INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106

A Xerox Education Company

A PHYSIOLOGICAL AND FINE STRUCTURAL STUDY OF PEROXIDASES WITHIN CORTICAL CELLS OF THE ABSCISSION LAYER OF *NICOTIANA TABACUM* FLOWER PEDICELS

by

EGBERT WINSTON HENRY

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1972

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

March 27, 1972
Date

Thomas E. Jensen
Chairman of Examining Committee
Prof. T. Jensen, Lehman College

May 4, 1972
Date

Louis G. Moriber
Executive Officer
Prof. L.G. Moriber

Jack A. Valdovinos
Prof. J. Valdovinos — Lehman College
Institution

J. Golubow
Prof. J. Golubow — Lehman College
Institution

Institution

George E. Wheeler
Prof. G. Wheeler — Brooklyn College
Institution

J. Kunkel
Prof. J. Kunkel — Boyce Thompson Institute
Institution

E. Witkus
Prof. E. Witkus — Fordham University
Institution

The City University of New York

Abstract

A PHYSIOLOGICAL AND FINE STRUCTURAL STUDY OF PEROXIDASES WITHIN CORTICAL CELLS OF THE ABSCISSION LAYER OF *NICOTIANA TABACUM L.* FLOWER PEDICELS

by

Egbert Winston Henry

Advisor: Professor Thomas E. Jensen

A study of cortical cells of the abscission layer of tobacco flower pedicels was conducted in an attempt to characterize peroxidase enzymes at the subcellular level. The diaminobenzidine staining technique, adapted to the electron microscope procedure, was used for localizing the sites of peroxidase and catalase reactive sites within cortical tissue. Cytochemical staining of abscission zone cortical tissue, using diaminobenzidine, revealed that control tissue has some peroxidase activity sites in the cell walls, intercellular spaces, microbodies, and within the single membrane-bound granular component of the chloroplast. Peroxidase staining within the cortical tissue was almost completely inhibited in the presence of 0.02M potassium cyanide, and microbody catalase staining was inhibited in the presence of the specific catalase inhibitor 3-amino-1,2,4-triazole. Ethylene-treated abscission zone cortical tissue showed a proliferation of rough endoplasmic

reticulum with preoxidase reactive sites. There was also increased density of peroxidase staining in the Golgi cisternae, Golgi vesicles, plasmodesmata, cell walls, middle lamella, and intercellular spaces, in ethylene-treated tissue. Mitochondria did not show peroxidase staining sites when the tissue was incubated in diaminobenzidine at a pH of 9.0; however, tissue incubated in diaminobenzidine at pH 6.0 showed a positive staining for cytochrome oxidase in the inner cristae mitochondrial membranes. Time course studies of ethylene-treated and control abscission zone cortical tissue revealed that the abscission zone tissue had a peak of peroxidase activity upon being exposed to ethylene for four hours, with the activity decreasing between four and five hours. Proximal and distal tissue immediately adjacent to the abscission zone had lower levels of peroxidase activity for the entire time course period of five hours of ethylene treatment. Peroxidase activity was determined spectrophotometrically using guaiacol as the substrate. Break-strength determinations of ethylene-treated cortical tissue indicated that there was a decrease in break-strength observable after three hours of applied exogeneous ethylene, with an almost exponential decrease in break-strength occuring between three hours and five hours, over the full time course period. The beginning of the decrease in break-strength coincided with the beginning of the rise in peroxidase activity (three hours) in ethylene-treated cortical tissue. Acrylamide gel electrophoresis of

cortical abscission zone control and ethylene-treated tissue, exposed to the gas for two, three, four, and five hours respectively, revealed two bands that stain positively for peroxidase using o-dianisidine as the oxidizable substrate. There was not an observable difference in the banding patterns of the control and ethylene-treated tissue over the five hour time course period of ethylene treatment. Ethylene may affect peroxidase levels within the tissue by its effect on lignin biosynthesis, lignin degradation, auxin destruction, auxin conjugation, and possibly by affecting the amount of peroxidase isozyme protein made in ethylene-treated tobacco flower pedicel tissue.

ACKNOWLEDGEMENTS

Sincere thanks and appreciation are tendered to Professor Thomas E. Jensen for help in choosing this research problem and for giving a great amount of helpful advice and suggestions in the preparation of the thesis. Thanks are extended to Professor Jack G. Valdovinos for allowing me to use all of his laboratory facilities and for his patience and helpful suggestions during the preparation of the thesis. I wish to thank Professor George Wheeler, Professor James Kuncle, Professor Eleanor Witkus, Professor Louis G. Moriber, and Professor Jules Golubow who were very helpful in offering many useful suggestions for the writing and discussion of this thesis. Thanks are extended to Professor Yaakov Shechter for permitting me to use his laboratory for the electrophoresis studies. Sincere appreciation is expressed to Miss Linda Sicko for help in assimilating the graphic data and to Mr. Lawrence Mwasi for help with the preparation of the photographs; sincere thanks are extended to Sharon and Alexander Pavlista for typing this thesis; sincere thanks are extended to all of the faculty in biology at Herbert H. Lehman College for encouragement and helpful advice during the time I was a graduate student.

I would like to express the deepest appreciation and

gratitude to my wife, Barbara J. Henry, for her patience and perseverance during my time in graduate school.

Sincere appreciation is extended to The Society of The Sigma XI for partial support of this research.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Figure Page

INTRODUCTION

Peroxidases are a class of enzymes that are present in most plant cells. Peroxidases may exist either in a soluble form or bound to cell walls or membranes (Jensen et al., 1960; Lipetz and Garro, 1965). Catalase has been characterized in terms of its role in catalyzing the degradation of hydrogen peroxide into water and oxygen; moreover, this enzyme comprises the major enzyme component of plant cell microbodies (Tolbert et al., 1968). Another peroxidase enzyme, indoleacetic acid oxidase, mediates the destruction of indoleacetic acid (Pilet and Galston, 1957). Peroxidases are believed to participate in thylakoid formation within chloroplasts (Pellegrini and Gerola, 1969) and in the process of senescence (Lee, 1911; Gahagan et al., 1968; Addicott, 1969).

Indoleacetic acid oxidase appears to consist of a thermostable fraction, possibly a phenolic peroxidase substrate, and a peroxidase, as a thermolabile fraction (Kenten, 1955). Indoleacetic acid oxidase may be ubiquitous in plant tissue, as is indoleacetic acid (Galston and Hillman, 1961). Since indoleacetic acid retards senescence in attached and detached leaves, auxin destruction, mediated by indoleacetic acid oxidase, may occur in abscission

(Addicott, 1969; Schwertner and Morgan, 1966).

The deposition of lignin and suberin in plant cell walls has been attributed to peroxidase activity (Van Fleet, 1942). Abscission involves a series of degradative processes that include the hydrolysis of pectin and cellulose, moreover, energy-requiring reactions may participate in the formation of ribonucleic acid and protein, this allowing for the de novo synthesis of new hydrolytic enzymes (Addicott, 1969).

Previous studies of microbodies in Nicotiana tabacum L. (Thornton and Thimann, 1964) have proposed a hydrolytic role for the enzyme-containing microbodies that participate in the changes occurring in the cell wall during abscission. Further studies on cortical tissue of Nicotiana tabacum L. revealed that rough endoplasmic reticulum is increased in natural abscission (Jensen and Valdovinos, 1968) and in ethylene-treated flower pedicels (Valdovinos, Jensen, and Sicko, 1971). Electrophoretic studies of peroxidase banding patterns (cultured tobacco cells, WR-132) are influenced by changes in temperature (De Jong et al., 1968). The extracts from cultured tobacco cells grown for 3 days at temperatures of 13 degrees C., 25 degrees C., and 35 degrees C., had specific peroxidase activities of 600, 42, and 20, respectively (De Jong et al., 1968). Acrylamide gel electrophoresis of leaf and stem tissue homogenates has been utilized to detect the presence of new peroxidase isozymes and/or any increase in previously existing

peroxidases (Sheen and Rebagay, 1970).

