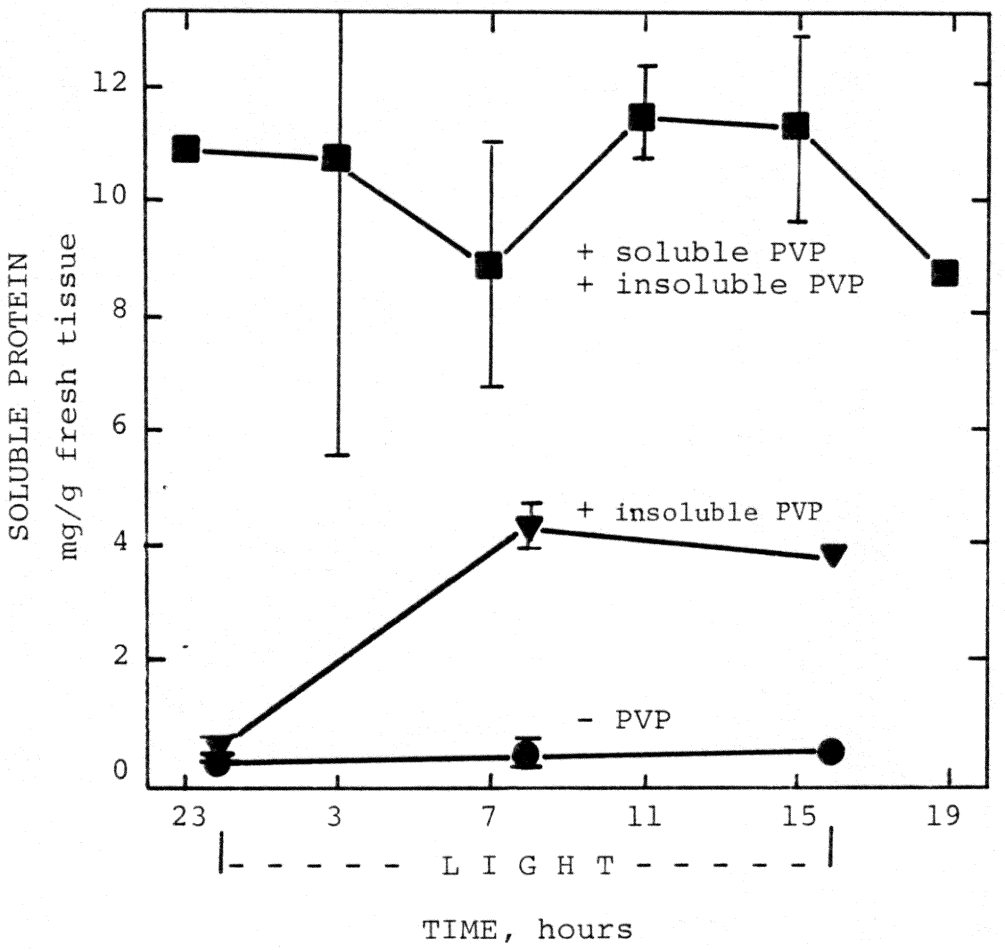
Soluble protein isolated from G. herbaceum C.B. 1697 leaf tissue as a function of harvest time and composition of medium

All extracts were prepared from two sequential homogenizations in 0.1 M potassium phosphate, pH 7.5 containing 0.01 M isoascorbate. Where indicated 0.5 g Polyclar AT/g fresh tissue was present. As designated, 1% (w/v) PVP-10 was added to the initial homogenization medium; during the second homogenization of the residue obtained by centrifugation, 0.5% PVP-10 was present in buffered isoascorbate. Range bars indicate the magnitude of variability.


No polyvinylpyrrolidone;


Insoluble polyvinylpyrrolidone;


Soluble + insoluble polyvinylpyrrolidone


SOLUBLE PROTEIN
mg/g fresh tissue
12
10
8
6
4
2
0
+ soluble PVP
+ insoluble PVP
+ insoluble PVP
- PVP
23
3
7
11
15
19
L I G H T
TIME, hours

compounds released during homogenization was likewise totally ineffective.

Hydrogen bonding of phenolic compounds by various grades of polyvinylpyrrolidone included in extraction media did improve results (FIGURE 6) due to decreased availability of tanning reagents for binding proteins or for oxidation to quinones which reduce protein solubility by covalent modification. Addition of insoluble PVP at a level of 0.5 g dry powder/g fresh tissue, as used by O'Sullivan and Wedding (147) for G. hirsutum leaf tissue, resulted in yields of protein which were quite variable but which increased during the light period. Yields as high as 4-5 mg/g tissue were observed at the midpoint and end of the light cycle, but levels of protein obtained from tissue harvested at the beginning of the photoperiod were little better than those observed in the absence of Polyclar AT. After several hours all of these extracts appeared brown, and higher levels of Polyclar AT failed to prevent or retard this browning reaction. Substitution of 5% PVP-10 for Polyclar AT routinely resulted in better yields of protein, but extracts browned rapidly and activity of oxidative enzymes decreased concomitantly. A combination of soluble and insoluble PVP at

concentrations used in preceeding experiments gave reproducible results. With this procedure yields of soluble protein on the order of 10 mg/g tissue were obtained at all times throughout a light/dark cycle (FIGURE 6); extracts were always green in color; and polymerization of phenols became obvious only after 15-20 hr at 5°.

While soluble PVP did not interfere with protein determinations and assays of oxidative enzymes used in this study, crude supernatants contained endogenous small molecular weight compounds, soluble PVP-phenol complexes, and isoascorbate which made direct analysis of these samples impossible. Immediate gel filtration was essential for rapid removal of these materials and resolved cotton extracts into three peaks of material which absorbed at 280 nm, FIGURE 7. Analyses of pooled fractions constituting each of these peaks revealed that Coomassie G-250-positive material and oxidative enzymes were associated only with the most rapidly migrating component, peak A. That this chromatography procedure successfully separated protein from interfering low molecular weight compounds was evident. The elution profile, resulting from chromatography of homogenization medium, indicated that soluble PVP and

FIGURE 7

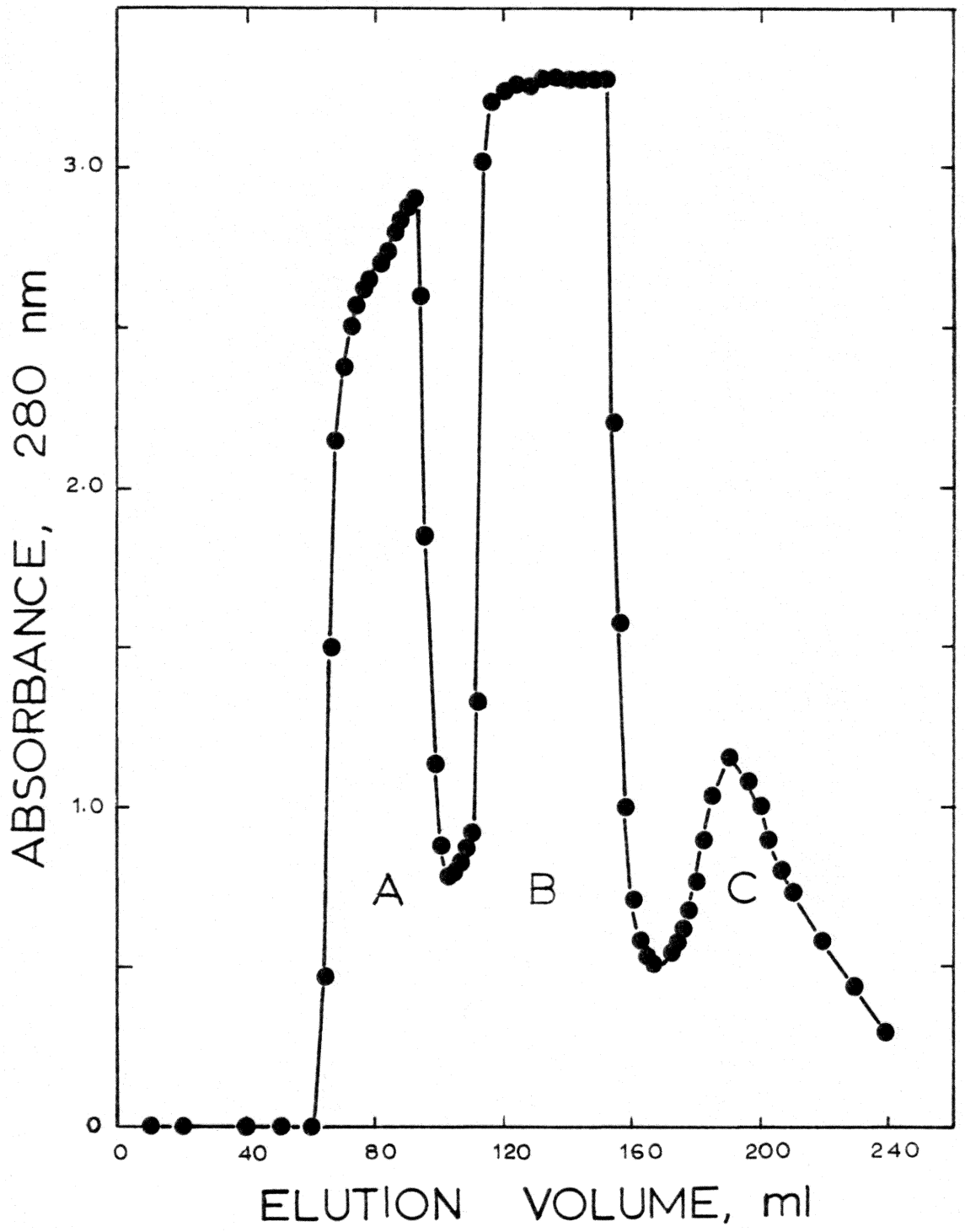
Elution profile for cotton leaf extracts chromatographed on Sephadex G-50

Initial homogenization was performed in 0.1 M Tris-Cl, pH 6.9 containing 0.01 M isoascorbate, 0.5 g Polyclar AT/g tissue and 1% (w/v) PVP-10; rehomogenization of the residue remaining after centrifugation was performed in buffered isoascorbate containing 0.5% (w/v) PVP-10. Supernatants from the initial homogenization and two rehomogenizations were combined, and the 45 ± 2 ml volume was chromatographed at 25° on a 2.5 x 45 cm Sephadex G-50 column at a flow rate of 3 - 4 ml/min. Eluate, collected in 2 ml fractions, was monitored spectrophotometrically at 280 nm.

Peak A: soluble protein

Peak B: soluble PVP, isoascorbate

Peak C: endogenous low molecular weight chemical species, material solubilized from Polyclar AT, compounds with affinity for the Sephadex


ABSORBANCE, 280 nm
3.0
2.0
1.0
0
A
B
C
0
40
80
120
160
200
240
ELUTION VOLUME, ml

isoascorbate eluted only in the region of peak B in FIGURE 7 and that peak C must arise from endogenous low molecular weight chemical species, material solubilized from Polyclar AT or compounds with affinity for the dextran.

The importance of a combination of soluble and insoluble PVP in homogenization media for isolating active enzymes from G. herbaceum C.B. 1697 leaf tissue is demonstrated in TABLE VII in which data for G. hirsutum appear for comparative purposes. For the "Coker 310" cultivar, good yields of protein and enzymatic activity were always achieved with Polyclar AT alone, and inclusion of soluble PVP in extraction media did not improve levels of either soluble protein or enzymatic activity. Glycolate oxidase could not be detected in "1697" samples prepared in the absence of PVP-10; in its presence, along with Polyclar AT, activities were observed to be comparable to those observed in "Coker 310" extracts. Levels of catalase and malate dehydrogenase were also 2-3 times higher in "1697" extracts prepared with both soluble and insoluble PVP while superoxide dismutase exhibited a relatively constant level of activity, independent of the presence of PVP-10.

Using an extraction medium composed of buffered

TABLE VII

Comparison of protein yields and enzyme levels in cotton leaf extracts prepared in the presence of soluble and insoluble PVP

Source of Leaf Tissue	*G. herbaceum* C.B. 1697		*G. hirsutum* Coker 310	
Content of PVP-10[a]	absent	present	absent	present
Enzyme Activity[b]				
		units/mg protein		
Superoxide Dismutase	nd[c]	20	20	10
Glycolate Oxidase	10	10	20	-
Catalase	100	330	320	-
Malate Dehydrogenase	2	4	5	3
		units/g tissue		
Superoxide Dismutase	nd	210	190	120
Glycolate Oxidase	50	70	190	-
Catalase	500	2900	3200	-
Malate Dehydrogenase	4	30	50	40
Soluble Protein[b,d] (mg/g tissue)	4	9	10	12

[a] Leaf tissue was homogenized in 0.1 M potassium phosphate, pH 7.5 containing 0.01 M isoascorbate and 0.5 g Polyclar AT/g tissue. Soluble PVP-10 was added as designated at a concentration of 1% (w/v).

[b] Averages of duplicate assays are reported for typical samples.

[c] Not detectable.

[d] Soluble protein was quantified by the Lowry procedure (134).

isoascorbate containing 0.5 g Polyclar AT/g tissue and 1% (w/v) PVP-10, levels of enzymes were evaluated as a function of harvest time during a 24 hr period, FIGURE 8. Within the range of experimental error, data revealed constant levels of superoxide dismutase, glycolate oxidase, catalase, peroxidase, malate dehydrogenase, and acid phosphatase throughout a diurnal period.

Using 5% (w/v) PVP-10 as a maximum concentration, yields of protein and oxidative enzymes were compared with those obtained from extracts prepared with 0.5% and 1% (w/v) PVP-10 in the initial homogenization medium, TABLE VIII. With the exception of peroxidase, enzyme activity and protein yields were greater at the higher concentrations of PVP-10. Although acid phosphatase increased significantly in direct correlation with PVP-10 content, other differences between 1% and 5% PVP-10 data fell within the range of tissue variability. Polyphenols appeared after 6-8 hr in extracts of 2-4 week-old leaf tissue prepared with 1% (w/v) PVP-10 in the initial homogenization medium. Elevation of the PVP-10 concentration to 2% (w/v) prevented this polymerization in cold extracts for 12-15 hr, but higher concentrations did not extend the time before the appearance

FIGURE 8

Levels of enzyme activities in G. herbaceum C.B. 1697 leaf extracts prepared at different times during a light/dark cycle

Soluble material from the initial homogenization of tissue in 0.01 M isoascorbate, 0.1 M potassium phosphate, pH 7.5 and 0.5 g Polyclar AT/g tissue was combined with the soluble material from a second homogenization of the residue. The concentration of PVP-10 was 1% and 0.5% (w/v) during the first and second extractions, respectively. Range bars indicate the magnitude of variability.

A. Levels of superoxide dismutase and glycolate oxidase.
B. Levels of catalase and peroxidase.
C. Levels of malate dehydrogenase and acid phosphatase.