Ethylene increases protein synthesis during the abscission process but does not cause de novo synthesis of new peroxidases in Coleus blumei Benth. explants; however, applied ethylene does cause the genesis of new peroxidase isozymes in sweet potato (Ipomoea batatas L. Lam.) root discs (Gahagan et al., 1968). In etiolated seedlings of Pisum sativum var. Alaska, ethylene is thought to affect the synthesis of new peroxidase isozymes at the translational step of protein synthesis (Ridge and Osborne, 1970). The development of the diaminobenzidine staining procedure allows one to investigate peroxidases from the stand point of its fine structural localization. This was done within the cortical tissue of the abscission zone areas of the flower pedicels. An attempt was made to compare the intensity of peroxidase or catalase enzyme activity sites, in flower pedicel abscission zone tissue treated with 5µl/l of ethylene, at various time intervals during the five hour period of ethylene treatment.

It is hoped that this research will help to elucidate more about the role of peroxidases in cortical tissue of Nicotiana tabacum L. flower pedicels. The localization of the sites of peroxidase activity within cortical cells and the determination of the physiological levels of peroxidase during the time course of ethylene treatment could help to ascertain whether there is de novo synthesis of new peroxidase isozymes during abscission. In addition, it could help to

determine if there is an increased mobilization of pre-existing enzymes that possibly migrate to particular cellular areas as the stages of senescence arise during the abcission process.

LITERATURE REVIEW

History of Peroxidases

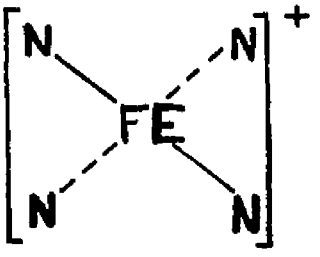
Thenard (1818) observed that a substance called hydrogen peroxide was capable of being decomposed by animal tissues with the liberation of gaseous oxygen. It was implied by Thenard that some type of catalyst was involved in mediating the following reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Schonbein (1855) determined that plant and animal tissues were capable of "activating" hydrogen peroxide, causing tincture of guaiacol to be oxidized and assume a blue color. Loew (1901) suggested the enzyme catalase was responsible for the degradation of hydrogen peroxide. Wolff and de Stoecklin (1910) purified a hemoglobin free erythrocyte catalase.

Battelli and Stern (1908) were the first investigators to study extensively the occurrence of peroxidase activity in parenchymatous cells of animal organs. They found that nearly all animal organ extracts they examined possessed peroxidase activity; however, the kidney tissue had the greatest ability to catalyze the peroxidation of formic acid.

Further characterization of catalase was achieved with its successful crystallization from lamb and horse liver by Dounce and Frampton (1930). Sumner and Gralen (1938) postulated a theoretical molecular weight of 248,000 for crystalline beef liver catalase. They defined the prosthetic group of both mammalian catalase and horseradish peroxidase as being a ferroprotoporphyrin or hemin with the following structure:



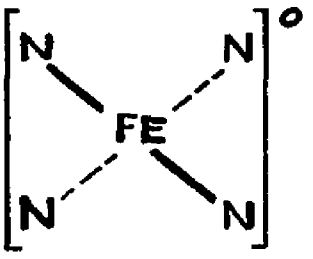
Review of Specific Characteristics of Various Peroxidases

Indoleacetic Acid Oxidase

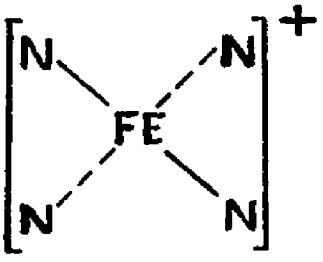
Indoleacetic acid oxidase was first noted by Thimann (1934) when he observed that water extracts of plants were able to cause rapid inactivation of the native auxins. In 1940, Larson described a thermolabile indoleacetic acid oxidase in extracts from pressed bean seedlings (Phaseolus vulgaris) as being able to inactivate indoleacetic acid from corn (Zea mays) seedlings (Larson, 1940). Subsequently, Tang and Bonner (1947) isolated an indoleacetic acid oxidase from etiolated epicotyls of Pisum sativum.

Indoleacetic acid oxidase has since been extracted from a wide variety of plants (Hare, 1964). In view of the rather ubiquitous nature of indoleacetic acid oxidase in plants, Galston and Hillman (1961) have proposed that it may occur with as much frequency as does indoleacetic acid.

Indoleacetic acid oxidase is characterized as a hemoprotein (Hare, 1964). The central portion of the iron-porphyrin complex may possess a zero net charge as in heme (ferroprotoporphyrin),



or may have a net positive charge as in hemin (ferriproto-porphyrin).



Electrophoretic studies indicate that indoleacetic acid oxidase is a small protein possessing a weak positive charge (Stutz, 1957). It was further characterized as being a light-activated flavoprotein, possibly linked with a

peroxidase (Galston and Baker, 1951). Further studies have revealed additional evidence implicating a peroxidase portion in the molecule (Kenten, 1955; Yamazaki and Souzu, 1960). The optimum temperature for indoleacetic acid oxidase activity in Pisum sativum is 25 degrees C (Tang and Bonner, 1947) and in bean (Wagenknecht and Burris, 1950) is between 6.2 and 6.7. However, pineapple indoleacetic acid oxidase has a much lower optimal pH of 3.5 to 4.0 (Ray and Thimann, 1955).

Catalase

It has been reported that beef liver catalase has a molecular weight of 250,000, according to Kiseler et al. (1967). Catalase is composed of 4 heme subunits of equal size, with molecular weights between 60,000 and 65,000 (Sund et al., 1967; Kiseler et al., 1967). These data are in agreement with X-ray diffraction and electron microscopic studies of purified crystals of beef liver catalase (Rossman and Labaw, 1967). The optimum pH for beef liver catalase is 7.0 and the enzyme is stable at 4 degrees C; freezing and lyophilization, however, cause it to become inactive (Tanford and Lovrien, 1962; Deisseroth and Dounce, 1967). Catalase has the highest turnover rate of any known enzyme, being able to decompose 44,000 molecules of hydrogen per second (White et al., 1964, p. 359).

Horseradish Peroxidase

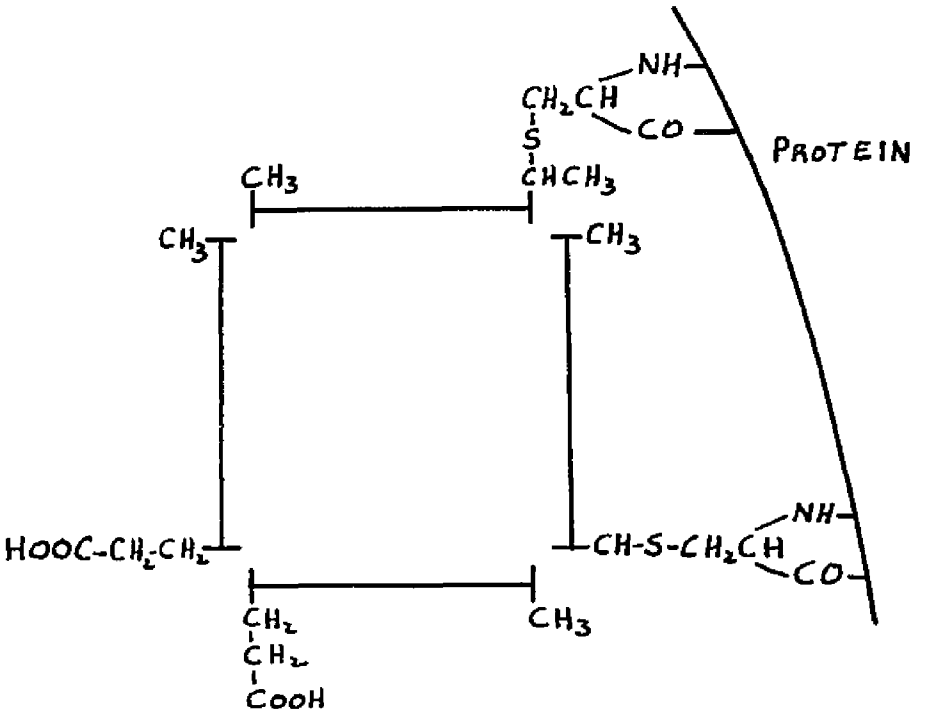
Horseradish peroxidase has a molecular weight of 40,000 in the purified crystalline form (Maehly, 1955). Each of the seven definable isozymes possesses the same prosthetic component (Shannon et al., 1966), with amino and neutral sugars comprising approximately 18% of the content of the enzyme. The active site of the enzyme contains both the apoprotein and heme parts of the molecule (Weiryb, 1966). The enzyme is stable and the lyophilized powder can retain almost complete activity if stored at 5 degrees C. Horseradish peroxidase reacts as follows:

(1) peroxidase + H_2O_2 $\longrightarrow$ compound (complex)

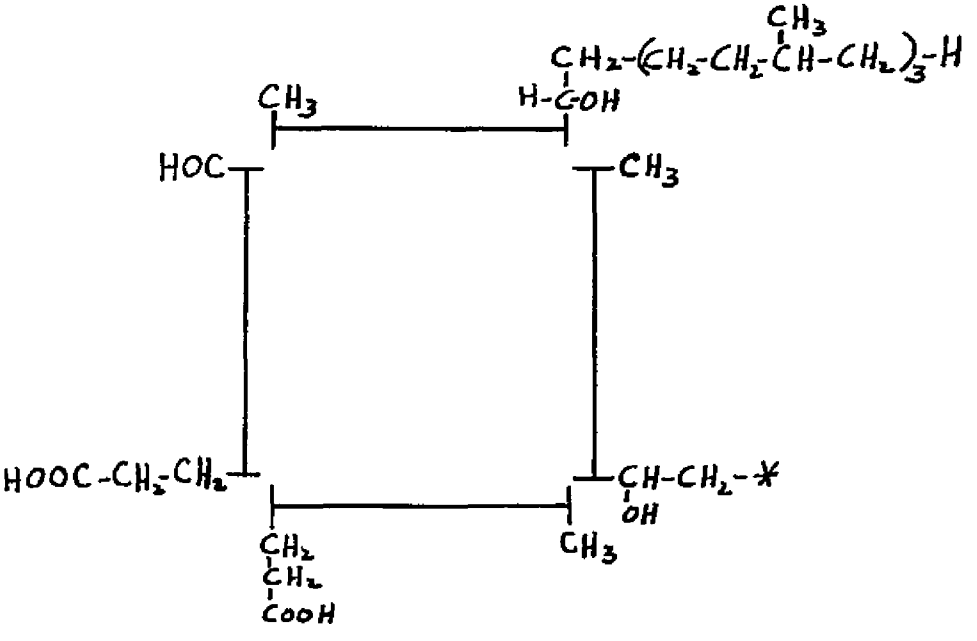
(2) compound (complex) + AH_2 (donor) $\longrightarrow$ peroxidase + H_2O + donor (oxidized)

Cytochrome Oxidase

Cytochrome oxidase was purified and crystallized from various animal tissue by Yonetani (1967). The prostetic group of cytochrome oxidase is heme according to Fruton and Simmonds (1958). Purified cytochrome oxidase has the following structure according to Fruton and Simmonds (1958), pp. 352.



CYTOCHROME C



CYTOCHROME OXIDASE

* PROTEIN ATTACHES HERE

The molecular weight of the cytochrome oxidase is approximately 75,000. Cytochrome oxidase is unique in that it contains some copper in addition to the iron (Bonner and Varner, 1965, p. 105). The usual ratio of copper to iron is one to one. The role of copper in the reaction of cytochrome oxidase with oxygen is unknown (Bonner and Varner, 1965, p. 105). The three types of cytochromes, called a, b, and c, can be identified according to their respective absorption maxima (Bonner and Varner, 1965, p. 104). The a cytochromes possess alpha-band maxima near 600 mμ, b cytochromes have maxima near 560 mμ, and c cytochromes have maxima near 550 mμ (Bonner and Varner, 1965, p. 104). Plant mitochondrial cytochrome c has a lower absorbance range (515 mu, 517 mμ, 547 mμ, and 549 mμ) than mitochondrial cytochrome c of animal origin (Bonner and Varner, 1965, pp. 105-107).

Cytochrome oxidase may really be a complex composed of cytochrome c and a. The oxidized ferric form of cytochrome oxidase readily combines with cyanide (White et al., 1964, p. 352).

Review of Assay Procedures for Peroxidases

Electrophoresis

The development of polyacrylamide gel electrophoresis made possible the simultaneous exploitation of differences in charge and molecular size in the separation of protein fractions (Ornstein, 1964). The gel pore size can be easily

adjusted to insure a maximum distance of separation between any two molecular species; also, molecules of various net charge can be separated over a rather wide pH range of between 3 and 11 (Chrambach and Rodbard, 1971). Acrylamide gel electrophoresis was first adapted for plant study to investigate variations in seed proteins of Leguminosea (Fox et al., 1964), Triticinae (Johnson and Hall, 1965) and in Brassica (Vaughan et al., 1966). Leaf protein variations were also studied in Collonsia (Desborough and Peloquin, 1966).

The polyacrylamide gel electrophoretic technique was used to study the taxonomic relationships within the genus Nicotiana, which comprises 64 species (Hart and Bhatia, 1967; Smith et al., 1970). Whitmore (1971) used the electrophoretic separation method to study peroxidase isozymes in wheat coleoptiles. Gel electrophoresis was used to compare the proteins of photoperiodically induced and vegetative cocklebur plants (Sherwood et al., 1971).

In acrylamide gel electrophoresis assays of peroxidase isozymes, individual peroxidase bands are developed as dark brown areas. The peroxidase substrates, guaiacol, o-dianisidine, benzidine-2HCl, pyrogallol, and catechol, are used in conjunction with added hydrogen peroxide, to give peroxidase band development (Gordon, 1968). Guaiacol has been reported to be superior to benzidine-2HCl for developing peroxidases isolated from the leaves of Nicotiana tabacum L. var. "Samsun NN" (van Loon, 1970).

Spectrophotometry

The spectrophotometric measurement of peroxidase activity was investigated in an effort to develop an accurate technique to assay catalase activity (Beers and Sizer, 1952). Catalase activity is measured spectrophotometrically at 240 nm in the presence of hydrogen peroxide at 25 degrees C. One unit of enzyme activity was defined as being equivalent to the decomposition of one micromole of hydrogen peroxide per minute at 25 degrees C (Beers and Sizer, 1952).

One unit of peroxidase activity (purified horseradish peroxidase) is defined as the amount of enzyme capable of decomposing one micromole of hydrogen peroxide per minute at 25 degrees C, in the presence of O-dianisidine as the hydrogen donor (Maehly and Chance, 1954).

Ponting and Joslyn (1948), while studying apple tissue peroxidases, modified the spectrophotometric assay procedure using guaiacol as the oxidizable substrate in the presence of hydrogen peroxide. Ridge and Osborne (1970) used the above procedure to investigate peroxidase activity in Pisum sativum var. Alaska segments and expressed enzyme activity in terms of the increase in absorbance over the linear portion of the curve (10 second readings) on a gram per fresh weight basis.

Gahagan et al. (1968) used a procedure similar to the one described above, except pyrogallol was utilized as the hydrogen donor in their assay of sweet potato (Ipomoea batatas L. Lam.) root disc peroxidases.

The spectrophotometric assay method for determining peroxidase activity is quite sensitive, as one is able to measure concentrations as low as 0.0016 mg/ml of peroxidase when purified horseradish peroxidase is used as a standard.

Development of the Fine Structural Localization of Peroxidase Activity

Diaminobenzidine

It was thought that catalase activity could not be detected histochemically since the reaction products of oxygen and water, derived from hydrogen peroxide, are not capable of being visualized (Lison, 1956). Alternative methods of catalase localization were proposed by Morikawa and Harada (1968) to develop sites of catalase activity with an immunologic fluorescent antibody technique. The fine structural localization of peroxidases was advanced when Hirai (1968) confirmed that the oxidized free radical produced by 3,3'-diaminobenzidine has a specific affinity for heme enzymes at their active sites.

At the light microscope level, it has been possible to demonstrate cytochrome oxidase activity in heart muscle with Nadi reagents (Burstone, 1959; Sabatini et al., 1963). However, at the electron microscope level, all of the Nadi reagents gave "droplet localization" which was related to the mitochondria of heart muscle but less prevalent in kidney and liver (Seligman et al., 1967).

The Nadi reagents, N-benzyl-p-phenylene-diamine and 1-naphthol, formed positive reaction sites for horseradish peroxidase at the electron microscope level (Seligman et al., 1967). However, the peroxide staining reaction product was still in the "droplet form." The term "droplet localization" was ascribed to the electron dense areas of osmium deposition sites of cytochrome oxidase activity in the presence of Nadi reagents (Sabatini et al., 1963). The localized sites of cytochrome oxidase activity were not compact but were flattened and surrounded by a peripheral lighter zone (Sabatini et al., 1963). The rounded halo-type of lighter outer zone around the osmium black reaction sites was described as "droplet localization" by Sabatini et al., (1963).