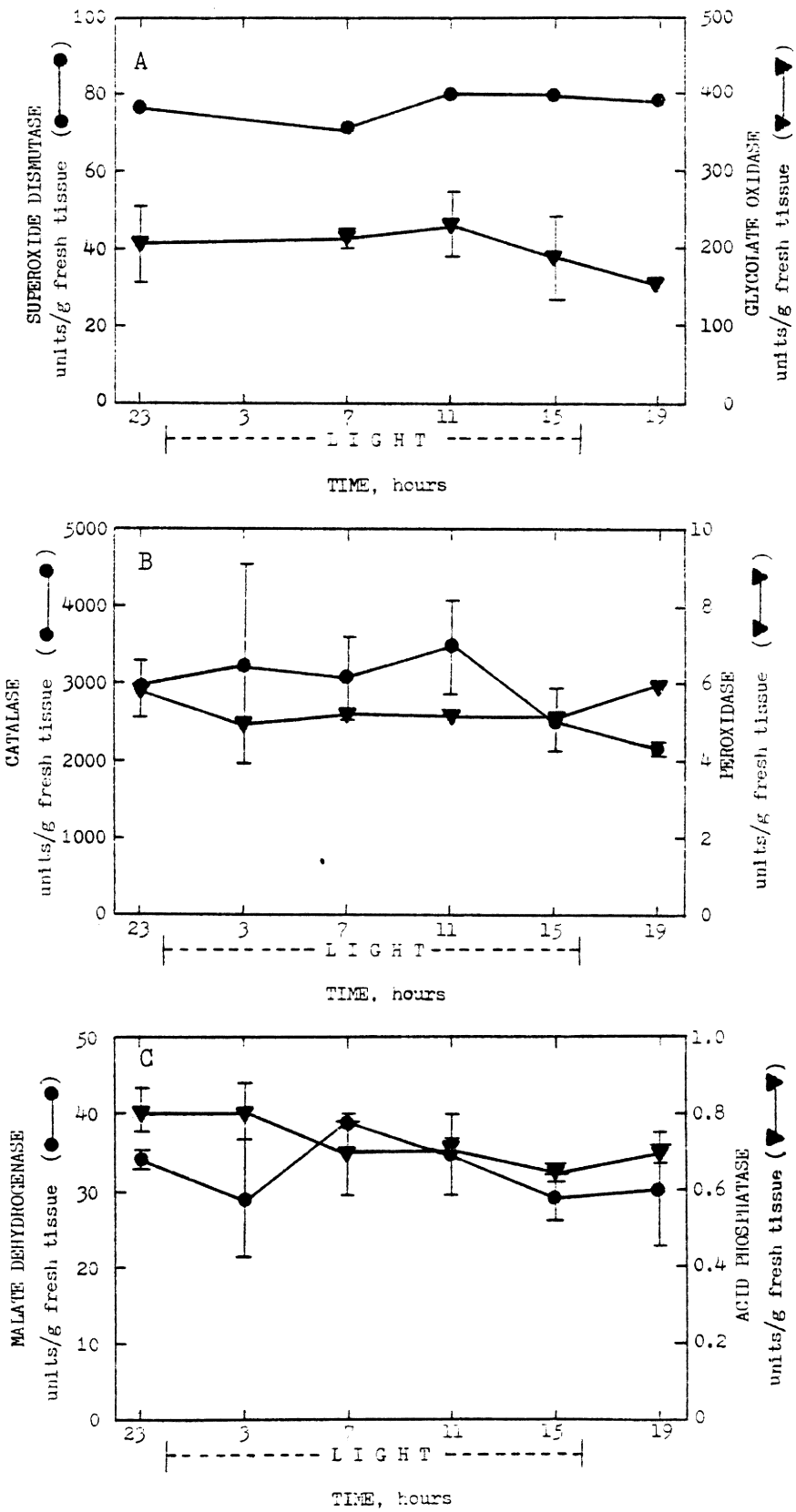

A
SUPEROXIDE DISMUTASE
units/g fresh tissue
GLYCOLATE OXIDASE
units/g fresh tissue
100
80
60
40
20
0
500
400
300
200
100
0
23
3
7
11
15
19
L I G H T
TIME, hours
B
CATALASE
units/g fresh tissue
PEROXIDASE
units/g fresh tissue
5000
4000
3000
2000
1000
0
10
8
6
4
2
0
23
3
7
11
15
19
L I G H T
TIME, hours
C
MALATE DEHYDROGENASE
units/g fresh tissue
ACID PHOSPHATASE
units/g fresh tissue
50
40
30
20
10
0
1.0
0.8
0.6
0.4
0.2
0
23
3
7
11
15
19
L I G H T
TIME, hours

TABLE VIII

Effect of soluble PVP concentration on protein yields and levels of enzyme activity in extracts from G. herbaceum C.B. 1697

Assay	Units	Concentration of PVP-10[a]		
		0.5%	1%	5%
Protein[b]	mg/ml extract	0.5	0.9	0.9
	mg/g tissue	6	10	12
Catalase	units/mg protein	350	380	360
	units/g tissue	2000	3700	4400
Glycolate Oxidase	units/mg protein	10	12	9
	units/g tissue	60	120	100
Peroxidase	units/mg protein	0.7	0.5	0.3
	units/g tissue	4	5	4
Acid Phosphatase	units/mg	0.1	0.1	0.2
	units/g	0.6	0.9	2.0

[a] Initial homogenization was performed in 25 ml 0.1 M potassium phosphate, pH 7.5 containing 0.01 M isoascorbate, 0.5 g Polyclar AT/g tissue, and the indicated amount of PVP-10. For all samples a single rehomogenization of the residue remaining after centrifugation was performed in buffered isoascorbate containing 0.5% PVP-10. The supernatant fractions were combined and chromatographed on Sephadex G-50 prior to analysis of the protein containing fractions. Data are reported for typical samples.

[b] Soluble protein was quantified by the Coomassie G-250 procedure (132).

of brown material was obvious.

Residue, consisting of tissue fragments and Polyclar AT, obtained after centrifugation was further homogenized to disrupt remaining intact tissue and to solubilize protein away from insoluble material. Evaluation of extracts obtained from each of 4 successive rehomogenization steps in buffered isoascorbate containing 0.5% (w/v) PVP-10 revealed that 90% of the total extracted protein and enzyme activity, with the exception of superoxide dismutase, was obtained as soluble material after 3 homogenizations, TABLE IX. Pooled supernatants from these first steps were therefore routinely cochromatographed, and protein eluted from the column was defined the soluble protein fraction, FIGURE 4.

For comparative purposes the procedure for grinding tissue in a mortar in the presence of Polyclar AT and liquid nitrogen, as described for other plant species having high levels of phenolic compounds (148), was evaluated for G. herbaceum C.B. 1697 leaf tissue. Although liquid nitrogen was a key factor in improving yields of protein with Polyclar AT alone, visible fragments of intact tissue remained even after prolonged grinding in the mortar. Crushing tissue in liquid nitrogen decreased the time required for

TABLE IX

Soluble protein and enzyme activity extracted by successive homogenizations of G. herbaceum C.B. 1697 leaf tissue

Assay	Homogenate[a]					1+2+3 as % of total
	1[b]	2[c]	3[c]	4[c]	5[c]	
Soluble Protein (mg/g tissue)	7	3	2	1	1	86
Enzyme Activity (units/g tissue)						
Glycolate Oxidase	153	56	4	nd[e]	nd	100
Catalase	2600	1200	400	200	100	93
Peroxidase	6	2	1	0.5	0.5	90
Superoxide Dismutase	53	55	82	106	70	52
Malate Dehydrogenase	20	9	3	2	1	91
Acid Phosphatase	0.8	0.4	0.3	0.1	0.1	88

[a] Data reported are for a typical sample of leaf tissue.

[b] Homogenate was prepared in 25 ml 0.1 M potassium phosphate, pH 7.5 containing 0.01 M isoascorbate, 1% (w/v) PVP-10, and 0.5 g Polyclar AT/g fresh tissue.

[c] Insoluble residue from a previous homogenization was rehomogenized in 15 ml 0.1 M potassium phosphate containing 0.01 M isoascorbate and 0.5% PVP-10.

[d] Soluble protein was quantified by the Coomassie G-250 procedure (132).

[e] Not detectable.

processing tissue with the Polytron, circumvented the vacuum infiltration step required to wet the pubescent leaf tissue, and minimized reaction between cellular components released by cell rupture prior to exposure to protective reagents.

While yield of protein and levels of oxidative enzymes were similar whether extracted, according to the optimized procedure (FIGURE 4), in 0.1 M potassium phosphate or 0.1 M Tris-Cl, the Tris-Cl system was selected for routine use as levels of acid phosphatase were to be quantified. Typical protein and enzymatic data for extracts from 1-2 month-old leaf tissue from G. herbaceum C.B. 1697 prepared according to this optimized protocol are reported in TABLE X. Data, expressed as averages of eight samples of tissue harvested from separate, equivalently-treated plants, reflect the superior yields and reproducibility achieved with the optimized extraction procedure.

Oxygen Effects on Levels of Protein and Oxidative Enzymes

Extracts from young and old G. herbaceum C.B. 1697 leaf tissue were compared, TABLE XI; 1-3 week-old leaves

TABLE X

Protein yields and enzyme levels observed in extracts from *G. herbaceum* C.B. 1697 prepared by the optimized extraction procedure

Assay	Yield [a]	
Soluble Protein, mg/g tissue[b]	15.4 ± 1.4	
Enzyme Activity	units/mg protein	units/g fresh tissue
Superoxide Dismutase	14 ± 2	211 ± 35
Glycolate Oxidase	19 ± 3	294 ± 60
Catalase	250 ± 17	3740 ± 467
Peroxidase	1.5 ± 0.3	22 ± 4
Malate Dehydrogenase	5.2 ± 0.7	79 ± 11
Acid Phosphatase	0.13 ± 0.04	1.9 ± 0.6

[a] Initial homogenization was performed in 25 ml 0.1 M Tris-Cl, pH 6.9 containing 0.01 M 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 to obtain the soluble protein fraction. Data are expressed as the average ± 1 standard deviation for 8 samples, each prepared from mature, 1 - 2 month old leaf tissue from separate, equivalently-treated plants.

[b] Soluble protein was quantified by the Coomassie G-250 procedure (132).

TABLE XI

Responses of old and young cotton leaves exposed to 75% O_2 for 48 hr: Activity of oxidative enzymes/g fresh tissue

Tissue Age[a]	1-2 months		1-3 weeks	
Atmosphere	Air	75±5% O_2 350±50 ppm CO_2	Air	75±5% O_2 350±50 ppm CO_2
Enzyme Activity, units/g tissue[b]				
Superoxide Dismutase	190	210	260	310
Glycolate Oxidase	260	200	400	890
Catalase	3600	3000	4300	6900
Peroxidase	20	20	20	20
Glutathione Reductase ($x10^{+2}$)	2	4	12	23
Malate Dehydrogenase	60	60	80	110
Acid Phosphatase	2	2	2	4
Soluble Protein, mg/g tissue[b,c]	11	11	18	18

[a] For each atmosphere, leaves of both ages were harvested from the same plant. Initial homogenization was performed in 25 ml 0.1 M Tris-Cl, pH 6.9 containing 0.01 M isoascorbate, 0.5 g Polyclar AT/g tissue and 1% PVP-10. Plants were exposed to 90-95% humidity throughout the course of the experiment.

[b] Averages of duplicate assays are reported for a typical sample.

[c] Soluble protein was quantified by the Coomassie G-250 procedure (132).

possessed higher levels of enzymatic activity consistent with higher levels of soluble protein observed in that tissue. Young tissue from plants exposed to atmospheres enriched to 75% oxygen exhibited increases in catalytic activity associated with glycolate oxidase, catalase, glutathione reductase, and acid phosphatase, (TABLE XI), but only glutathione reductase activity was elevated in 1-2 month-old leaves exposed to the higher oxygen tension. Protein levels in the two tissue types appeared insensitive to the changed environmental conditions. Similar trends in catalytic activity expressed on the basis of protein were also observed.

The greater apparent response of younger leaf tissue to altered levels of environmental oxygen was the basis for selection of that tissue type for evaluation of time-related changes in levels of enzymes during exposure to 75% oxygen. Plants were acclimated in air in vinyl chambers used for controlled atmosphere studies in order to evaluate the effects of these experimental chambers on the enzyme pattern in leaf tissue. Obvious changes in peroxidase (+89%), catalase (-30%), glycolate oxidase (-42%), and soluble protein (-27%) were observed in the air control during the total 5 day experimental period, TABLE XII. During the same

TABLE XII

Changes in levels of oxidative enzymes in cotton leaf tissue examined at different times during acclimation to 90-95% humidity and exposure to 75% O_2

Chamber	Soluble Protein[a] mg/g tissue	Enzyme Activity, units/g tissue			
		Superoxide Dismutase	Glycolate Oxidase	Catalase	Peroxidase
Preacclimation[b]	11±1.6	160±32	510±23	4160±599	9±0.8
Control[c]					
Acclimation: Air					
24 hr[d]	11±1.6	200±54	550±113	4300±1854	11±2.2
72 hr[e]	10±4.2	230±172	440±103	3320±1085	15±2.4
Treatment: Air					
24 hr[f]	9±1.0	230±149	380±38	3610±891	16±1.4
48 hr[e]	9±1.9	250±134	310±77	3320±749	17±4.0
72 hr[e]	8±1.8	250±101	320±79	3000±709	17±6.0
Experimental[c]					
Acclimation: Air					
24 hr[e]	11±1.3	200±31	500±45	4020±385	10±1.5
72 hr[e]	9±2.6	240±102	400±57	3070±639	15±1.3
Treatment: 75±5% O_2, 350±50 ppm CO_2					
24 hr[f]	10±2.4	290±189	390±49	3340±1284	15±2.5
48 hr[e]	12±0.8	240±126	500±222	3700±1588	19±4.5
72 hr[e]	9±1.9	270±127	460±120	2940±1400	20±6.7

[a] Soluble protein was prepared from 2-4 week old leaf tissue according to the optimized extraction procedure, FIGURE 4, and was assayed by the Coomassie G-250 technique (132).

[b] Data are for plants maintained in air at 70-80% humidity. n=6

[c] Plants, transferred to vinyl chambers, were exposed to 90-95% humidity during the acclimation and treatment periods.

[d] n=2

[e] n=4

[f] n=3

period, peroxidase increased 188% from its preacclimation specific activity, 0.8 ± 0.2, to a maximum of 2.3 ± 1.1 mg protein units.

In this and other experiments, oxygen-dependent changes became apparent after 48 hr. Statistical analyses of data obtained at this time point indicated significant increases in specific activities of glutathione reductase (+95%) and malate dehydrogenase (+30%), (TABLE XIII).

In this experiment, after plants were introduced into the vinyl chambers, humidity increased to 95% due to the high moisture content of the potting material and to transpiration by the plants. Prolonged exposure to this high humidity during acclimation and treatment caused young leaves to lose their turgidity, curl downward, and become chlorotic. These responses, observed in both air control and oxygen-treated plants, and the altered levels of enzyme activity were unexpected responses to increased humidity. When humidity in vinyl chambers was carefully maintained at the preacclimation level of 70-80%, no apparent physiological changes were observed, even after two weeks.

In a final experiment under these low humidity conditions, responses of enzymes related to the 75% oxygen

TABLE XIII

Changes in levels of oxidative enzymes in cotton leaf tissue exposed to 75% O_2 for 48 hr under 90-95% humidity

Atmosphere[a]	Air	75±5% O_2[b] 350±50 ppm CO_2	Air	75±5% O_2[b] 350±50 ppm O_2
Enzyme Activity[c]	units/g tissue		units/mg protein	
Superoxide Dismutase	250 ± 134	190 ± 139	30 ± 19.4	17 ± 12.6
Glycolate Oxidase	310 ± 77	500 ± 222	36 ± 14.5	44 ± 20.7
Catalase	3320 ± 749	3700 ± 1588	370 ± 137	320 ± 149
Peroxidase	17 ± 4.0	19 ± 4.5	2.0 ± 0.70	1.7 ± 0.44
Glutathione Reductase ($x10^{+3}$)	62 ± 8	121 ± 18***	7 ± 2.5	11 ± 2.0*
Glyoxylate Reductase	0.25 ± 0.03	0.26 ± 0.04	0.027 ± 0.007	0.022 ± 0.005
Malate Dehydrogenase	77 ± 10.3	100 ± 4.8***	8 ± 0.9	9 ± 0.3
Acid Phosphatase	2.4 ± 0.75	3.3 ± 1.29	0.3 ± 0.13	0.3 ± 0.12
Soluble Protein, mg/g tissue	9 ± 1.9	12 ± 0.8*	9 ± 1.9	12 ± 0.8*

[a] Plants were exposed to 90-95% humidity throughout the acclimation and exposure periods.

[b] Plants were acclimated in air for 72 hr prior to exposure to the high oxygen atmosphere.