Graham and Karnovsky (1965) introduced the diaminobenzidine staining technique to demonstrate peroxidase active sites. Diaminobenzidine is able to undergo oxidation in the presence of peroxidase and hydrogen peroxide, to give a dark brown osmiophilic reaction product resolvable at the light microscope level (Graham and Karnovsky, 1965).

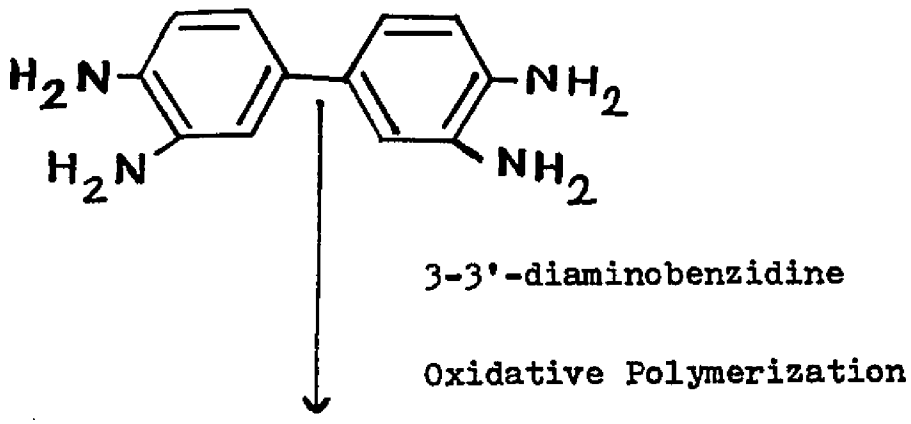
A procedure for staining microbodies at the fine structural level, based on the oxidation of 3,3'-diaminobenzidine peroxidase substrate, was reported by Novikoff and Goldfischer, 1968; Hirai, 1968; Fahimi, 1969. Novikoff and Goldfischer (1968) modified the basic diaminobenzidine staining procedure to demonstrate that microbodies stained more intensely at the alkaline pH of 9.0 while mitochondria stained better at a pH of 6.0. Goodman and Tephly (1968)

found methanol peroxidatic oxidation by rat hepatic microbody catalase to give maximum diaminobenzidine response at pH 8.3. Chance (1952) had earlier reported that the catalase-hydrogen peroxide complex is pH dependent. Fahimi (1969) found that catalase oxidized some substrates faster than others at an alkaline pH. The pH optima of other microbody enzymes have been reported: Mahler et al., (1955) found a pH value of 9.0 for urate oxidase; Dixon and Kleppe (1955), D-amino acid oxidase, pH 8.5; Blanchard et al., (1946), L-alpha-hydroxy oxidase, pH 8.0; and glycolate oxidase, pH 8.8, Kun et al., (1954).

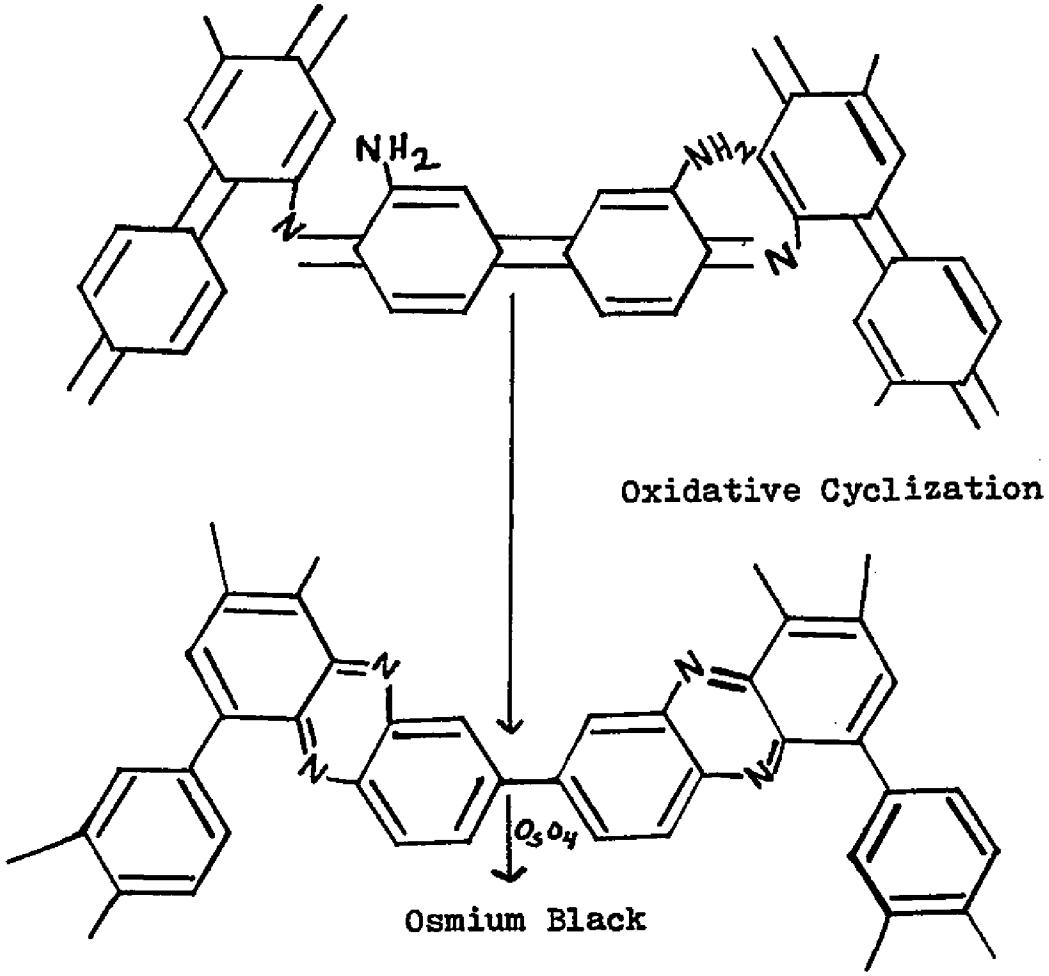
The Mechanism of Action of Diaminobenzidine

Seligman et al., (1968) proposed a reaction sequence for the diaminobenzidine staining reaction. The initial step involves the oxidative polymerization of diaminobenzidine to an indamine polymer. The final step in the reaction sequence is the addition of osmium tetroxide to give the final reaction product of unknown structure called "osmium black" (Seligman et al., 1968). Osmium black is probably not a single uniform compound and may vary according to the number of sulfur-containing organic reductants present within the molecule (Hanker et al., 1967). These authors suggested that osmium black may be composed of coordinated polymers of osmium, including organic sulfur ligands and hydrated osmium oxides. Osmium blacks are ideally suited for electron microscopy because they are usually insoluble in tissue dehydration solvents and have light scattering properties which produce

high contrast on electron micrographs (Hanker et al., 1967).



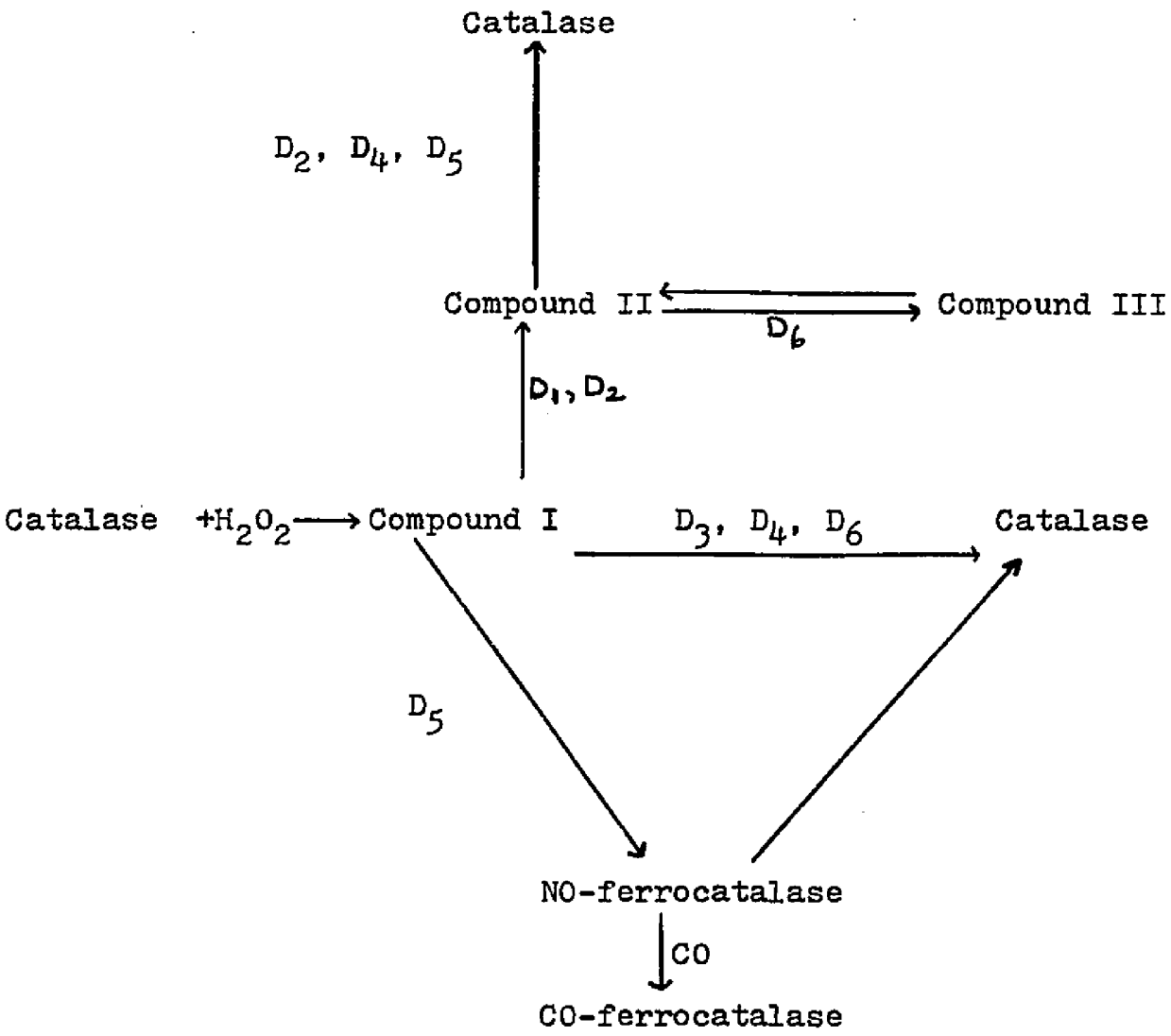
The second step in the reaction sequence involves the addition of quinoid to the primary amine, resulting in oxidative cyclization to phenazine polymer.



Inhibitors Used in the Diaminobenzidine Staining Reaction

Aminotriazole

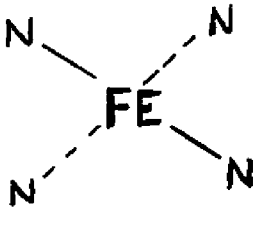
Keilin and Hartree (1954) proposed a sequence of reactions to explain catalase activity in the presence of hydrogen peroxide:



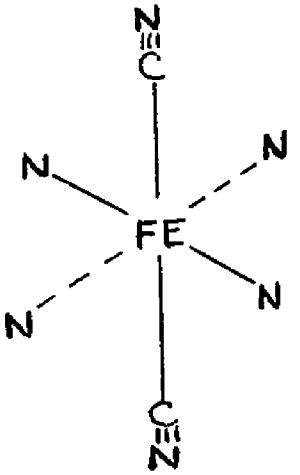
The hydrogen donors are D_1 (ascorbate or ferrocyanide), D_2 (phenols), D_3 (alcohols or formate), D_4 (sodium nitrite), D_5 (azide or hydroxylamine), and D_6 (hydrogen peroxide). The compound, 3-amino-1,2,4-triazole, may be covalently bound to a histidyl residue of the enzyme molecule (Agrawal and Margoliash, 1969; Margoliash and Agrawal, 1969). Several investigators have routinely used 0.02M concentrations of 3-amino-1,2,4-triazole in the diaminobenzidine staining procedure to demonstrate inhibition of catalase activity (Novikoff and Goldfischer, 1968; Beard and Novikoff, 1968; Hirai, 1968; Fahimi, 1968; Vigil, 1969; Frederick and Newcomb, 1969).

Potassium Cyanide

Ferroprotoporphyrin, the heme component of peroxidase, can combine with cyanide to form cyanide ferroprotoporphyrin complex:



Ferroprotoporphyrin



Cyanide Ferroporphyrin

Fine structural examination of tobacco leaves (Nicotiana tabacum L.) by Frederick and Newcomb (1969), revealed non-uniformity in the ability of potassium cyanide to inhibit staining in the diaminobenzidine reaction medium. However, Hirai (1969) achieved complete inhibition of rat hepatic cell peroxidase with 0.02M potassium cyanide. Fahimi (1969) found partial inhibition of rat liver microbody catalase with 0.01M potassium cyanide. Vigil (1970) obtained complete inhibition of catalase activity in diaminobenzidine stained microbodies.

The degree of inhibition in the diaminobenzidine stained procedure is dependent upon several variables, especially the temperature of incubation, length of incubation period, and most important, the ability of the inhibitory agent to fully penetrate the tissue (Frederick and Newcomb, 1969; Vigil, 1970).

The Novikoff and Goldfischer (1968) modification of Graham and Karnovsky's (1965) diaminobenzidine stain was used by several investigators to study the fine structural localization of peroxidases in plant tissue (Vigil, 1969; Frederick and Newcomb, 1969; Vigil, 1970).

Diaminobenzidine Staining of Tissue

Animal

Novikoff and Goldfischer (1968) modified the original diaminobenzidine staining procedure to demonstrate that rat

liver microbody catalase stains most intensely at an alkaline pH of 9.0, while mitochondria (inner and intramembrane cristae) stain deepest at a Ph of 6.0.

Fahimi (1970) utilized the diaminobenzidine staining reaction to demonstrate that peroxidase activity in rat liver Kupffer cells was localized in the cisternae of the endoplasmic reticulum and Golgi apparatus.

Anatomical and Physiological Aspects of Abscission Cells of Tobacco and Other Plant Species

Bornman et al., (1967) defined the abscission zone of cotton (Gossypium hirsutum) petioles as the "...zone in which morphological and physiological changes associated with the abscission of a part or organ are centered." Bornman et al., (1967) further defined the separation layer or abscission layer: "In the abscission zone, the cells immediately involved in the separation of a part or organ either through breakdown and/or by fracture of the tissue across the cells and their walls or along the common wall between adjacent cells."

In tobacco flower pedicels, the actual processes of separation occur between cells that are located several tiers distal to the center of the abscission zone (Yager, 1957). The first fine structural examinations of abscission zone tissue of cotton (Gossypium hirsutum) were made by Leinweber and Hall (1959) and by Bornman et al., (1967). The first

fine structural examinations of abscission zone tissue of tomato (Lycopersicon esculentum Mill) and tobacco (Nicotiana tabacum L.) were made by Jensen and Valdovinos (1967).

When viewed under the light microscope, the tobacco (Nicotiana tabacum L.) abscission zone has an indentation, made up of epidermal tissue, which demarcates the separation layer and projects into the pedicel tissue (Jensen and Valdovinos, 1967). At the fine structural level, the indentation of epidermal tissue contains branches that occur parallel to the middle lamella in the areas between cell walls (Jensen and Valdovinos, 1967).

The dimensions of these extending branches, that emanate from the main indentation, measure approximately 200 mu at the widest points and 20 mu near the ends (Jensen and Valdovinos, 1967). The line of indentation is viewed as being continuous with the cuticle, which measures approximately 90 mu in thickness.

Plasmalemma

The flower pedicel abscission zone tissue contains frequent invaginations of the plasmalemma, fibrillar in consistency, and having a density similar to that of the cell wall (Jensen and Valdovinos, 1967).

Plasmodesmata

Cell walls of tobacco flower pedicel tissue contains plasmodesmata that are not complete, with thin walls, from

cell to cell. The plasmodesmata measure approximately 40 mμ in diameter (Jensen and Valdovinos, 1967).

Microbodies

The abscission zone tissue of tobacco flower pedicels contain microbodies with crystalloid cores measuring between 900 mμ and 1,500 mμ while those of tomato pedicels average between 300 mμ and 500 mμ in size (Jensen and Valdovinos, 1967). The microbodies were single membrane-bound and contained crystalloid cores. These cores are cuboidal in shape and consist of parallel sheets of osmiophilic material (Jensen and Valdovinos, 1967).