[c] Data are expressed as the average ± 1 standard deviation for 2 samples, each prepared from mature, 2-4 week old leaf tissue, harvested at the modpoint of the photoperiod from separate, equivalently-treated plants. Where data for the air and 75% O_2 atmospheres are significantly different, asterisks indicate the atmosphere producing the higher level of protein or enzymatic activity and denote the P value as follows: * $0.01 < P < 0.05$, ** $0.005 < P < 0.01$, *** $P < 0.005$.

[d] Soluble protein prepared according to FIGURE 4 was quantified by the Coomassie G-250 procedure (132).

atmosphere were observed, TABLE XIV. While protein levels were significantly lower (-15%) in oxygen-treated tissue, increases in specific activity of glutathione reductase (+250%), acid phosphatase (+100%), peroxidase (+57%), and glycolate oxidase (+27%) were significant. In contrast, catalase activity per g fresh tissue was significantly lower (-32%) in tissue exposed to the high-oxygen tension for 48 hr, and glyoxylate reductase, malate dehydrogenase and both cyanide-sensitive and insensitive superoxide dismutases were independent of the atmosphere to which plants were exposed. Greater standard deviations for these data and those in TABLE XIII, as compared to results for old tissue reported in TABLE X, arises from the greater variability in tissue age within the defined age limits.

Since oxygen treatment appeared not to alter levels of superoxide dismutase, electrophoresis of extracts was performed; polyacrylamide disc gels stained for superoxide dismutase activity indicated 5 distinct electrophoretic forms of superoxide dismutase having relative mobilities 0.31, 0.46, 0.53, 0.61, and 0.86; only one band, that having an R_f value of 0.61, was insensitive to 10^{-3} M cyanide in the staining solutions, FIGURES 9 and 10. Extracts

TABLE XIV

Changes in levels of oxidative enzymes in cotton leaf tissue exposed to 75% O_2 for 48 hr under 70-80% humidity

Atmosphere[a]	Air	75±5% O_2[b] 350±50 ppm CO_2	Air	75±5% O_2[b] 350±50 ppm CO_2
Enzyme Activity[c]	units/g tissue		units/mg protein	
Superoxide Dismutase[d]	350±47*	290±79	26±3.2	26±7.1
cyanide sensitive	250±47	190±61	18±3.1	18±5.7
cyanide insensitive	100±23	90±24	7±2.0	9±2.0
Glycolate Oxidase	300±50	300±50	22±2.6	28±3.7***
Catalase	3300±771***	2260±245	240±44*	210±16
Peroxidase	9±1.2	11±1.8**	0.7±0.10	1.1±0.17***
Glutathione Reductase ($x10^{+3}$)	18±6	51±13***	1.3±0.4	4.6±0.9***
Glyoxylate Reductase	0.07±0.03	0.07±0.01	0.005±0.002	0.006±0.001
Malate Dehydrogenase	57±9.1*	47±8.9	4±0.4	4±0.3
Acid Phosphatase	1.3±0.20	2.4±0.17***	0.1±0.01	0.2±0.02***
Soluble Protein, mg/g tissue[c,e]	13±1.4**	11±1.4	13±1.4**	11±1.4

[a] Plants were exposed to 70-80% relative humidity throughout the acclimation and exposure periods.

[b] Plants were acclimated in air for 24 hr prior to exposure to the high oxygen atmosphere.

[c] Data are expressed as the average ± 1 standard deviation for 8 samples, each prepared from mature, 2-4 week old leaf tissue harvested at the midpoint of the photoperiod from separate, equivalently-treated plants. Where data from the air and 75% O_2 atmospheres are significantly different, asterisks indicate the atmosphere producing the higher level of protein or enzymatic activity and denote the P value as follows: * $0.01 < P < 0.05$, ** $0.005 < P < 0.01$, *** $P < 0.005$.

[d] Cyanide-insensitive superoxide dismutase activity was quantified by the inclusion of 10^{-3} M cyanide in the assay mixture. Cyanide-sensitive activity was calculated from the difference between total and cyanide-insensitive superoxide dismutase activities.

[e] Soluble protein prepared according to FIGURE 4 was quantified by the Coomassie G-250 procedure (132).

FIGURE 9

Electrophoretically distinct forms of superoxide dismutase in cotton leaf tissue

Following electrophoresis in the direction cathode to anode as indicated, superoxide dismutase activity was localized on 7% polyacrylamide disc gels using the NBT activity stain in the presence, gel A, and absence, gel B, of 10^{-3}M cyanide. Bands as depicted illustrate relative positions, and sizes are not representative of bands as they appear on gels.

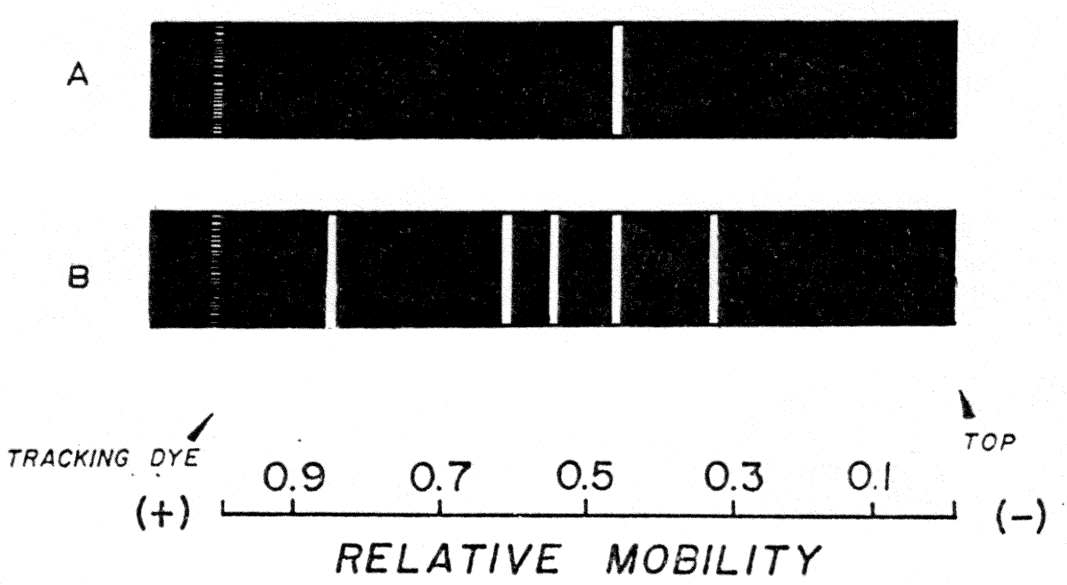
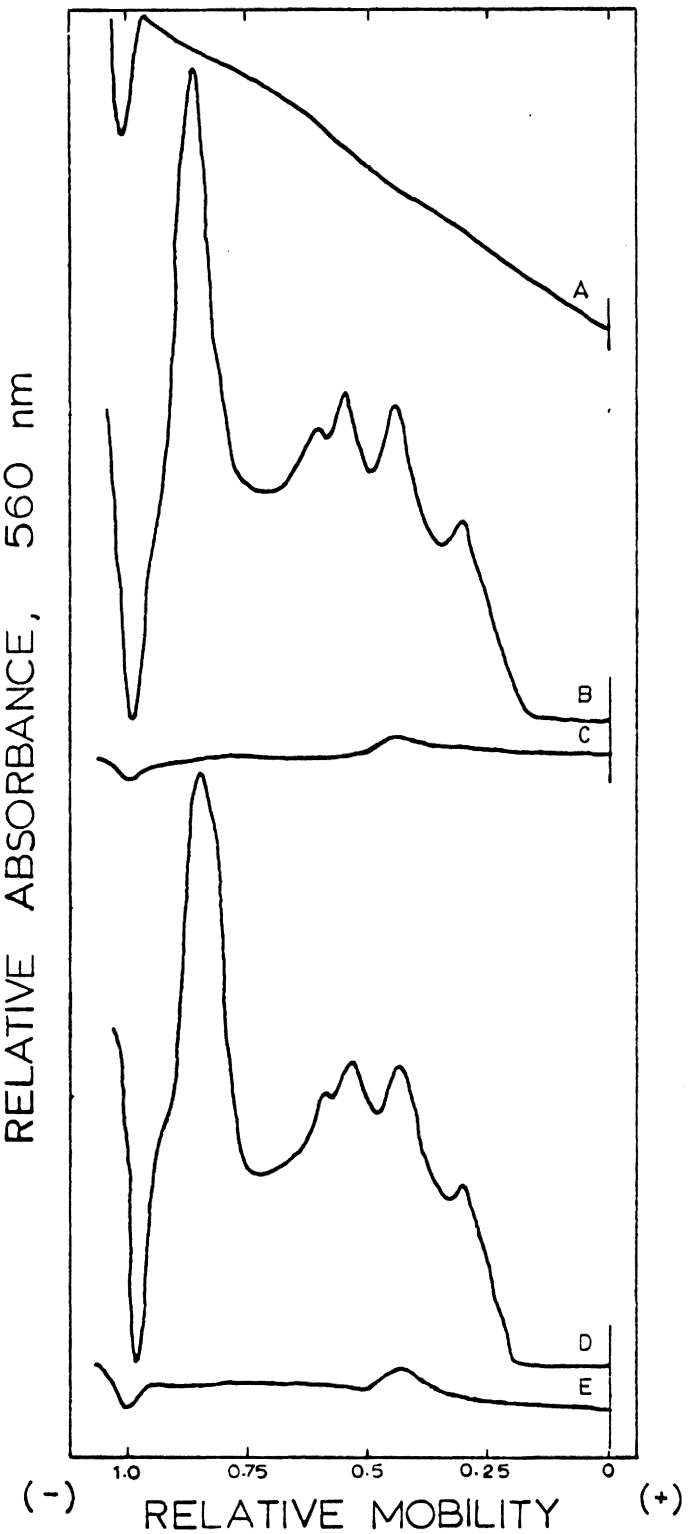

A
B
TRACKING DYE
TOP
0.9
0.7
0.5
0.3
0.1
(+)
(-)
RELATIVE MOBILITY

FIGURE 10

Spectrodensitometric scans of disc gels stained for superoxide dismutase in extracts from tissue exposed to ambient and elevated oxygen environments

Appropriate volumes (85-120 µl) of extracts from G. herbaceum C.B. 1697 leaf tissue exposed to ambient or 75% atmospheric oxygen, each containing 50 µg of protein in 5% (w/v) sucrose, were layered on gels. Following electrophoresis, performed in the direction cathode to anode, superoxide dismutase was localized using the NBT activity stain in the presence and absence of 10^{-3} M cyanide as indicated. As presented, positive peaks represent achromatic regions on gels having a dark blue background as measured with a Schoeffel model SD 3000 Spectrodensitometer. For curves A, B, and D, sensitivity = 0.4 absorbance; for curves C and E, sensitivity = 1.0 absorbance.

A: Blank, $\pm$ 10^{-3} M cyanide
B: Air control
C: Air control, 10^{-3} M cyanide
D: 75% oxygen, 48 hr
E: 75% oxygen, 48 hr, 10^{-3} cyanide


RELATIVE ABSORBANCE, 560 nm
A
B
C
D
E
1.0
0.75
0.5
0.25
0
(-)
RELATIVE MOBILITY
(+)

from air controls and tissue exposed to 75% oxygen produced staining patterns which were qualitatively and quantitatively identical when scanned spectrodensitometrically, FIGURE 10.

Because previous data indicated that approximately 50% of the superoxide dismutase in cotton leaf tissue was associated with the insoluble fraction, pellet 3, (FIGURE 4, TABLE IX), protein in this fraction, obtained from tissue exposed to 75% oxygen for 48 hr or from corresponding air controls, was solubilized by inclusion of 0.2% Triton X-100 during rehomogenization. Protein analysis indicated percentage yields which correlated with those for superoxide dismutase activity in the corresponding homogenates, TABLE XV. However, utilization of Triton X-100 during homogenizations 4, 5, and 6 (TABLE XV) solubilizes pigment-protein complexes as evidenced by the dark green color of the soluble fractions and contributes to the 10% higher yield of total protein (TABLE XV) as compared to that obtained in the absence of Triton (TABLE IX).

Consistent with observations of the soluble protein fraction, homogenates 1 + 2 + 3, superoxide dismutase in Triton extracts, homogenates 4 + 5 and 6, from oxygen-

TABLE XV

Evaluation 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 75% O_2 for 48 hr

Assay	Homogenate[a] 1 + 2 + 3[b]	4 + 5[c]	6[c]
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 ppm 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 ppm CO_2			
units/g tissue	290 ± 79	50 ± 1	10 ± 2
% of total	83	14	3

[a]Initial homogenization was performed in 0.1 M Tris-Cl, pH 6.9 containing 0.01 isoascorbate, 2% PVP-10, and 0.5 g Polyclar AT/g tissue. Rehomogenization of the insoluble residue from a previous homogenization was performed in 0.1 M Tris-Cl, pH 6.9 containing 0.01 M isoascorbate and 0.5% PVP-10. Homogenizations 4, 5, and 6 were performed in the presence of 0.2% Triton X-100.

[b]Data are averages of 8 samples ± 1 standard deviation.

[c]Data are averages of 2 samples ± 1 standard deviation.

[d]Soluble protein was quantified by the Coomassie G-250 assay (132).

exposed tissue was not elevated above that in extracts from air controls, TABLE XV. Moreover, the percentage of superoxide dismutase activity in each homogenate was independent of the atmosphere to which tissue was exposed. Superoxide dismutase activity in these homogenates could be completely eliminated by boiling aliquots for 40 min, but total insensitivity of this activity to 10^{-3} M cyanide and, in fact, a higher level of activity in the presence of cyanide suggested solubilization of proteins such as cytochrome oxidase which interfere with the rate of cytochrome c reduction during assay of extracts (149). Analysis of superoxide dismutase activity on disc gels following electrophoresis revealed that the predominant form of superoxide dismutase present in these extracts was the cyanide-sensitive form having a relative mobility of 0.86.