Chloroplasts

An appreciable number of chloroplasts present in tobacco and tomato flower pedicels contain a granular component bound to a single membrane. These chloroplasts measure approximately 20-24 mμ in diameter. Stetler and Laetsch (1969) observed a granular component bound to a single membrane to occur within the chloroplasts of light and dark grown tobacco leaves and shoot tissue of Nicotiana tabacum L. "Maryland Mammoth." Gerola and Dassu (1960) originally used the term "opaque body" to describe the granular body present within the chloroplasts of Jerusalem artichoke (Helianthus tuberousus). Israel and Stewart (1967) used the term "prethylakoid body" to describe a similar body in cultured cells of Daucus carota. The granular bodies were

observed in the chloroplasts of dark-grown material when they were exposed to light. Following the light treatment, fragmentation of the body occurred, which apparently led to the formation of thylakoids.

Srivastava (1966) observed a granular component bound to a single membrane within the chloroplasts of cambial cells of Fraxinus americana. He used the term "intralamellar inclusion" to describe this body which appeared to be lipoprotein in nature. The body described by Srivastava is appreciably more electron dense than those reported in other species by the investigators cited above. Newcomb, (1967) observed a granular component bound to a single membrane within the leucoplasts of Phaseolus vulgaris root tip cells. The material within the single membrane-bound granular component observed by Jensen and Valdovinos (1967) in tobacco abscission zone tissue also resembles the body described by Newcomb (1967).

Several different functions of the granular body have been suggested. Marinos (1967) cites food storage as a principal function. Israel and Stewart (1967) and Gerola and Dassu (1960) report that the body breaks up in dark-grown material to form thylakoids. Stetler and Laetsch (1969) report that the body may have a role lamellar formation.

Pellegrini and Gerola (1969) observed the lamellae emanating from the opaque body of plastids in both light and dark-grown plants. It is their opinion that the opaque body

within the plastids of etiolated plants is similar to the prolamellar body of etioplasts described in Jerusalem artichoke by Gerola and Dassu (1960).

Review of Cytoplasmic Changes Accompanying Abscission In Flower Pedicels of Tobacco (based on Jensen and Valdovinos, 1968, except where otherwise stated).

Cell Wall

During the early stages of abscission, the middle lamellar region of the cell wall begins to undergo cell separation (between adjoining cells). As the middle lamellar region breaks down there is also a parallel disintegration of the primary wall. As cell wall disintegration progresses, there is an increased plasticity and swelling of the cell wall. Also, as cell wall dissolution becomes more advanced, some of the cell walls of the separating cells collapse together.

Microbodies

It was found that unpollinated flowers, if examined three days after anthesis, contained microbodies with crystalloid cores, having a cross-hatched appearance. Also, some microbodies had a matrix that became more osmiophilic, while in others the continuity of the single membrane disappeared. A possible function for the microbody in abscission may be to furnish enzymes involved in the dissolution processes

that accompany abscission.

Endoplasmic Reticulum

As disintegration of the middle lamella region becomes more advanced, there is an increased proliferation of segments of endoplasmic reticulum. Unpollinated tobacco flower pedicels have rough endoplasmic reticulum. This rough endoplasmic reticulum is quite different from the smooth endoplasmic reticulum observable in abscission zone tissue at anthesis and three or four days afterwards if pollination has occurred. It was suggested that the rough endoplasmic reticulum may be involved in moving metabolites out of the cell dissolution area or participate in the synthesis of enzymes that play an active role in the abscission process.

The Nucleus

Cortical cells of tobacco (Nicotiana tabacum L.) abscission zones have nucleoli that vary in structure, in tissue observed three days after anthesis, where pollination has been prevented. Some of these nucleoli appear to be condensed while others have only amorphous zones remaining as the fibrillar and granular portions have been degraded. These observations agree with those of Gueskens and Bernard (1966), in that the amorphous portion of the nucleolus is inactive and the fibrillar and granular portions represent areas of RNA synthesis. In this view, the absence of the inner nucleolar elements would indicate a possible decrease

in the protein synthesis normally required for maintaining the integrity of the separation layer.

Review of the Effects of Ethylene on Abscission

A large portion of the evidence for ethylene having a role in the abscission process has come from investigations on bean (Phaseolus vulgaris) explants (Addicott, 1970). There is a sudden release of ethylene after the explants have been severed, possibly as a response to the wounding of the plant tissue (Rubinstein and Abeles, 1965; Jackson and Osborne, 1970). When the accumulation is measured on a time course period, it appears that the ethylene build-up affects the time of onset of abscission. Also, it is suggested that ethylene production occurs at a particular stage of cellular senescense, thereby initiating biochemical changes which start the reaction sequences of the abscission process (Rubinstein and Abeles, 1965; Abeles, 1967; Jackson and Osborne, 1970; Dela Fuente and Leopold, 1968).

In studies of citrus fruits, it was found that detached fruits exhibited an increase in the release of ethylene as the onset of abscission became evident (Lewis et al., 1968). Jackson and Osborne (1970) have presented convincing evidence that ethylene can be a natural inducer of leaf abscission.

Exogeneously applied ethylene can induce and accelerate abscission in fruits, flowers, petioles, and leaf blades

(Burg and Burg, 1968). In bean explants there appear to be two stages of sensitivity to applied ethylene. During the first stage the tissue is insensitive to ethylene and the major stages of senescence begin to occur in the distal parts of the bean explants. The second stage brings about a rapid response to ethylene, evidenced by an acceleration of abscission. Also, withdrawal of ethylene decreases the abscission rate to that of the control (Dela Fuente and Leopold, 1968). Pectinase activity becomes more pronounced as the second stage proceeds (Morre, 1968), and there is also a corresponding rise in cellulase activity (Horton and Osborne, 1967). A further observation is the rapid decline in break-strength across the abscission zone as stage two continues (Cracker and Abeles, 1969).

Several studies of explants, petioles, and stems of Pisum sativum, Coleus blumei, and Gosspium hirsutum reveal that exogeneously applied ethylene can inhibit polar auxin transport, cause an increase in indoleacetic acid oxidase activity, and bring about a decrease in the level of diffusable auxin (Burg and Burg, 1968; Hall and Morgan, 1964; Morgan et al., 1968; Valdovinos et al., 1967; Osborne and Mullins, 1969; Ernest and Valdovinos, 1971).

Valdovinos et al., (1972) did time course experiments, using 5 µl/l of exogeneously applied ethylene to treat tobacco (Nicotiana tabacum L.) flower pedicels over a five hour period. Tissue samples were collected at two, three, and five hour intervals for fine structural examination.

Ethylene-exposed flower pedicels reveal an increased accumulation of rough endoplasmic reticulum in the cortical tissue after two hours of treatment. There is a continual increase in rough endoplasmic reticulum occurring between the third and fifth hours after treatment (Valdovinos et al., 1971).

After five hours of ethylene treatment, the single membrane-bound microbodies appear to lose integrity. There does not appear to be any change in the structure of the crystalloid cores during the applied ethylene time course period (Valdovinos et al., 1972).

During cell wall degradation stages, there is an increase in fibrous material, vesicular structures and either striated or non-striated electron dense bodies in the wall areas during the ethylene exposure period (Valdovinos et al., 1972).

The authors did not observe any appreciable change in the structure of mitochondria, nuclei, chloroplasts, or microbody crystalloid cores during the time course of ethylene treatment (Valdovinos et al., 1972).

The break-strength required to bend the pedicels, after two hours of ethylene exposure, does not cause breakage; however, there is a degree of bending across the separation layer. The pedicels begin to break across the abscission layer after 2.5 hours of applied ethylene and require a break-strength of 40 grams for breakage. Between 2.5 and 5 hours of ethylene treatment, the break-strength across the

separation layer decreases exponentially to 5 grams at 5 hours (Valdovinos et al., 1972).

The specific role for ethylene in abscission has yet to be clearly defined. It has been suggested that, in a two stage sequence, the role of auxin may predominate in the first stage, with ethylene assuming a major role in the second stage (Addicott, 1970). However, the problem is compounded by the many different events (cell wall dissolution, increase in rough endoplasmic reticulum, and the increase in pectinase and cellulase enzymes) that occur during the abscission process (Addicott, 1970).