DISCUSSION

Selection of Experimental System

Diploid cotton, G. herbaceum C.B. 1697, leaf tissue was selected for evaluation of oxygen-dependent changes in enzyme levels following a survey of superoxide dismutase levels in leaf tissues exposed to 45% oxygen for 1-2 weeks (98). Though environmental parameters were not rigidly controlled in those experiments and insufficient data were available for statistical analysis, superoxide dismutase activity appeared to be elevated in leaves from G. herbaceum plants exposed to the supraatmospheric concentration of oxygen. The apparent increase in superoxide dismutase activity in this tissue was consistent with observations of induction of superoxide dismutase in Chlorella (116), bacterial cells (110,111), yeast (115), and animal cells (112-114). However, superoxide dismutase activity in G. hirsutum cv Coker 310 leaf tissue treated in an identical manner, and in seedling maize leaves exposed to as much as 70% oxygen for the same period of time, remained constant. It therefore became necessary to more carefully quantify superoxide dismutase activity in G. herbaceum leaves and,

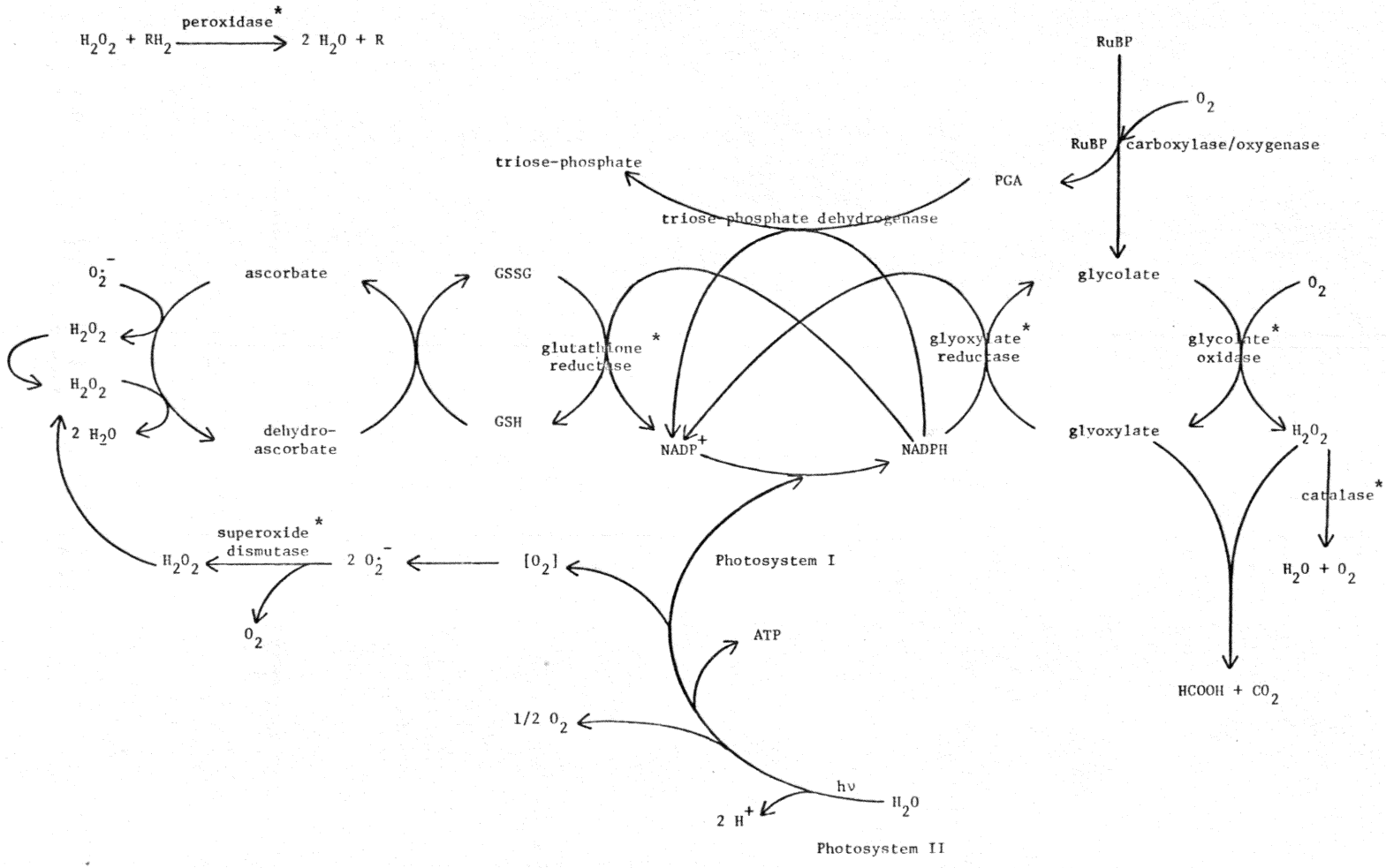
further, to investigate a variety of enzymes which might function to protect photosynthetic tissues against the toxic effects of oxygen and its metabolites.

Enzymes - Since chloroplasts both produce and utilize oxygen, and, with the cytosol, peroxisomes, and mitochondria, are integrated into the metabolism of oxygen, attention was focused upon selected enzymes which catalyze reactions involving superoxide or hydrogen peroxide or enzymes which might act indirectly to alter levels of these chemical species, FIGURE 11. Under conditions in which oxygen is present in high concentrations and limited quantities of $NADP^+$ are available to accept electrons during photosynthesis, increased utilization of molecular oxygen as an electron acceptor was proposed to give rise to concentrations of superoxide which might be sufficiently elevated that a concomitant increase in the level of superoxide dismutase would be necessary for efficient disproportionation of the free radical. In the absence of glutathione peroxidase in plant tissues (83,84) and localization of catalase restricted to peroxisomes (59,65,73,85), removal of hydrogen peroxide, generated in the chloroplast during disproportionation of superoxide, may be accomplished by an ascor-

FIGURE 11

Aspects of oxygen metabolism in higher plants

Those enzymes designated with an asterisk were selected for quantitative measurements of activity and the sensitivity of this activity to elevated concentrations of oxygen.


peroxidase*
$H_2O_2 + RH_2 \rightarrow 2\ H_2O + R$
RuBP
O_2
RuBP carboxylase/oxygenase
triose-phosphate
PGA
triose-phosphate dehydrogenase
O_2^-
ascorbate
GSSG
glutathione reductase*
glyoxylate reductase*
glycolate
O_2
glycolate oxidase*
H_2O_2
H_2O_2
$2\ H_2O$
dehydro-ascorbate
GSH
$NADP^+$
NADPH
glyoxylate
H_2O_2
catalase*
superoxide dismutase*
H_2O_2
$2\ O_2^-$
$[O_2]$
Photosystem I
$H_2O + O_2$
O_2
ATP
$HCOOH + CO_2$
$1/2\ O_2$
hν
H_2O
$2\ H^+$
Photosystem II

bate-mediated, reduced glutathione-dependent reduction sequence (FIGURE 11) which occurs nonenzymatically at pH > 7.5, values observed in the chloroplast during photosynthesis (80,81). If this proposed mechanism functions to maintain chloroplastic hydrogen peroxide concentrations at tolerable levels and perhaps assists in the removal of superoxide, reduced glutathione must be restored, and glutathione reductase levels could fluctuate in response to changing needs for reduced glutathione as regulated by endogenous levels of hydrogen peroxide and superoxide.

Chloroplastic levels of glyoxylate reductase and triose-phosphate dehydrogenase, as well as glutathione reductase, may reflect mechanisms whereby plants insure availability of $NADP^+$ as the preferred electron acceptor during photosynthetic electron transport, minimizing production of superoxide (FIGURE 11). With fluctuations in photorespiratory activity, perhaps involving changes in levels of glycolate oxidase, alterations in glyoxylate concentrations might influence the availability of $NADP^+$ through concomitant changes in glyoxylate reductase activity. Quantification of glyoxylate reductase was therefore considered important. Since triose-phosphate

dehydrogenase was judged to be subject to a flux of electrons similar to that encountered by glyoxylate reductase, and because glyoxylate reductase was found to be quite insensitive to an increase in oxygen tension (TABLES XII, XIV), there was no apparent need to monitor triose-phosphate dehydrogenase. Moreover, the decision not to examine this enzyme for a potential, oxygen-dependent increase in activity was based upon recognition of the light activation of this enzyme (150) as well as the limitation imposed by availability of PGA which was not expected to increase under the experimental conditions. Decomposition of extrachloroplastic hydrogen peroxide by peroxidase and both production of hydrogen peroxide by glycolate oxidase and utilization of hydrogen peroxide by catalase in the peroxisome indicated a need to also monitor these enzymes in leaves exposed to altered concentrations of environmental oxygen.

As indicated in FIGURE 11, ribulose bisphosphate carboxylase/oxygenase catalyzes the incorporation of oxygen into glycolate without the uptake or release of $O_2^{\cdot -}$ or H_2O_2 (25). Moreover, oxygenase activity increases in a hyperbolic manner with increasing assay concentrations of oxygen, but the enzyme does not become saturated, even at 100%

oxygen (15,27). Since oxygen occurs in air at a concentration which is far below the K_m (0.75 mM), the enzyme should have the capacity to accomodate oxygen concentrations of 75% (15). The level of this enzyme was therefore not expected to fluctuate under the experimental conditions, and thus oxygenase activity was not evaluated in these studies.

Since enzymes are often induced or derepressed by their substrates, determination of responses of enzymes which do not directly utilize oxygen or its intermediate reduction products was important for evaluating specificity of the effect of a supraatmospheric oxygen concentration on enzyme levels in leaf tissue. Acid p-nitrophenylphosphatase, an hydrolytic enzyme, was selected as such a marker enzyme. However, because cellular distribution of this enzyme includes the chloroplast (151) which plays such an active role in oxygen metabolism, it was felt that an extrachloroplastic enzyme should also be evaluated, and NADH-specific malate dehydrogenase was selected for this purpose.

Tissue - Success of comparative studies of enzyme levels in leaf tissue depended upon utilization of genetically uniform, biochemically similar tissue. Genetic

contribution to tissue variability was minimized by generating plants by vegetative propagation. Environmental parameters were then manipulated to insure that physiological status of normal plants was as nearly identical as possible. A constant temperature of 30°, 70% humidity and a 16 hr day were established to promote good, vegetatative growth, and light was provided at the maximum output for the growth chambers. Continued production of consistent quality tissue demonstrated the ability of Hoagland's nutrient to support long-term growth of cotton plants and to provide tissue suitable for quantitative enzyme analyses. Further, it was demonstrated that Huffman's nutrient (145), used in nutritional studies with cotton, was not suitable for long-term growth required in these studies. Analysis of leaf tissue from these plants, which exhibited obvious symptoms of nutritional stress, revealed both manganese and iron to be present at adequate levels (TABLE VI); however a lower calcium/magnesium ratio in this tissue, combined with a decrease in the pH of the potting medium which was attributed to the NH_4^+ content of Huffman's nutrient, was consistent with the poor growth and condition of these plants (152,153).

Selection of young cotton leaf tissue as appropriate for evaluation of effects of high oxygen tensions on oxygen metabolism was based upon levels of oxidative enzymes observed in air-grown leaves and responsiveness of these enzymes to increases in atmospheric oxygen (TABLE XI). Because enzymes undergo changes in levels of activity during development of a tissue, it was necessary to harvest leaves which, though still young, were sufficiently mature that time-dependent changes in enzyme levels during exposure to elevated concentrations of oxygen could be attributed to environemntal conditions and not to developmental changes.

Plants grown in relatively inert potting media supplied with nutrient solutions are subject to rapid accumulation of soluble salts, but a high moisture content of the potting medium can ameliorate detrimental effects of high salt conditions (154). The need to restrict moisture content of potting material to maintain humidity at a constant level when pots were transferred to vinyl chambers required that soluble salts be present at a tolerable level (1000-2000 ppm)[5]. That this levels was not exceeded was assured

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[5] Donohue, S. J., Personal communication.

by use of newly propagated plants, recently transplanted into fresh potting medium and supplied with 20% Hoagland's nutrient.

Environment - For the studies reported here, an atmospheric oxygen concentration of 75% was selected since 45% oxygen utilized in previous experiments produced little or no change in the level of superoxide dismutase (98) and because oxygen concentrations in excess of 60% are necessary for saturation of glycolate oxidase (15). Carbon dioxide was maintained at a concentration of 350±50 ppm so that changes in enzyme levels would reflect only effects of the increase in oxygen in the atmosphere. An exposure period of 48 hr was established after time-course evaluations at 24 hr intervals (TABLE XII) indicated this to be the first time point at which differences in enzyme levels between control and oxygen-treated tissue were statistically significant (TABLE XIII).

The need to be attentive to humidity as an environmental parameter was made evident by observations of humidity-dependent alterations in enzyme levels in cotton leaf tissue, TABLE XII. Relative humidity during the

experimental period was therefore maintained at the level (70%) used during normal culture of plants.

Extraction of Protein: Requirements for Protective Reagents

A major contribution made by this study is the successful quantitative extraction of soluble protein and measurement of enzyme activity from cotton leaf tissue. Representative data, presented in TABLE XVI provide comparative yields of soluble protein from animal, bacterial, and plant tissue. For cotton, virtually no protein was obtained in the absence of protective reagents. Other researchers have also experienced difficulty in isolating protein and active enzymes from this tissue. Herrero and Hall (158) and Morgan and Hall (159) reported problems in demonstrating indoleacetic acid oxidase activity in aqueous extracts from cotton leaves, and absence of detectable activity was shown to be due to an endogenous inhibitor which functioned in a manner identical to gossypol, a phenolic substance found in cotton leaves (159). For the Upland cottons, Barber and Hassid (160) found that presence of soluble PVP during extraction was effective in isolating active cellulose-synthesizing particles from

TABLE XVI

Soluble protein yields from different sources

TISSUE	mg PROTEIN/g TISSUE	REFERENCE
Liver	120-130[a]	(155)
E. coli	75-80[a]	(156)
Spinach, Leaves	9-17[a]	(157)
Cotton, Leaves		
Total Protein	24-28[b]	
Buffer Extract	<0.1	
Sonicate + Isoascorbate[c]	1-2	
Homogenate + Isoascorbate[c]	0.5-1	
Homogenate + Isoascorbate[c] + Polyclar AT	1-4	
Homogenate + Isoascorbate[c] + Polyclar AT + PVP-10	10-15	

[a] Values were obtained from published enzyme purifications in which tissue was extracted with aqueous buffer in the absence of added protective reagents.

[b] Total protein was estimated by Kjeldahl nitrogen analysis performed by Dr. Dale Wolf.

[c] Tissue disruption was accomplished in the presence of the indicated protective reagent(s).

cotton bolls, whereas, in the absence of PVP, no polysaccharide synthesis by the cell-free preparation could be detected. O'Sullivan and Wedding (146) successfully used insoluble PVP to prepare protein from G. hirsutum leaf tissue for studies of isozymes of malate dehydrogenase; anion exchange resins have been used effectively to isolate ribulose bisphosphate carboxylase/oxygenase from the same tissue (161). Isolation of the carboxylase/oxygenase from other plant species in the presence of soluble PVP and dithiothreitol was found to increase yields of activity by up to 50% (162). Attempts to extract protein from leaf tissue from G. herbaceum C.B. 1697 revealed that procedures, satisfactory for Upland cottons (146,160,161) were not adequate for this Old World species (TABLE VI,XVI) (163) and yielded less than 20% of the total leaf protein detected by Kjeldahl analysis. This may be attributed to the characteristically higher concentration of gallocatechins and possibly aldehyde quinones in Old World cottons (128).

Reviews of the literature on extracting protein from plant tissues (164-166) indicate that a wide range of materials can protect plant extracts against phenolic compounds released during homogenization, but the degree

of protection afforded varies with plant species, and no single compound or combination of compounds is universally applicable. While contamination of insoluble PVP with the soluble reagent during isolation of protein from plant tissues has been carefully avoided by other workers (145, 166), a mixture of the two forms during homogenization (FIGURE 4) was absolutely essential to maximize reproducible yields of protein and active enzymes from G. herbaceum C.B. 1697 leaf tissue (TABLES VII,X,XII,XIV). Phenols, bound by insoluble PVP present in excess concentration, are quickly removed from homogenates by centrifugation, but insolubility of Polyclar AT may result in microenvironments devoid of protective effects during tissue disruption. On the other hand, soluble PVP which is uniformly dispersed in the medium and therefore more readily available to compete efficiently with protein for binding phenols may, by virtue of solubility properties, allow these bound phenols to further react with proteins. However, protective effects provided by solubility of PVP-10, combined with the ability of PVP to disrupt protein-phenol complexes (167), may account for the observation that when both soluble and insoluble reagents are

used during homogenization of tissue, they complement each other, acting cooperatively or synergistically, to give improved yields of soluble protein and active enzymes. In extracts prepared with the combination of soluble and insoluble PVP, maximal yields and a slow rate of browning following gel filtration were observed as a result of removal of insoluble Polyclar AT-phenol complexes by centrifugation and soluble PVP-phenol complexes and free phenolic compounds by gel filtration, leaving high levels of unmodified proteins in solution contaminated only by a small quantity of soluble PVP-phenol-protein and protein-phenol complexes. Rhodes (164) has reported that, even under favorable conditions, most protein preparations from plant tissues contain some attached phenolic or quinone molecules. Including the antioxidant isoascorbate in the homogenization medium and crushing tissue in liquid nitrogen to minimize reactions between proteins and phenolic compounds prior to addition of protective reagents also contributed to improved quality of the protein extracts. Utilization of this optimized protocol for protein extraction (FIGURE 4), combined with the stability of enzymatic activity in these extracts, provided assurance that conclusions based

on quantitative comparisons were valid.