MATERIALS AND METHODS

Plant Growth Conditions

Tobacco plants (Nicotiana tabacum L. cv, "Little Turkish") were grown in the greenhouse under a normal photoperiod of 14 hours with minimum temperatures of 25 degrees C during the day and 20 degrees C at night. Under natural short-day conditions, the plants were grown under 14 hours per day of light from cool-white fluorescent and incandescent lamps at an intensity of 20,000 lux and at a temperature of 25 ±2 degrees C.

Preparation of Control Tissue For Time Course Studies

Leafy branched stems of tobacco, each containing ten or more flower pedicels, each 10 mm long, were severed from the main plant stem and re-cut under distilled water, immediately prior to being placed in 500 ml plastic beakers, each containing 150 ml of one-half strength Hoagland's nutrient solution (Arnon and Hoagland, 1940).

The plastic beakers, each containing several whole flower pedicels, were placed in a Sherer Gillette controlled

environment chamber, model CL25-7HL, containing a constant light source of incandescent and fluorescent light, giving a constant light intensity of 20,000 lux, 50 ±5% relative humidity, and a temperature of 25 degrees C. Groups of flower pedicels were withdrawn from the control growth chamber at one, two, three, three and one-half, four, four and one-half, and five hours, for use in the subsequent physiological and electron microscopic examinations. Some flower pedicel segments (10 mm in length) were collected directly from the greenhouse plants and not put into the growth chamber.

Preparation of Ethylene-Treated Tissue For Time Course Studies

The flower pedicel tissue was prepared in the same manner as described above for the control time course studies. However, the ethylene treatment chamber was provided with a continuous flow of air (30 liter/min.) containing 5µl/l of ethylene. The air flowing through the chamber was first passed through an ethylene scrubbing chamber device. The scrubbing canister contained Purafil which is composed of activated Al_2O_3 impregnated with $KMnO_4$ in the form of pellets measuring one-eighth inch in diameter. Purafil was obtained from Marbon Division, Borg-Warner Corporation, Washington, West Virginia. The canister was made of degreased galvanized pipe (10 by 40 cm) fitted with circles of screen wire in drilled pipe caps that were fitted with regular gas

outlets. The tape threads were covered with Teflon rather than oil to provide for easy disassembly for refilling and to protect the Purafil from contamination. The plants were exposed to 5 µl/l of ethylene gas over the time course period of the experiment. Beakers containing at least 10 flower pedicels were withdrawn from the ethylene chamber at one, two, three, three and one-half, four, four and one-half, and five hour intervals for use in the subsequent physiological and electron microscopic studies.

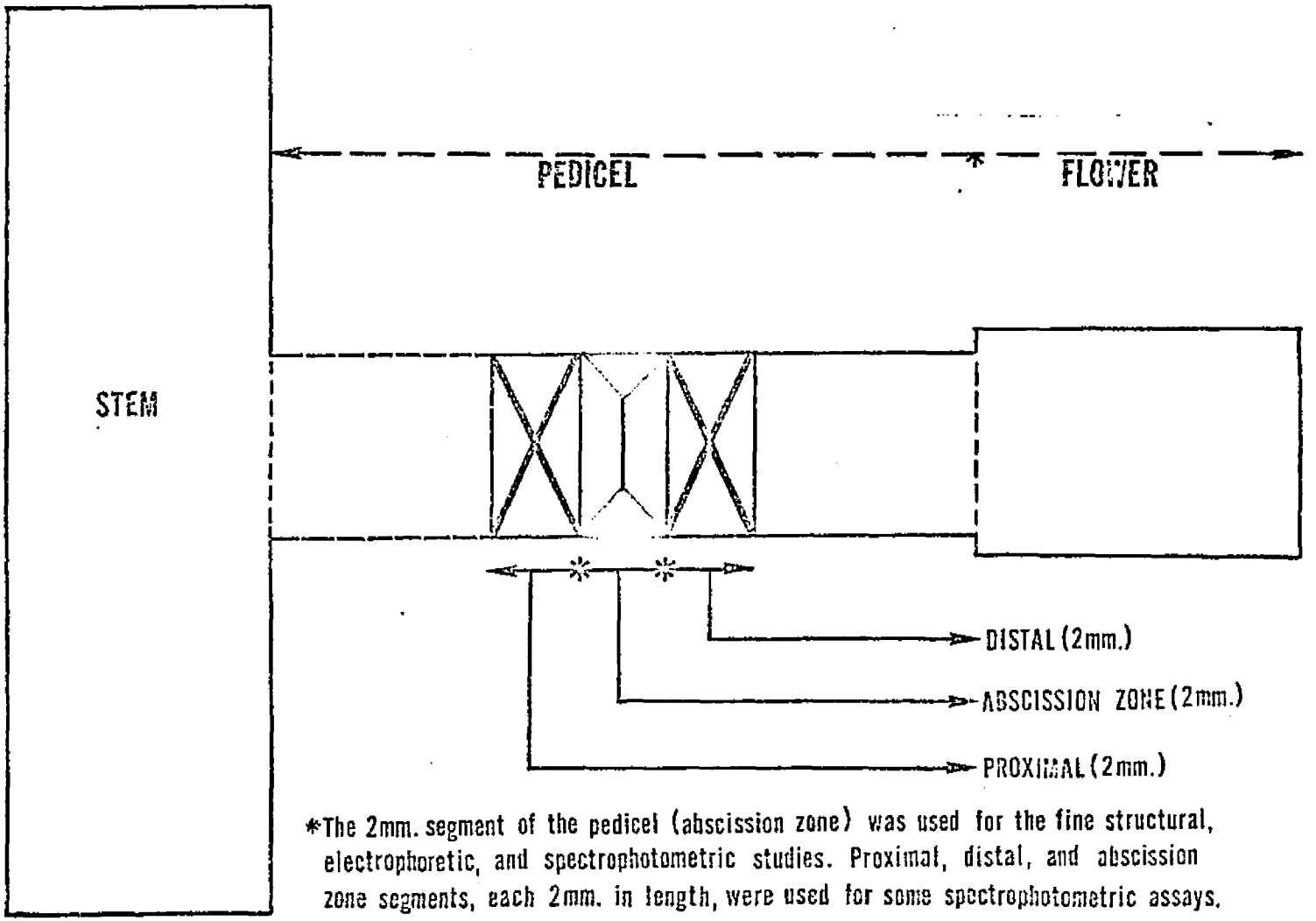
Preparation of Tissue For Electron Microscopic Examination

Two millimeter long segments of flower pedicel tissue, containing the separation zone, were excised (Figure 1). A calibrated constant-width knife, composed of two double-edged razor blades, fastened together with a 2 millimeter spacer between the blades, was used for cutting the segments. The harvested tissue was immediately fixed in 3% glutaraldehyde (Sabatini et al., 1963) in 0.1M phosphate buffer at a pH of 7.2 for 1 hour at 4 degrees C. The tissue was then rinsed six times in buffer (six separate rinses over a period of 24 hours). Other stem segments were collected and fixed for 2 hours at room temperature in a mixture of 2% glutaraldehyde buffered with 0.05M collidine plus 0.06M sucrose at pH 7.3--7.4. The tissue sections were then washed for 1.5 hours in collidine buffer (Mollenhauer and Totten, 1970).

Fig. 1. A diagram of pedicel tissue of Nicotiana tabacum L. showing the portion of the flower pedicel used in these studies. The portion of pedicel used for experiments shown in Figure 2 comprised a 6 mm long pedicel segment including distal (2 mm), abscission zone (2 mm), and proximal (2 mm) portions. Two mm segments of proximal, abscission zone, and distal pedicel tissue were assayed separately for the experiments illustrated in Figures 3 through 8. Two mm segments of the pedicel (abscission zone) were used for the five structural and electrophoretic studies.

FIGURE 1

DIAGRAM OF PEDICEL TISSUE OF NICOTIANA TABACUM L.*



*The 2mm. segment of the pedicel (abscission zone) was used for the fine structural, electrophoretic, and spectrophotometric studies. Proximal, distal, and abscission zone segments, each 2mm. in length, were used for some spectrophotometric assays.