Under conditions in which protein was inadequately protected against the detrimental effects of phenols, increasing yields of soluble protein from beginning to end of the photoperiod (FIGURE 6) probably reflect altered phenol content rather than diurnal changes in the level of protein. This observation is consistent with reports of a light-activated reversal of indoleacetic acid oxidase inhibition by phenolic compounds and of a change in inhibitor concentration as a function of day length (160). Moreover, the higher concentration of PVP-10 required for successful extraction of protein from young leaves probably reflects the greater content of phenolic compounds in this tissue (128,168).

Data from protein and enzyme analyses of extracts prepared from 1-2 month-old tissue (TABLE XIV) demonstrate the excellent reproducibility which can be achieved using the optimized procedure (FIGURE 4) for extraction of protein from G. herbaceum C.B. 1697 leaf tissue. For protein, the optimal range of 2-3 units about the mean (TABLES IX, XIV) is a more realistic estimate of variability than range bars in FIGURE 6 for extracts for which the extraction

procedure was not optimized. Moreover, standard deviations of 10-20% of the mean for these data (TABLES IX, XIV) also indicate the absence of an effect of chamber position (FIGURE 3). Greater variability in data for 2-4 week-old tissue exposed to 95% humidity (TABLE XII) is probably related to variability in water accumulation among harvested leaves and the contribution this water makes to fresh tissue weight. On the other hand, the higher yield of protein obtained from 1-3 week-old leaves (TABLE XI) is most likely a function of the age of the tissue. Overall, however, protein yields are similar to those reported for spinach and represent approximately 50% of the total leaf protein estimated by Kjeldahl nitrogen analysis (TABLE XVI).

Quantitative Analysis of Oxidative Enzyme Levels as Influenced by Protective Reagents

Protein extracts prepared from G. herbaceum C.B. 1697 leaf tissue in the presence of both soluble and insoluble polyvinylpyrrolidone contained glycolate oxidase and exhibited significantly improved yields of catalase and malate dehydrogenase compared to enzyme levels observed in

extracts prepared with insoluble PVP alone (TABLE VII). However, quantitative determinations of superoxide dismutase activity indicated that addition of PVP-10 to the Polyclar AT-containing homogenization medium had little effect on the yield of superoxide dismutase from this tissue. Apparently superoxide dismutase was either not affected by phenols or some factor related to the procedure and only marginally affected by the addition of PVP-10 at a concentration of 1% (w/v) was responsible for a low yield of this enzyme. While differential sensitivity of enzymes to phenolic compounds may account for these observations, affinity of PVP for copper-containing proteins (169) may have resulted in association of the major form of superoxide dismutase in plants (102,104,107,170-172) with the insoluble, Polyclar AT-containing fraction. Although superoxide dismutase was not monitored in the evaluation of effects of PVP-10 concentration on enzyme yields, TABLE VIII, competition between soluble and insoluble PVP for the Cu-Zn superoxide dismutase may account for the slight increase in superoxide dismutase activity observed upon inclusion of 1% PVP-10 during homogenization, TABLE VII. After initial extraction in an homogenization medium

containing Polyclar AT and 1% (w/v) PVP-10, nearly 50% of the extracted superoxide dismutase appeared in the third and fourth washes of the particulate fraction, (TABLE IX). That only 15% of the superoxide dismutase appeared in these washes when the procedure was repeated using 2% (w/v) PVP-10 (TABLE XV), substantiates this competition hypothesis. Distribution studies, which suggest that less than 15% of the superoxide dismutase in higher plant tissues is membrane associated, lend further support. However, constant levels of superoxide dismutase in extracts from control and oxygen-exposed tissue, TABLE XV, demonstrate that the homogenization procedure effectively extracts superoxide dismutase from G. herbaceum leaf tissue.

Effects of Atmospheric Water and Oxygen on Protein and Enzyme Levels

During acclimation of G. herbaceum plants to environmental conditions, including 95% humidity, inside vinyl chambers, appearance of cellular edema was indicative of accumulation of water in the leaves. Decreasing yields of soluble protein per g fresh tissue (TABLE XI) were consistent with an increasing contribution of water to fresh

tissue weight, and after a 6 day exposure period, a 25-35% decrease in the dry weight/fresh weight ratio corresponded with a similar decrease in protein yield. Since specific activities of glycolate oxidase and catalase remained essentially unchanged, decreases in glycolate oxidase (-37%) and catalase (-28%) activities expressed as a function of fresh tissue weight (TABLE XII) are probably also related to this humidity effect. However, the 188% increase in the specific activity of peroxidase during this same period represents a change in the level of this enzyme in response to the elevation in humidity, and development of chlorosis, followed by leaf drop, implicate senescence phenomena in the alteration in peroxidase levels (142,173). These observations indicate a need to carefully regulate humidity around plants during experimental procedures so that treatment conditions do not differ significantly from growth conditions and alterations in health of the tissue are related only to the parameter being evaluated.

In 75% oxygen studies carried out at 70% humidity, visible symptoms of environmental stress were absent from both control and oxygen-treated tissue. Apparent good health of leaves, combined with reports of protection

against toxic effects of oxygen afforded bacteria, blue-green and green algae, and rats by oxygen-dependent increases in superoxide dismutase activity (75,110-115), suggested an oxygen-dependent increase in superoxide dismutase in cotton leaves. Similar electrophoretic patterns for superoxide dismutase in extracts from oxygen-treated and control tissues (FIGURES 9,10) and a failure to observe an increase in the level of activity of either the cyanide-sensitive or insensitive superoxide dismutases in maize (98) and G. herbaceum C.B. 1697 (TABLES XI, XIII, XIV) leaf tissues exposed to greater-than-normal levels of atmospheric oxygen suggests that higher plants possess adequate constitutive levels of the enzyme, utilize additional mechanisms for removal of superoxide, or regulate superoxide production.

It has been proposed that superoxide dismutase is essential for protection of aerobic organisms against toxic effects of oxygen (45,174-176), yet identity of the chemical species responsible for oxygen-dependent changes in levels of superoxide dismutase remains obscure, although superoxide is considered a likely candidate. Utilization of oxygen as an electron acceptor may impose upon

the higher plant a high concentration of superoxide, even under normal growth conditions, and endogenous production of oxygen during photosynthetic oxidation of water may provide a natural mechanism for increasing intracellular oxygen concentrations. Sensitivity of chloroplastic components to photooxidative damage (74,76) may therefore necessitate high levels of superoxide dismutase activity, and, in fact, specific activities of superoxide dismutase in crude extracts from higher plants (20-25 units/mg protein) (105,171,177) are similar to, or greater than, those present in extracts from eukaryotic algae (15-25 units/mg protein) (104,116), and microorganisms (0-30 units/mg protein) (75,178-181). Additionally, the 2-3-fold higher specific activity of the Cu-Zn superoxide dismutase purified from higher plant sources, TABLE XVII, and the high content (50-75%) of this form of superoxide dismutase in land plants (105) may permit higher plants to tolerate large fluctuations in atmospheric oxygen, even of the magnitude experienced in experiments reported here. Perhaps the absence of the Cu-Zn superoxide dismutase in eukaryotic algae (105) and the lower concentration of oxygen in buffered aqueous media [5% O_2 (v/v)] (194) compared to

TABLE XVII

Specific activities of purified, homogeneous superoxide dismutases from prokaryotic and eukaryotic sources

FORM	SOURCE	SPECIFIC ACTIVITY[a]	REFERENCE
Cu-Zn			
	Spinach	9300	(107)
	Green Pea	6400	(169)
	Wheat Germ	4700	(170)
	Neurospora crassa	2700	(182)
	Saccharomyces cerevisiae	3300	(183)
	Chicken Liver Cytosol	3300	(184)
	Bovine Erythrocytes	3300	(134)
	Human Erythrocytes	3000	(134)
Mn			
	Red Algae	3200	(185)
	Chicken Liver Mitochondria	3400	(184)
	Escherichia coli	3800	(186)
	Thermus aquaticus	2700	(187)
Fe			
	Plectonema boryanum	2900	(188)
	Pseudomonas ovalis	4000	(189)
	Escherichia coli	2500	(190)
	Desulfovibrio desulfuricans	2060	(191)
	Chromatium vinosum	2250	(192)
	Thiobacillus denitrificans	1940	(193)

[a] Specific activities (units/mg enzyme) were determined according to McCord and Fridovich (133).

air [21% O_2 (v/v)] accounts for induction of superoxide dismutase in Chlorella, but not in cotton or maize leaves.

As summarized then in TABLE XVIII, exposure of G. herbaceum C.B. 1697 to 75% oxygen resulted in alterations in levels of certain enzymes and soluble protein. Prominent features of these results, however, are the essentially unchanged level of superoxide dismutase and a very large, statistically significant increase in glutathione reductase level in extracts from oxygen-exposed tissue. On the basis of its unparalled sensitivity to the increase in atmospheric oxygen, a 100-200% increase in activity, glutathione reductase appears to be a likely participant in the protection of cells against oxygen or its intermediate reduction products. Although the specific activity of glutathione reductase in cotton leaf extracts is relatively low, it compares favorably with data reported for glutathione reductase in crude protein extracts from other plant tissues, TABLE XIX; its activity, expressed as a function of fresh tissue weight is consistent with data obtained for evergreens (123); and it is stable in extracts prepared according to the optimized procedure for this tissue (FIGURE 4). Moreover, glutathione reductase in G.

TABLE XVIII

Oxygen-dependent changes in enzyme levels in G. herbaceum C.B. 1697 leaf tissue exposed to 75% O_2 for 48 hr

	Change (%)[a]	
Humidity	95%[b]	70%[c]
Glutathione Redutase	+95***	+183***
Catalase	unchanged	-32***
Peroxidase	unchanged	+22**
Malate Dehydrogenase (NADH)	+30***	unchanged
Acid Phosphatase	unchanged	+85**
Soluble Protein	unchanged	-15**
Superoxide Dismutase	unchanged	unchanged
Glyoxylate Reductase	unchanged	unchanged
Glycolate Oxidase	unchanged	unchanged

[a] Changes were calculated using enzyme activities, expressed as units/g 2-4 week-old fresh tissue, in extracts obtained from control tissue and tissue exposed to 75% oxygen for 48 hr. Asterisks define those enzymes for which statistically significant changes in activity occurred as a result of the high-oxygen treatment: ** $0.005 < P < 0.01$; *** $P < 0.005$.

[b] Means (n=4) used in calculations of % changes were obtained from TABLE XIII. Control conditions: 95% humidity, 21% oxygen.

[c] Means (n=8) used in calculations of % change were obtained from TBALE XIV. Control conditions: 70% humidity, 21% oxygen.

TABLE XIX

Specific activities of glutathione reductase in crude protein extracts from higher plant tissues

Tissue	Specific Activity (units/mg protein)	Reference
G. herbaceum C.B. 1697[a]	0.004	
Spinach Leaf[b]	0.023	(195)
Sunflower Leaf[b]	0.003	(195)
Cabbage Leaf[b]	0.004	(195)
Parsley Leaf & Stem[b]	0.008	(195)
Tomato Leaf & Stem[b]	0.003	(195)
Spinach Chloroplast Stroma[c]	0.016	(141)
Wheat Germ[b]	0.024	(195)
Pea Seedling[c]	0.030	(196)
Etiolated Pea Seedling[b]	0.004	(195)

[a] Protein was estimated using the Coomassie G-250 dye-binding assay (132). Specific activity represents the average of data for samples from experiments summarized in TABLES X, XI, XIII, and XIV for air-grown tissue less than 1 month in age.

[b] Protein was estimated from the dry weight of dialyzed tissue extract (195).

[c] Protein was estimated using the Lowry procedure (196).

herbaceum C.B. 1697 leaf tissue was the only enzyme to exhibit an increase in activity after 24 hr of exposure of plants to 75% oxygen (TABLE XII), and a 2-3-fold increase in glutathione reductase activity was observed routinely in extracts from leaves exposed to the increase in atmospheric oxygen for 48 hr (TABLES XI-XIV).

In view of its presence in an organelle subject to a flux of reactive oxygen species and its sensitivity to oxygen in the environment, glutathione reductase may indeed function in maintaining reactive oxygen species at tolerable levels (FIGURE 11). Absence of response of superoxide dismutase in leaf tissue exposed to elevated atmospheric oxygen concentrations may be related to protection which accrues from participation of glutathione reductase in the regeneration of reduced glutathione. By providing reduced glutathione for utilization in the nonenzymatic GSH:ascorbate sequence for reduction of superoxide and hydrogen peroxide (FIGURE 11), glutathione reductase not only supplements protection provided by superoxide dismutase, but may also minimize the potential for hydrogen peroxide inhibition (197,198) of the predominant Cu-Zn superoxide dismutase (102) localized within the chloroplast. In the

absence of catalase (59,65,73,85) and peroxidase (83,84, 150) from this organelle, the compensatory role of reduced glutathione in removal of chloroplastic hydrogen peroxide is likely a major factor in the turnover of the glutathione pool. The increase in glutathione reductase activity in response to high oxygen tensions also insures availability of reduced glutathione for protection of sulfhydryl enzymes against an increase in oxidative conditions within the chloroplast (199,200). Thus for the plant system, glutathione reductase may be equally as important as superoxide dismutase in protecting cellular components against harmful effects of oxygen, and its observed response under the experimental conditions contributes toward the understanding of oxygen metabolism in leaf tissue.

It has been shown (201) that, upon exposure to high pressure oxygen (17 atm) at pH 7.0 in the presence of heavy metal ions, glutathione becomes oxidized at a rate ($k = 0.242$ μmoles/min) which is approximately 4 times the rate at which ascorbate is oxidized ($k = 0.058$ μmoles/min). Moreover, within the chloroplast, glutathione and ascorbate are present at concentrations of 3.5 mM and 2.5 mM, respectively (61) while, at 1 atm pressure, oxygen in

buffered aqueous media occurs at a concentration of 2.5 mM, the solubility of oxygen in this medium at 0° with respect to an atmosphere of 100% oxygen (194). Limited solubility of oxygen and its subsequent effect on oxygen concentration, combined with the comparatively higher concentration of chloroplastic glutathione and the potential for direct reduction of oxygen by heavy metal-catalyzed autoxidation of reduced glutathione (199,200), may result in an intrachloroplastic oxygen concentration which is lower than expected, based on the concentration of oxygen in the atmosphere, and which is insufficient to cause an induction of superoxide dismutase.

As a consequence of the turnover of reduced glutathione and the dynamic flow of electrons through intermediates to $NADP^+$, increased utilization of reducing equivalents from NADPH by glutathione reductase for regeneration of reduced glutathione may simultaneously reduce the possibility for enhanced production of superoxide under high oxygen tensions by insuring availability of the preferred electron acceptor, $NADP^+$. Elevated levels of glutathione reductase, but unaltered levels of the chloroplastic NADPH-specific glyoxylate reductase in oxygen-exposed tissue (TABLES XIV, XIII)

suggests that, if superoxide production were regulated by levels of $NADP^+$ in the chloroplast, the primary mechanism involves the indirect participation of glutathione reductase, not glyoxylate reductase (FIGURE 11). Further, carbon moving through glycolate and also turned over through glyoxylate reductase is likely not increased in these experiments to a point beyond that which can normally be accomodated in the cell since there is no increase either in peroxisomal enzymes or in glyoxylate reductase (TABLES XIV, XVIII).

Significantly lower levels of catalase activity in extracts from oxygen-treated cotton leaf tissue (TABLES XIV, XVIII) were consistent with the decreased catalase levels observed in higher plants subjected to high pressure oxygen (121) and may be attributed to oxygen-induced, senescence-like phenomena (142,173). Should a proteinaceous catalase inhibitor of the type observed in maize scutella (202) occur in cotton leaf tissue, an oxygen-dependent increase in that inhibitor might also account for the observed decrease in catalase activity. On the other hand, the increase in oxygen tension may have caused an alteration in oxygen metabolism that did not require the

protective action of catalase. Indeed, oxygen-toxicity studies have, in several instances, failed to demonstrate increased resistance to detrimental effects of oxygen, even when catalase was induced (111,119).

Hydrogen peroxide generated by glycolate oxidase in the peroxisome may be removed nonenzymatically by action with glyoxylate to produce formate and carbon dioxide (FIGURE 11) (65). Under normal growth conditions this reaction may be responsible for destruction of H_2O_2 not removed by catalase and may function under conditions in which abnormally high concentrations of glyoxylate and hydrogen peroxide are present. The observation that activity of glycolate oxidase/g tissue was unaffected (TABLE XIV) by exposure of leaves to 75% oxygen, combined with the decrease in catalase level, suggests that problems related to oxygen metabolism under these conditions do not require additional capacity in the flow of metabolites processed by the peroxisomes.

With distribution of peroxidase among numerous cellular compartments, including the nucleus, mitochondria, cell wall, golgi, and cytoplasm (86,87,150), the increase in level of this enzyme in tissues exposed to 75% oxygen may be symptomatic of increased concentration of hydrogen

peroxide in any one or several of these cellular locations. While superoxide dismutase, also found in mitochondria and in the cytosol, may generate H_2O_2 during disproportionation of superoxide, the slight decrease in activity of this enzyme is not consistent with the large, increase in peroxidase activity. However, the increase in peroxidase activity may be similar to a disease response (109,150) caused by oxygen-related tissue damage, not apparent upon visual examination of the leaves, or a senescence response induced by the high oxygen tension (142,173). Alternatively, the higher level of peroxidase activity in extracts from oxygen-treated tissue may reflect release of peroxidase from membrane structures to which it is normally closely associated.

The increase in acid phosphatase(s) activity in these same extracts (TABLES XIV, XVIII) indicate that oxygen effects on higher plant metabolism are not restricted to oxidative enzymes. Though no explanation for the response has been offered, Buchanan, et al. (203) have demonstrated an activation of spinach acid phosphatase under oxidative conditions, data consistent with the in vivo observations reported in TABLES XIV and XVIII. Although most of the acid phosphatase of leaves is localized in the vacuole (203),

some may be associated with chloroplasts (151), mitochondria, lysosomes, golgi, endoplasmic reticulum, and the cell wall (204). As with peroxidase, exposure of tissue to elevated oxygen may facilitate extraction of acid phosphatase from insoluble cellular components.

Consistent with its selection as a marker enzyme, NADH-specific malate dehydrogenase in cotton leaf tissue was insensitive to the elevation in atmospheric oxygen, and constant levels of this enzyme (TABLE XIV, XVIII), as well as superoxide dismutase and NADPH-dependent glyoxylate reductase, indicate that not all enzymes in leaf tissue exposed to the high oxygen tension undergo alterations in levels of activity. Increased respiratory activity which accompanies senescence events, combined with distribution of this form of malate dehydrogenase among peroxisomes, the cytosol, and particularly mitochondria (151) may be responsible for the increase in malate dehydrogenase activity in extracts from leaves subjected to the dual stress of 95% humidity and 75% oxygen (TABLES XII, XIII, XVIII).

The decrease in soluble protein in extracts from cotton leaf tissue exposed to 75% oxygen (TABLE XIV, XVIII) makes evaluation of ribulose bisphosphate carboxylase/

oxygenase in these extracts a matter of primary interest. Initially not thought to be an issue, an oxygen-dependent decrease in the amount of this enzyme may occur. Since ribulose bisphosphate carboxylase/oxygenase constitutes approximately 50% of the total soluble protein in leaf tissue (205), a decrease in the level of this one enzyme may have an important impact upon interpretation of enzymatic involvement in oxygen metabolism. Moreover, the inconsistent response of glycolate oxidase under the experimental conditions (TABLE XIV) indicates the further need to evaluate effects of longer exposure periods upon levels of both soluble protein and enzymatic activity.

As mentioned, metabolism of oxygen in leaf tissue occurs in multiple cellular locations. Absence of an oxygen effect on glycolate oxidase and a decrease in catalase activity in extracts from leaves exposed to 75% oxygen at 70% humidity (TABLE XVIII) are important aspects in the appreciation of interactive metabolism outlined in FIGURE 11. Data presented here indicate that peroxisomal enzymes related to the photorespiratory pathway are not sensitive to high concentrations of oxygen. However, responsiveness of glutathione reductase implicate the chloroplast as the

intracellular site most sensitive to oxygen, and while effects of oxygen concentrations less than 21% on activity of ribulose bisphosphate carboxylase/oxygenase have been evaluated (9,10,15-22), this research has made apparent the need to explore effects of supraatmospheric oxygen concentrations on this enzyme.

In addition to activation of acid phosphatase(s) by oxidized glutathione (203), Wolosiuk and Buchanan (150,206) have reported light-dependent activation of several regulatory enzymes in spinach chloroplasts by the ferredoxin/thioredoxin reductase system and subsequent deactivation of these regulatory enzymes in the dark by soluble oxidants such as dehydroascorbate and oxidized glutathione. Although small molecular weight compounds are rapidly removed from extracts in the present experiments, it is important to recognize that such activation and deactivation processes may occur during processing of tissue so that observed *in vitro* levels of enzymatic activity may not be representative of *in vivo* levels.

SUMMARY

Success in evaluating effects of a 75% oxygen atmosphere, containing an ambient concentration of carbon dioxide, on levels of protein and oxidative enzymes in G. herbaceum C.B. 1697 leaf tissue depended upon the development of a procedure that, with uniform tissue, gives reproducible quantities of soluble protein and active enzymes. Analysis of oxidative enzymes, successfully extracted in a buffered isoascorbate homogenization medium containing both soluble and insoluble polyvinylpyrrolidone, revealed that 2-4 week-old leaves were more sensitive to environmental conditions than older tissue, and changes in enzymatic activity were observed in response to both high humidity and a high oxygen tension.

Under otherwise typical growth conditions, 75% oxygen produced a 2-3 fold increase in glutathione reductase activity, but had no effect on levels of superoxide dismutase. Increases in peroxidase (+22%) and acid phosphatase (+85%) and a 32% decrease in catalase occurred simultaneously. Combined with a decrease in soluble protein (-15%), constant levels of malate dehydrogenase and glyoxylate reductase, as well as superoxide dismutase,

indicated that a general alteration in metabolic activity did not occur, but that certain enzymes responded specifically to the elevation in atmospheric oxygen concentration.

REFERENCES

1. Hutchins, L. M. (1926) Studies on the oxygen-supplying powers of the soil together with quantitative observations on the oxygen-supplying power requisite for seed germination. Plant Physiol. 1, 95-150.

2. Boveris, A. and Chance, B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem. J. 134, 707-716.

3. Barber, D. A., Ebert, M. and Evans, N. T. S. (1962) The movement of ^{15}O through barley and rice plants. J. Exp. Bot. 13, 397-403.

4. Greenwood, D. J. (1967) Studies on the transport of oxygen through the stems and roots of vegetable seedlings. New Phytol. 66, 337-347.

5. Letey, J., Stolzy, L. G. and Blank, G. B. (1962) Effect of duration and timing of low soil oxygen content on shoot and root growth. Agron. J. 54, 34-37.

6. Van't Woudt, B. D. and Hagan, R. M. (1957) Crop responses at excessively high soil moisture levels. In Drainage of Agricultural Lands (Luthin, J. N., Ed.), pp 514-612, American Society of Agronomy, Madison.

7. Healy, M. T. and Armstrong, W. (1972) The effectiveness of internal oxygen transport in a mesophyte (Pisum sativum L.) Planta 103, 302-309.

8. Chollet, R. and Ogren, W. L. (1975) Regulation of photorespiration in C_3 and C_4 species. Bot. Rev. 41, 137-179.

9. Quebedeaux, B. and Hardy, R. W. F. (1975) Reproductive growth and dry matter production of Glycine max (L.) Merr. in response to oxygen concentration. Plant Physiol. 55, 102-107.

10. Quebedeaux, B. and Hardy, R. W. F. (1973) Oxygen as a new factor controlling reproductive growth. Nature 243, 477-479.

11. Elstner, E. F. and Konze, J. R. (1974) Light-dependent ethylene production by isolated chloroplasts. FEBS Lett. 45, 18-21.

12. Solomos, T. (1977) Cyanide-resistant respiration in higher plants. Ann. Rev. Plant Physiol. 28, 279-298.

13. Viil, J., Laisk, A., Oja, V. and Parnik, T. (1977) Enhancement of photosynthesis caused by oxygen under saturating irradiance and high CO_2 concentrations. Photosynthetica 11, 251-259.

14. Warburg, O. (1920) Über die Geschwindigkeit der photochemischen Kohlensäurezersetzung in lebenden Zellen. II. Biochem. Z. 103, 188-217.

15. Andrews, T. J., Lorimer, G. H. and Tolbert, N. E. (1973) Ribulose diphosphate oxygenase. I. Synthesis of phosphoglycolate by Fraction-1 protein of leaves. Biochemistry 12, 11-18.

16. Bowes, G., Ogren, W. L. and Hageman, R. H. (1971) Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. Biochem. Biophys. Res. Commun. 45, 716-722.

17. Bowes, G. and Ogren, W. L. (1972) O_2 inhibition and other properties of soybean ribulose 1,5-diphosphate carboxylase. J. Biol. Chem. 247, 2171-2176.

18. Badger, M. R. and Andrews, T. J. (1974) Effects of CO_2, O_2, and temperature on a high affinity form of ribulose diphosphate carboxylase-oxygenase from spinach. Biochem. Biophys. Res. Commun. 60, 204-210.

19. Ogren, W. L. and Bowes, G. (1971) Ribulose diphosphate carboxylase regulates soybean photorespiration. Nature New Biol. 280, 159-160.

20. Goldsworthy, A. (1976) Photorespiration. Oxford/Carolina Biology Readers Series 80. Carolina Biological Supply Company, Burlington, North Carolina.

21. Laing, W. A., Ogren, W. L. and Hageman, R. H. (1974) Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2 and O_2 and ribulose 1,5-diphosphate carboxylase. Plant Physiol. 54, 678-685.

22. Ku, S. -B. and Edwards, G. E. (1977) Oxygen inhibition of photosynthesis. I. Temperature dependence and relation to O_2/CO_2 solubility ratio. Plant Physiol. 59, 986-990.

23. Steiger, H. M., Beck, E. and Beck, R. (1977) Oxygen concentration in isolated chloroplasts during photosynthesis. Plant Physiol. 60, 903-906.

24. Vartapetyan, B. B. (1964) Polarographic investigation of oxygen transport in plants. Soviet Plant Physiol. (Engl.) 11, 659-665.

25. Lorimer, G. H., Andrews, T. J. and Tolbert, N. E. (1973) Ribulose diphosphate oxygenase. II. Further proof of reaction products and mechanism of action. Biochemistry 12, 18-23.

26. Viil, J. and Parnik, T. (1978) Control of glycolate pathway by light and oxygen. Z. Pflanzenphysiol. 88, 219-226.

27. Jackson, W. A. and Volk, R. J. (1970) Photorespiration. Ann. Rev. Plant Physiol. 21, 385-432.

28. Goldsworthy, A. (1970) Photorespiration. Bot. Rev. 36, 321-340.

29. Krause, G. H., Lorimer, G. H., Heber, U. and Kirk, M. R. (1978) Photorespiratory energy dissipation in leaves and chloroplasts. In Photosynthesis 77, Proceedings of the Fourth International Congress on Photosynthesis (Hall, D. O., Coombs, J. and Goodwin, T. W., Eds.), pp 299-310, Biochemical Society, London.

30. Lorimer, G. H. and Andrews, T. J. (1973) Plant photorespiration -- an inevitable consequence of the existence of atmosphere oxygen. Nature 243, 359-360.

31. Kaler, V. L., Klinger, Y. E., Lokter, A. V. and Vecher, A. S. (1977) Coupling of glycolate metabolism with chlorophyll synthesis in plants. Soviet Plant Physiol. (Engl.) 24, 21-24.

32. Krause, G. H., Kirk, M., Heber, U. and Osmond, C. B. (1978) O_2-dependent inhibition of photosynthetic capacity in intact isolated chloroplasts and isolated cells from spinach leaves illuminated in the absence of CO_2. Planta 142, 229-233.

33. Osmond, C. B. and Björkman, O. (1972) Effects of 0_2 on photosynthesis. Simultaneous measurements of oxygen effects on net photosynthesis and glycolate metabolism in C_3 and C_4 species of Atriplex. Carnegie Inst. Washington Yearbook 71, 141-148.

34. Mehler, A. H. (1951) Studies on reaction of illuminated chloroplasts. I. Mechanisms of the reduction of oxygen and other Hill reagents. Arch. Biochem. Biophys. 33, 65-77.

35. Radmer, R. J. and Kok, B. (1976) Photoreduction of 0_2 primes and replaces $C0_2$ assimilation. Plant Physiol. 58, 336-340.

36. Allen, J. F. (1975) Oxygen reduction and optimum production of ATP in photosynthesis. Nature 256, 599-600.

37. Egneus, H., Heber, U., Matthiesen, U. and Kirk, M. (1975) Reduction of oxygen by the electron transport chain of chloroplasts during assimilation of carbon dioxide. Biochim. Biophys. Acta 408, 252-268.

38. Forti, G. and Gerola, P. (1977) Inhibition of photosynthesis by azide and cyanide and the role of oxygen in photosynthesis. Plant Physiol. 59, 859-862.

39. Forrester, M. L., Krotkov, G. and Nelson, C. D. (1966) Effect of oxygen on photosynthesis, photorespiration and respiration in detached leaves. I. Soybean. Plant Physiol. 41, 422-427.

40. Chapman, F. A. and Graham, D. (1974) The effect of light on the tricarboxylic acid cycle in green leaves. I. Relative rates of the cycle in the dark and the light. Plant Physiol. 53, 879-885.

41. Schonbaum, G. R., Bonner, W. D., Jr., Storey, B. T., and Bahr, J. T. (1971) The respiratory chain of plant mitochondria. I. Electron transport between succinate and oxygen in skunk cabbage mitochondria. Plant Physiol. 44, 115-128.

42. Wilson, S. B. (1978) Energy conservation associated with cyanide-insensitive respiration in plant mitochondria. Biochim. Biophys. Acta 223, 383-387.

43. Berry, J. A., Osmond, C. B. and Lorimer, G. H. (1978) Fixation of $^{18}O_2$ during photorespiration. Kinetic and steady state studies of the photorespiratory carbon oxidation cycle with intact leaves and isolated chloroplasts of C_3 plants. Plant Physiol. 62, 954-967.

44. Fridovich, I. (1974) Superoxide dismutase. In Advances in Enzymology 41, (Meister, H., Ed.), pp 35-98, John Wiley and Sons, New York.

45. Fridovich, I. (1974) Superoxide and evolution. In Horizons in Biochemistry and Biophysics (Quagliariello, E., Palmieri, F., and Singer, T. P., Eds.), pp 1-37, Addison-Wesley, Reading, Massachusetts.

46. Koppenol, W. H. and Butler, J. (1977) Mechanism of reactions involving singlet oxygen and the superoxide anion. FEBS Lett. 83, 1-6.

47. Fong, K. -L., McCay, P. B., Poyer, J. L., Misra, H. P. and Keele, B. B. (1976) Evidence for superoxide dependent reduction of Fe^{3+} and its role in enzyme generated hydroxyl radical formation. Chem. Biol. Interact. 15, 77-89.

48. Haber, F. and Weiss, J. (1934) The catalytic decomposition of hydrogen peroxide by iron salts. Proc. Roy. Soc. London Ser. A, 147, 332-351.

49. Mayeda, E. A. and Bard, A. J. (1974) Singlet oxygen: the suppression of its production in dismutation of superoxide ion by superoxide dismutase. J. Amer. Chem. Soc. 96, 4023-4024.

50. Schaap, A. P., Thayer, A. L., Foler, G. R., Goda, K. and Kimura, T. (1974) Singlet molecular oxygen and superoxide dismutase. J. Amer. Chem. Soc. 96, 4025-4026.

51. Loschen, G., Azzi, A., Richter, G. and Flohé, L. (1974) Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett. 42, 68-72.

52. Kellogg, E. W. and Fridovich, I. (1975) Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J. Biol. Chem. 250, 8812-8817.

53. Asada, K. and Kiso, K. (1973) The photo-oxidation of epinephrine by spinach chloroplasts and its inhibition by superoxide dismutase: evidence for the formation of superoxide radicals in chloroplasts. Agr. Biol. Chem. 37, 453-454.

54. Allen, J. F. and Hall, D. O. (1973) Superoxide reduction as a mechanism of ascorbate-stimulated oxygen uptake by isolated chloroplasts. Biochem. Biophys. Res. Commun. 52, 856-862.

55. Elstner, E. F. and Heupel, A. (1976) Oxygen activation by isolated chloroplasts from Euglena gracilis. Isolation and purification of a fluorescent compound that catalyzes monovalent oxygen reduction. Arch. Biochem. Biophys. 173, 614-622.

56. Elstner, E. F. and Kramer, R. (1973) Role of superoxide free radical ion in photosynthetic ascorbate oxidation and ascorbate mediated photophosphorylation. Biochim. Biophys. Acta 314, 340-353.

57. Epel, B. L. and Neumann, J. (1973) The mechanism of oxidation of ascorbate and Mn^{2+} by chloroplasts: the role of the radical superoxide. Biochim. Biophys. Acta 325, 520-529.

58. Asada, K., Kiso, K. and Yoshikawa, K. (1974) Univalent reduction of molecular oxygen by spinach chloroplasts on illumination. J. Biol. Chem. 249, 2175-2181.

59. Asada, K. and Kiso, K. (1973) Initiation of aerobic oxidation of sulfite by illuminated spinach chloroplasts. Eur. J. Biochem. 33, 253-257.

60. Harbour, J. R. and Bolton, J. R. (1975) Superoxide formation in spinach chloroplasts: electron spin resonance detection by spin trapping. Biochem. Biophys. Res. Commun. 64, 803-807.

61. Asada, K., Takahashi, M. -A., Tanaka, K. and Nakano, Y. (1977) Formation of active oxygen and its fate in chloroplasts. In Biochemical and Medical Aspects of Active Oxygen (Hayaishi, O. and Asada, K., Eds.), pp 45-63, University Park Press, Baltimore.

62. Takahama, U. and Nishimura, M. (1975) Formation of singlet molecular oxygen in illuminated chloroplasts. Effects on photoinactivation and lipid peroxidation. Plant Cell Physiol. 16, 737-748.

63. Halliwell, B. (1978) The chloroplast at work. A review of modern developments in our understanding of chloroplast metabolism. Progr. Biophys. Molec. Biol. 33, 1-54.

64. Rich, P. R. and Bonner, W. D., Jr. (1978) The sites of superoxide anion generation in higher plant mitochondria. Arch. Biochem. Biophys. 188, 206-213.

65. Rich, P. R., Boveris, A., Bonner, W. D., Jr. and Moore, A. L. (1976) Hydrogen peroxide generation by the alternate oxidase of higher plants. Biochem. Biophys. Res. Commun. 71, 695-703.

66. Boveris, A., Sanchez, R. A. and Beconi, M. T. (1978) Antimycin- and cyanide-resistant respiration and superoxide anion production in fresh and aged potato tuber mitochondria. FEBS Lett. 92, 333-338.

67. Grodzinski, B. and Butt, V. S. (1976) Hydrogen peroxide production and the release of carbon dioxide during glycolate oxidation in leaf peroxisomes. Planta 128, 225-231.

68. Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Shuman, M. and Sullivan, P. A. (1969) The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochem. Biophys. Res. Commun. 36, 891-897.

69. Halliwell, B. (1978) Lignin synthesis: the generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols. Planta 140, 81-88.

70. Gross, G. G., Janse, C., and Elstner, E. F. (1977) Involvement of malate, monophenols and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (Armoracia lapathifolia Gilib.). Planta 136, 271-276.

71. Tolbert, N. E., Yamazaki, R. K. and Oeser, A. (1970) Localization and properties of hydroxypyruvate and glyoxylate reductases in spinach leaf particles.

J. Biol. Chem. 245, 5129-5136.

72. Abeles, F. B., Leather, G. R. and Forrence, L. E. (1978) Plant chemiluminescence. Plant Physiol. 62, 696-698.

73. Brennan, T. and Frenkel, C. (1977) Involvement of hydrogen peroxide in the regulation of senescence in pear. Plant Physiol. 59, 411-416.

74. Halliwell, B. (1978) Biochemical mechanisms accounting for the toxic action of oxygen on living organisms: the key role of superoxide dismutase. Cell Biol. Internat. Reports 2, 113-128.

75. Abeliovich, A., Kellenberg, D. and Shilo, M. (1974) Effect of photoxidative conditions on levels of superoxide dismutase in Anacystis nidulans. Photochem. Photobiol. 19, 379-383.

76. Farrington, J. A., Ebert, M., Land, E. J., and Fletcher, K. (1973) Bipyridylium quaternary salts and related compounds. V. Pulse radiolysis studies of the reaction of paraquat radical with oxygen. Implications for the mode of action of bipyridyl herbicides. Biochim. Biophys. Acta 314, 372-381.

77. Matthews-Roth, M. M., Wilson, T., Fujimori, E. and Krinsky, N. J. (1974) Carotenoid chromophore length and protection against photosensitization. Photochem. Photobiol. 19, 217-222.

78. Krinski, N. I. (1966) The role of carotenoid pigments as protective agents against photosensitized oxidations in chloroplasts. In Biochemistry of Chloroplasts. I. (Goodwin, T. W., Ed.), pp 423-430, Academic Press, New York.

79. Walker, D. A., Ludwig, L. J. and Whitehouse, D. G. (1970) Oxygen evolution in the dark following illumination of chloroplasts in the presence of added manganese. FEBS Lett. 6, 281-284.

80. Halliwell, B. and Foyer, C. H. (1978) Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta 139, 9-17.

81. Foyer, C. H. and Halliwell, B. (1976) The presence of glutathione and glutathione reductase in chloro-

plasts: a proposed role in ascorbic acid metabolism. Planta 133, 21-25.

82. Elstner, E. F., Stoffer, C. and Heupel, A. (1975) Determination of superoxide free radical ion and hydrogen peroxide as products of photosynthetic oxygen reduction. Z. Naturforsch 30 C, 53-56.

83. Flohé, L. and Günzler, W. A. (1976) Glutathione-dependent enzymatic oxidoreduction reactions. In Glutathione: Metabolism and Function, Kroc Foundation Series 6 (Arias, I. M. and Jakoby, W. B., Eds.), pp 17-34, Raven Press, New York.

84. Flohé, L. and Menzel, A. (1971) The influence of GSH upon light-induced high-amplitude swelling and lipid peroxide formation of spinach chloroplasts. Plant Cell Physiol. 12, 325-333.

85. Halliwell, B. (1974) Superoxide dismutase, catalase, and glutathione peroxidase: solutions to the problems of living with oxygen. New Phytologist 73, 1075-1086.

86. Raa, J. (1973) Cytochemical localization of peroxidases in plant cells. Physiol. Plant. 28, 132-133.

87. Brinkman, F. G. and Sminia, T. (1977) Histochemical location of catalase in peroxisomes and peroxidase in cell walls and golgi bodies of cells in differentiating potato tuber tissue. Z. Pflanzenphysiol. 84, 407-412.

88. Fridovich, I. (1975) Superoxide dismutases. Ann. Rev. Biochem. 44, 147-159.

89. Rotilio, G., Bray, R. C. and Fielden, E. M. (1972) A pulse radiolysis study of superoxide dismutase. Biochim. Biophys. Acta 268, 605-609.

90. Forman, H. J. and Fridovich, I. (1973) Superoxide dismutase: a comparison of rate constants. Arch. Biochem. Biophys. 158, 396-400.

91. Rigo, A., Viglino, P. and Rotilio, G. (1975) Kinetic study of O_2^- dismutation by bovine superoxide dismutase. Evidence for saturation of the catalytic sites by O_2. Biochem. Biophys. Res. Commun. 63, 1013-1018.

92. Weser, U., Paschen, W., and Younes, M. (1975) Singlet oxygen and superoxide dismutase (cuprein). Biochem. Biophys. Res. Commun. 66, 769-777.

93. Hodgson, E. K. and Fridovich, I. (1975) The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: chemiluminescence and peroxidation. Biochemistry 14, 5299-5303.

94. del Río, L. A., Sevilla, F., Gomez, M., Yañez, J. and López, (1978) Superoxide dismutase: an enzyme system for the study of micronutrient interactions in plants. Planta 140, 221-225.

95. Arron, G. P., Henry, L., Palmer, J. M. and Hall, D. O. (1976) Superoxide dismutase in mitochondria from Helianthus tuberosus and Neurospora crassa. Biochem. Soc. Transact. 4, 618-620.

96. Giannopolitis, C. N. and Ries, S. K. (1977) Superoxide dismutase. I. Occurrence in higher plants. Plant Physiol. 59, 309-314.

97. Beauchamp, C. O. and Fridovich, I. (1973) Isozymes of superoxide dismutase from wheat germ. Biochem. Biophys. Acta 317, 50-64.

98. Foster, J. G. (1976) Comparison of Superoxide Dismutase Levels in Extracts from Plant Tissue Grown in Normal and Elevated Concentrations of Oxygen. Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

99. Lumsden, J. and Hall, D. O. (1974) Soluble and membrane-bound superoxide dismutase in a blue-green alga (Spirulina) and spinach. Biochem. Biophys. Res. Commun. 58, 35-41.

100. Doll, S., Lutz, C. and Ruppel, H. G. (1976) Biochemische und cytologische Untersuchen zur Chloroplasten-entwicklung. Z. Pflanzenphysiol. 80, 166-176.

101. Lumsden, J. and Hall, D. O. (1975) Superoxide dismutase in photosynthetic organisms provides an evolutionary hypothesis. Nature 257, 670-672.

102. Jackson, C., Dench, J., Moore, A. L., Halliwell, B., Foyer, C. H. and Hall, D. O. (1978) Subcellular localization and identification of superoxide dismutase in the leaves of higher plants. Eur. J.

Biochem. 91, 339-344.

103. Elstner, E. F. and Heupel, A. (1975) Lamellar superoxide dismutase of isolated chloroplasts. Planta 123, 145-154.

104. Asada, K., Kanematsu, S., Takahashi, M. and Kona, Y. (1976) Superoxide dismutases in photosynthetic organisms. In Iron and Copper Proteins: Advances in Experimental Medicine and Biology 74 (Yasunoba, K. T., Mower, H. F., and Hayaishi, O., Eds.), pp 551-564, Plenum Press, New York.

105. Asada, K., Kanematsu, S. and Uchida, K. (1977) Superoxide dismutases in photosynthetic organisms: absence of the cuprozinc enzyme in eucaryotic algae. Arch. Biochem. Biophys. 179, 243-256.

106. Lumsden, J. and Hall, D. O. (1975) Chloroplast manganese and superoxide. Biochem. Biophys. Res. Commun. 64, 595-602.

107. Asada, K., Urano, M. and Takahashi, M. (1973) Subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. Eur. J. Biochem. 36, 257-266.

108. Tolbert, N. E., Yamazaki, R. K. and Oeser, A. (1970) Localization and properties of hydroxypyruvate and glyoxylate reductase in spinach particles. J. Biol. Chem. 245, 5129-5136.

109. Filner, P., Wary, J. L. and Varner, J. E. (1969) Enzyme induction in higher plants. Science 165, 358-367.

110. Puget, K. and Michelson, A. M. (1974) Isolation of a new copper-containing superoxide dismutase: bacteriocuprein. Biochem. Biophys. Res. Commun. 58, 830-838.

111. Gregory, E. M. and Fridovich, I. (1973) Induction of superoxide dismutase by molecular oxygen. J. Bacteriol. 114, 543-548.

112. Crapo, J. D. and Tierney, D. F. (1974) Superoxide dismutase and pulmonary oxygen toxicity. Amer. J. Physiol. 226, 1401-1407.

113. Liu, J., Simon, L. M., Phillips, J. R. and Robin, E. D. (1977) Superoxide dismutase (SOD) activity in hypoxic mammalian systems. J. Appl. Physiol.: Respir. Environ. Exercise Physiol. 42, 107-110.

114. Stevens, J. B. and Autor, A. P. (1977) Oxygen-induced synthesis of superoxide dismutase and catalase in pulmonary macrophages of neonatal rats. Lab Invest. 37, 470-477.

115. Gregory, E. M., Goscin, S. A. and Fridovich, I. (1974) Superoxide dismutase and oxygen toxicity in a eucaryote. J. Bacteriol. 117, 456-460.

116. Pulich, W. M. (1974) Resistance to high oxygen tension, streptonigrin, and ultraviolet irradiation in the green alga Chlorella sorokiniana strain ORS. J. Cell Biol. 62, 904-907.

117. Gregory, E. M., Yost, F. J., Jr. and Fridovich, I. (1974) Superoxide dismutase of Escherichia coli: intracellular localization and functions. J. Bacteriol. 115, 987-991.

118. Crapo, J. D., Sjostrom, K. and Drew, R. T. (1978) Tolerance and cross-tolerance using NO_2 and O_2. I. Toxicology and biochemistry. J. Appl. Physiol.: Respir. Environ. Exercise Physiol. 44, 364-369.

119. Gregory, E. M. and Fridovich, I. (1973) Oxygen toxicity and the superoxide dismutase. J. Bacteriol. 114, 1193-1197.

120. Hassan, H. M. and Fridovich, I. (1978) Regulation of the synthesis of catalase and peroxidase in Escherichia coli. J. Biol. Chem. 253, 6445-6450.

121. Pritchard, G. G. and Hudson, M. A. (1967) Changes in catalase activity in higher plants and fungi treated with oxygen at high pressure. Nature 214, 945-946.

122. Ray, L. E. and Cherry, J. P. (1977) Effects of hyperoxia on glutathione reductase activity, membrane proteins, and esterases of rabbit erythrocytes. Aviation, Space, Environ. Med. 48, 649-653.

123. Esterbauer, H. and Grill, D. (1978) Seasonal variation of glutathione and glutathione reductase in needles of Picea-Abies. Plant Physiol. 61, 119-121.

124. Berger, J. (1969) The World's Major Fibre Crops: Their Cultivation and Manuring, pp 17-18, Conzett and Huber, Zurich.

125. El-Sharkawy, M. A. and Hesketh, J. D. (1964) Effects of temperature and water deficit on leaf photosynthetic rates of different species. Crop Sci. 4, 514-518.

126. Baker, D. N. (1965) Effects of certain environmental factors on net assimilation in cotton. Crop Sci. 5, 53-56.

127. Ackerson, R. C. and Krieg, D. R. (1977) Stomatol and mitochondrial regulation of water use in cotton, corn, and sorghum. Plant Physiol. 60, 850-853.

128. Bell, A. A., Stipanovic, R. D., O'Brien, D. H. and Fryxell, P. A. (1978) Sesquiterpenoid aldehyde quinones and derivatives in pigment glands of Gossypium. Phytochemistry 17, 1297-1305.

129. Hoagland, D. R. and Arnon, D. (1938) The water-culture method for growing plants without soil. Univ. Calif. Agr. Expt. Station Circular 347.

130. Donohue, S. J. and McCoy, D. E. (1977) Laboratory procedures, soils testing laboratory, Agronomy Department, Virginia Polytechnic Institute and State University, Va. Polytech. Inst. and State Univ. Extension Bull. MA-143.

131. Perkin-Elmer (1964) Analytical Methods for Atomic Absorption Spectrophotometry. Norwalk, Connecticut.

132. Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

133. Sedmak, J. J. and Grossberg, S. E. (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. 79, 544-552.

134. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193, 265-273.

135. McCord, J. M. and Fridovich, I. (1969) Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049-6055.

136. Beauchamp, C. O. and Fridovich, I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276-287.

137. Davis, B. J. (1964) Disc Electrophoresis II. Method and application to human serum proteins. Annals New York Acad. Sci. 121, 404-427.

138. Beers, R. F., Jr. and Sizer, I. W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133-140.

139. Cohen, H. J. (1973) The use of diaminobenzidine for spectrophotometric and acrylamide gel detection of sulfite oxidase and its applicability to hydrogen peroxide-generating enzymes. Anal. Biochem. 53, 208-222.

140. Nickel, R. S. and Cunningham, B. A. (1969) Improved peroxidase assay method using leuco 2.3',6-trichloroindophenol and application to comparative measurements of peroxidatic catalysis. Anal. Biochem. 27, 292-299.

141. Schaedle, M. and Bassham, J. A. (1977) Chloroplast glutathione reductase. Plant Physiol. 59, 1011-1012.

142. Reed, R. E. (1974) Partial Purification and Characterization of Aspartate Aminotransferase from Seedling Oat Tissue. Ph.D. Dissertation, p 19 Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

143. DeLeo, P. and Sacher, J. A. (1970) Control of ribonuclease and acid phosphatase by auxin and abscisic acid during senescence of Rhoeo leaf sections. Plant Physiol. 46, 806-811.

144. Dixon, W. J. and Massey, F. J. (1969) Introduction to Statistical Analysis. McGraw-Hill, Inc. New York.

145. Huffman, W. E. D., Jr. and Allaway, W. H. (1973) Growth of plants in solution culture containing

low levels of chromium. Plant Physiol. 52, 72-75.

146. Sabbe, W. E. and MacKenzie, A. J. (1973) Plant analysis as an aid to cotton fertilization. In Soil Testing and Plant Analysis (Walsh, L. M. and Beaton, J. D., Eds.), pp 298-313, Soil Science Society of American, Inc., Madison.

147. O'Sullivan, S. A. and Wedding, R. T. (1972) Malate dehydrogenase isoenzymes from cotton leaves. Plant Physiol. 49, 117-123.

148. Loomis, W. D. and Battaile, J. (1966) Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry 5, 423-438.

149. Markossian, K. A., Poghossian, A. A., Paitian, N. A. and Nalbandyan, R. M. (1978) Superoxide dismutase activity of cytochrome oxidase. Biochem. Biophys. Res. Commun. 81, 1336-1343.

150. Wolosiuk, R. A. and Buchanan, B. B. (1978) Activation of chloroplast NADP-linked glyceraldehyde-3-phosphate dehydrogenase by the ferredoxin/thioredoxin system. Plant Physiol. 61, 669-671.

151. Parish, R. W. (1972) The intracellular location of phenol oxidases, peroxidase and phosphatases in the leaves of sugar beet (Beta vulgaris L. subspecies vulgaris). Eur. J. Biochem. 31, 446-455.

152. Shear, C. B. (1975) Calcium-related disorders of fruits and vegetables. Hortscience 10, 361-365.

153. Simon, E. W. (1978) The symptoms of calcium deficiency in plants. New Phytologist. 80, 1-15.

154. Waters, W. E., Llewelyn, W., Geraldson, C. M. and Woltz, S. S. (1973) The interpretation of soluble salt procedures as influenced by salinity testing procedure and soil media. Proc. Trop. Reg. Amer. Soc. Hort. Sci. 17.

155. Rajkamar, T. V., Woodfin, B. M. and Rutler, W. J. (1966) Aldolase B from (adult) rabbit liver. In Methods in Enzymology 9 (Colowick, S. P. and Kaplan, N. O., Eds.), pp 491-498, Academic Press, New York.

156. Fish, D. C. and Blumenthal, H. J. (1966) 2-keto-3-deoxy-D-glutarate aldolase. In Methods in Enzymology 9 (Colowick, S. P. and Kaplan, N. O., Eds.), pp 529-534, Academic Press, New York.

157. Wishnick, M. and Lane, M. D. (1971) Ribulose diphosphate carboxylase from spinach leaves. In Methods in Enzymology 23 (San Pietro, A., Ed.), pp 570-577, Academic Press, New York.

158. Herrero, F. A. and Hall, W. C. (1960) General effects of ethylene on enzyme systems in the cotton leaf. Physiol. Plant. 13, 736-750.

159. Morgan, P. W. and Hall, C. W. (1963) Indolacetic acid oxidizing enzymes and inhibitors from light-grown cotton. Plant Physiol. 38, 365-370.

160. Barber, G. A. and Hassid, W. Z. (1965) Synthesis of cellulose by enzyme preparations from the developing cotton boll. Nature 207, 295-296.

161. Wong, W. W. L., Benedict, C. R., McGrath, T. and Kohel, R. J. (1977) The fractionation of stable carbon isotopes by RuDP carboxylase. Plant Physiol. 59 (supp.) 42, abstr. 233.

162. Ku, S. B., Gutierrez, M. and Edwards, G. E. (1974) Localization of the C_4 and C_3 pathways of photosynthesis in the leaves of Pennisetum purpureum and other C_4 species. Insignificance of phenol oxidase. Planta 119, 267-278.

163. Foster, J. G. and Hess, J. L. (1979) Isolation of soluble protein from cotton leaf tissue and determination of oxidative enzyme levels. Proc. Beltwide Cotton Physiol. Conf., in press.

164. Rhodes, M. J. C. (1976) The extraction and purification of enzymes from plant tissues. In Regulation of Enzyme Synthesis and Activity in Higher Plants. (Smith, H., Ed.), pp 245-269, Academic Press, London.

165. GAF Corporation, Dyestuff and Chemical Division. Polyvinylpyrrolidone in the removal of phenolic compounds in the isolation of plant enzymes. Technical Report Chemfo #66.

166. Loomis, W. D. (1969) Removal of phenolic compounds during the isolation of plant enzymes. In Methods in Enzymology 13 (Lowenstein, J. M., Ed.), pp 555-563, Academic Press, New York.

167. Goldstein, J. L. and Swain, T. (1965) The inhibition of enzymes by tannins. Phytochemistry 4, 185-192.

168. Towers, G. H. N. (1974) Enzymological aspects of flavanoid and lignin biosynthesis and degradation in plants. In Biochemistry Series One 11, (Northcote, D. H., Ed.), pp 247-276, Butterworth and Co., Ltd., London.

169. Jones, J. D., Hulme, A. C. and Wooltortor, L. S. C. (1964) Mitochondrial preparations from the fruit of the apple. II. Oxidative phosphorylation. Phytochemistry 3, 201-212.

170. Sawada, Y., Ohyama, T. and Yamazaki, I. (1972) Preparation and physicochemical properties of green pea superoxide dismutase. Biochim. Biophys. Acta 268, 305-312.

171. Beauchamp, C. O. and Fridovich, I. (1973) Isozymes of superoxide dismutase from wheat germ. Biochim. Biophys. Acta 317, 50-64.

172. Giannopolitis, C. N. and Ries, S. K. (1977) Superoxide dismutases. II. Purification and quantitative relationship with water-soluble protein in seedlings. Plant Physiol. 59, 315-318.

173. Kar, M. and Mishra, D. (1976) Catalase, peroxidase, and polyphenolase activities during rice leaf senescence. Plant Physiol. 57, 315-319.

174. Fridovich, I. (1978) The biology of oxygen radicals. The superoxide radical is an agent of oxygen toxicity; superoxide dismutases provide an important defense. Science 201, 875-880.

175. McCord, J. M., Keele, B. B., Jr. and Fridovich, I. (1971) An enzyme-based theory of obligate anaerobiosis: The physiological function of superoxide dismutase. Proc. Natl. Acad. Sci. U.S.A. 68, 1024-1027.

176. Fridovich, I. (1975) Superoxide dismutases. Ann. Rev. Biochem. 44, 147-159.

177. Baker, J. E. (1976) Superoxide dismutase in ripening fruits. Plant Physiol. 58, 644-647.

178. Kanematsu, S. and Asada, K. (1978) Superoxide dismutase from an anaerobic photosynthetic bacterium, Chromatium vinosum. Arch. Biochem. Biophys. 185, 473-482.

179. Gregory, E. M., Moore, W. E. C. and Holdeman, L. V. (1978) Superoxide dismutase in anaerobes: survey. Appl. Environ. Microbiol. 35, 988-991.

180. Misra, H. P. and Keele, B. B., Jr. (1975) The purification and properties of superoxide dismutase from a blue-green alga. Biochim. Biophys. Acta 379, 418-425.

181. Britton, L. and Fridovich, I. (1977) Intracellular localization of the superoxide dismutases of Escherichia coli: a reevaluation. J. Bacteriol. 131, 815-820.

182. Misra, H. P. and Fridovich, I. (1972) The purification and properties of superoxide dismutase from Neurospora crassa. J. Biol. Chem. 247, 3410-3414.

183. Goscin, S. A. and Fridovich, I. (1972) The purification and properties of superoxide dismutase from Saccharomyces cerevisiae. Biochim. Biophys. Acta 289, 276-283.

184. Weisiger, R. A. and Fridovich, I. (1973) Superoxide dismutase. Organelle specificity. J. Biol. Chem. 248, 3582-3592.

185. Misra, H. P. (1977) Purification and properties of superoxide dismutase from a red alga, Porphyridium cruentum. J. Biol. Chem. 252, 6421, 6423.

186. Keele, B. B., Jr., McCord, J. M. and Fridovich, I. (1970) Superoxide dismutase from Escherichia coli B. A new manganese-containing enzyme. J. Biol. Chem. 245, 6176-6181.

187. Sato, S. and Harris, J. I. (1977) Superoxide dismutase from Thermus aquaticus. Isolation and characterisation of manganese and apo enzymes. Eur. J. Biochem. 73, 373-381.

188. Misra, H. P. and Keele, B. B., Jr. (1975) The purifi-

cation and properties of superoxide dismutase from a blue-green alga. Biochim. Biophys. Acta 379, 418-425.

189. Yamakura, F. (1976) Purification, crystallization and properties of iron-containing superoxide dismutase from Pseudomonas ovalis. Biochim. Biophys. Acta 422, 280-294.

190. Yost, D. F., Jr. and Fridovich, I. (1973) An iron-containing superoxide dismutase from Escherichia coli. J. Biol. Chem. 248, 4905-4908.

191. Hatchikian, E. C. and Henry, Y. A. (1977) An iron-containing superoxide dismutase from the strict anaerobe Desulfovibrio desulfuricans (Norway 4). Biochimie 59, 153-161.

192. Kanematsu, S. and Asada, K. (1978) Superoxide dismutase from an anaerobic photosynthetic bacterium Chromatium vinosum. Arch. Biochem. Biophys. 185, 473-482.

193. Baldensperger, J. B. (1978) An iron-containing superoxide dismutase from the chemolithothrophic Thiobacillus denitrificans "RT" strain. Arch. Microbiol. 119, 237-244.

194. Kester, D. R. (1975) Dissolved gases other than CO_2. In Chemical Oceanography 1 (Riley, J. P. and Skirrow, G., Eds.), pp 498-556, Academic Press, London.

195. Anderson, D. G., Stafford, H. A., Conn, E. E. and Vennesland, B. (1952) The distribution in higher plants of TPN-linked enzyme systems capable of reducing glutathione. Plant Physiol. 27, 675-684.

196. Mapson, L. W. and Isherwood, F. A. (1963) Glutathione reductase from germinated peas. Biochem. J. 86, 173-211.

197. Hodgson, E. K. and Fridovich, I. (1975) The inactivation of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. Biochemistry 14, 5294-5298.

198. Bray, R. C., Cockle, S. A., Fielden, E. M., Roberts, P. B., Rotilio, G. and Calabrese, L. (1974) Reduction and inactivation of superoxide dismutase

by hydrogen peroxide. Biochem. J. 139, 43-48.

199. Haugaard, N. (1968) Cellular mechanisms of oxygen toxocity. Physiol. Rev. 48, 311-373.

200. Jocelyn, P. C. (1972) Biochemistry of the SH group. Academic Press Inc., London.

201. Barron, E. S. G. (1955) Oxidation of some oxidation-reduction systems by oxygen at high pressures. Arch. Biochem. Biophys. 59, 502-510.

202. Sorenson, J. G. and Scandalios, J. G. (1975) Purification of a catalase inhibitor from maize by affinity chromatography. Biochem. Biophys. Res. Commun. 63, 239-246.

203. Buchanan, B. B., Crawford, N. A., and Wolosiuk, R. A. (1979) Activation of plant acid phosphatases by oxidized glutathione and dehydroascorbate. Plant Science Lett. 14, 245-251.

204. Hall, J. L., Flowers, T. J., and Roberts, R. M. (1974) Plant Cell Structure and Metabolism. Longman Group, LTD, London.

205. Akazawa, T. (1970) The structure and function of Fraction-1 protein. Regulatory aspects of photosynthetic CO_2-fixation in chloroplasts. Progr. Phytochem. 2, 107-141.

206. Wolosiuk, R. A. and Buchanan, B. B. (1977) Thioredoxin and glutathione regulate photosynthesis in chloroplasts. Nature 266, 565-567.

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EVALUATION OF OXIDATIVE ENZYMES IN LEAF TISSUE FROM INTACT COTTON PLANTS EXPOSED TO DIFFERENT OXYGEN CONCENTRATIONS

by

Joyce Geraldine Foster

(ABSTRACT)

Success in evaluating effects of a 75% oxygen atmosphere, containing an ambient concentration of carbon dioxide, on levels of protein and oxidative enzymes in cotton, Gossypium herbaceum L. C.B. 1697, leaf tissue was dependent upon generation of genetically uniform and physiologically similar leaves. Sufficient quantities of 2-4 week old leaves for experimental procedures were obtained from plants grown in a 1:1 perlite:vermiculite potting medium supplied with 20% Hoagland's nutrient and maintained in a controlled environment growth chamber under the following conditions: 16 hr day/8 hr night cycle, irradiances of 200-300 $\mu E\ m^{-2}\ sec^{-1}$, 70% humidity, and 30°.

Maximum yields of soluble protein (10-12 mg/g tissue) and active enzymes were obtained when freshly harvested leaves were crushed in liquid nitrogen and then homogenized

in 0.1 M Tris-Cl, pH 6.9, containing 0.01 M isoascorbate and polyvinylpyrrolidone [2% (w/v) PVP-10 and 0.5 g dry Polyclar AT/g tissue]. Routinely 90% of the solubilized protein was obtained following centrifugation and chromatography on Sephadex G-50.

Introduction of cotton plants into vinyl chambers of 95% humidity resulted in foliar symptoms of physiological stress, including chlorosis, cellular edema, leaf curling, and loss of turgidity. Of the oxidative enzymes analyzed, catalase activity decreased 30%, glycolate oxidase activity decreased 42%, and peroxidase increased 89% while soluble protein decreased 27% in plants maintained in the high humidity for 5 days. Exposure of plants to 75% oxygen, 350 ppm carbon dioxide for 48 hr under these conditions resulted in a 70% increase in glutathione reductase and a 25% increase in malate dehydrogenase. Other enzymes, superoxide dismutase, catalase, peroxidase, glyoxylate reductase, and acid phosphatase, appeared to be independent of the oxygen treatment.

When humidity was carefully controlled at 70%, treatment with 75% oxygen, 350 ppm carbon dioxide resulted in a 180% increase in glutathione reductase, an 85% increase in

acid phosphatase, and a 22% increase in peroxidase. In the same experiment catalase decreased by 32% and total soluble protein decreased by 15%. Activities of glyoxylate reductase and both cyanide-sensitive and insensitive superoxide dismutases appeared to be unaffected by the 75% oxygen treatment after 48 hr.

Obviously the impact of humidity as a component of the regulation of metabolism in leaf tissue must be considered. It is concluded that oxidoreductase activities located in the chloroplast, peroxisome, and cytosol are not equally sensitive to high oxygen tensions, and effects of environmental oxygen are not restricted to oxidative enzymes.