Diaminobenzidine Treatment of Growth Chamber and Greenhouse Tissue

The Novikoff and Goldfischer (1968) in situ diaminobenzidine peroxidase staining procedure was used in this investigation. Each individual 2 mm section of flower pedicel tissue was cut into four smaller sections before transfer to the diaminobenzidine incubation staining medium. The incubation medium contained 10 mg of 3,3'-diaminobenzidine tetrachloride (DAB) available from the Sigma Chemical Company, St. Louis, Missouri, 0.1 ml of 3% hydrogen peroxide and 5 ml of 2-amino-2-methyl-1,3-propandiol buffer (0.05M, pH 9.0. See Appendix A, Schedule V).

Segments of flower pedicels, fixed and sliced as described above, were incubated for 1 hour at 37 degrees C in (a)complete diaminobenzidine staining medium; (b)complete medium without diaminobenzidine; and (c)complete medium minus hydrogen peroxide. The tissue sections were rinsed in 0.05M propandiol buffer, pH 9.0. Segments of flower pedicels were also incubated in the above mentioned media at pH 6.0. See Appendix A, Schedule V.

Inhibitors

Segments (2 mm long) of flower pedicel tissue were incubated in complete medium for 1 hour at 37 degrees C with either (a)0.02M 3-amino-1,2,4-triazole (Aldrich Chemical

Company) or (b) 0.02M potassium cyanide. The respective flower pedicel segments were then rinsed in 0.05M propandiol buffer, pH 9.0. See Appendix A, Schedule V.

Embedding Procedure for Examination of Tissue At the Fine Structural Level

After the tissue segments were incubated in the different diaminobenzidine media, they were rinsed briefly in 0.05M propandiol buffer, pH 9.0 and stained for 1 hour at 4 degrees C with 2% osmium tetroxide in 0.1M phosphate buffer, pH 7.2. Other tissue sections were pre-fixed in 2% paraformaldehyde for 1 hour at 4 degrees C, rinsed at least five times with 0.1M phosphate buffer, pH 7.2, and then fixed in 1% osmium tetroxide for 24 hours at 4 degrees C. The tissue was dehydrated in a graded ethanol series, followed by treatment with propylene oxide, and embedded in Epon 812 (Luft), 1961); see Appendix A, Schedule IV.

Preparation of Tissue Sections for Electron Microscopy

Cortical tissue from several different depths of the abscission zone were collected for sectioning. A Dupont diamond knife was used to cut silver-grey sections of approximately 0.05μ in thickness on a LKB μltramicrotome. The sections were collected on clean 300-mesh copper grids. Some unstained sections were viewed and photographed with the

electron microscope to determine the actual intensity of in situ staining and other sections were stained with either lead salts (Millinog, 1961; Karnovsky, 1961; Watson, 1958) or uranyl acetate in methanol (Stempak and Ward, 1964), either together or separately. A Hitachi 11 E electron microscope was used. The micrographs were taken in the magnification range of 2,000 to 22,000X on Kodak contrast plates. The negatives were routinely enlarged to 2.8 to 4.2 times using Kodak F-3, F-4, or F-5 Kodabromide paper.

Spectrophotometric Assay for Peroxidase

Groups of 10 tissue sections (2 mm long) were collected from the control and ethylene treatment chambers. A separate group of 6 mm flower pedicel segments were further subdivided into three equal parts, representing proximal, distal, and abscission zone segments. After excision from the flower pedicels, the tissue was weighed and immediately put into pre-chilled mortars at 4 degrees C. The tissue samples were ground with a mortar and pestle at 4 degrees C for an average of five minutes in 4 ml of 0.01M phosphate buffer, pH 6.0. The tissue homogenates were centrifuged at 2,000 g for 10 minutes at 4 degrees C to remove cellular debris. The respective supernatant solutions were used for the peroxidase assays (Ridge and Osborne, 1970). Other tissue samples were taken from greenhouse plants to assay the peroxidase levels in plants grown under normal

greenhouse conditions.

Spectrophotometric determinations were measured at a wavelength of 460 nm, using a Beckman model DU spectrophotometer. The standard solution consisted of 1.3 ml of 0.1N hydrogen peroxide and 1.3 ml of 0.05% guaiacol plus 0.8 ml of distilled water (Ridge and Osborne, 1970). The experimental solution contained 1.3 ml of 0.05% guaiacol plus 0.8 ml of enzyme supernatant prepared as described above (Ridge and Osborne, 1970). See Appendix A, Schedule VI.

The Determination of Peroxidase Activity

The enzyme supernatant solution was added at time zero and the change in absorbance was read at five second intervals, over a period of three minutes. The linear portion of the curve was over the range between 70 and 150 seconds and the final relative peroxidase activity was expressed in terms of units per gram fresh weight of tissue (Ridge and Osborne, 1970; Gahagan et al., 1968). See Appendix A, Schedule IX.

Soluble Protein Determination

The supernatant solutions from the respective tissue homogenates, prepared as described above, were used for the protein assay. The measurement of protein content of the tissue homogenate supernatant solutions was determined by

the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard; see Appendix A, Schedule VII.

Electrophoresis

About 250 segments were pooled to give groups of 40 segments of flower pedicels (2 mm long) of Nicotiana tabacum L. which were collected from control and ethylene chambers at two, three, four, and five hour intervals. The respective tissue samples were immediately put into a pre-chilled mortar at 4 degrees C. The individual tissue samples were ground with mortar and pestle at 4 degrees C in 1 ml of 0.8% sodium chloride and 0.1% sodium nitrate, pH 5.9 (Smith et al., 1970). The separate homogenates were centrifuged at 4 degrees C for one hour at 5,000 g to remove cellular debris. The respective supernatant solutions were used for the electrophoretic assays.

A Canalco vertical polyacrylamide gel electrophoresis apparatus (Canalco Company, Rockville, Maryland) was used in this study. Electrophoretic gel or separating gel (see Appendix A, Schedule VIII), using ammonium persulfate as a catalyst, was added to a tube height of 12.0 cm. A 7.5% stacking gel, using riboflavin as a catalyst, was added to a tube height of 13.5 cm. The sample gel in the remainder of the tube, using riboflavin as a catalyst, contained 0.2 ml of enzyme supernatant solution. The gels were run in 0.1M Tris-Glycine buffer, pH 8.5, for one and one-half hours, with

a current of 5 milliamps per tube (Sheen, 1970). See Appendix A, Schedule VIII.

Flower Pedicel Break-Strength Assay

Flower pedicels were prepared as described above for control and ethylene growth chamber treatments. Stems, containing 10 or more pedicels each, were withdrawn from the control and ethylene treatment chambers at intervals of two, three, three and one-half, four, four and one-half, and five hours. Each average measurement represents the break-strengths of ten separate flower pedicels. Individual break-strength determinations were made with a flower pedicel excised 5 mm proximal to the separation layer. The break-strength is defined as the amount of force, in grams, necessary to cause complete separation of the cell layers comprising the abscission zone (Dela Fuente and Leopold, 1968). The force, in grams, required to cause complete separation across the abscission zone, was measured with a laboratory balance by holding the proximal end of the flower pedicel (severed 1 mm from the stem) against the pan of the balance and pressing the distal portion of the flower pedicel against the balance pan at a 45° angle. The amount of force required to cause complete breakage across the abscission zone was expressed in grams. Ten flower pedicels were used for each respective determination with the standard error indicated by the uncolored circles superimposed on the vertical

line through each datum point.

Determination of Standard Error

The standard deviation was determined for each ethylene-treated and control tissue sample over the time course period of the experiment, each determination representing the spectrophotometric absorbance readings expressed in terms of relative units of peroxidase activity per g. fresh weight (ten separate tissue samples for each respective time and treatment period). The standard deviation for each time interval and treatment period was calculated as follows:

1. The arithmetical mean ($\bar{x}$) was determined by adding all of the individual observations (X_i) and dividing the total by the number of individual samples (N).
2. The individual deviation from the mean $(X_i-\bar{x})^2$ was calculated.
3. Each individual deviation from the mean $(X_i-\bar{x})^2$ was squared so that positive values could be obtained.
4. The sum of the squared deviations from the mean was obtained ($\Sigma(X_i-\bar{x})^2$).
5. The standard deviation(s) of the sample was calculated, using the formula

$$s=\sqrt{\frac{\Sigma(X_i-\bar{x})^2}{N-1}}$$

The standard deviation enables one to measure the possible error of the estimate. It is also possible to estimate

the sample mean by an interval. The value $\frac{s}{\sqrt{N}}$ is the standard error of the sample mean. The ethylene-treated plants were compared with the untreated controls for peroxidase activity, measured in relative units per g. fresh weight. The standard error was computed for each tissue sample (representing ten separate determinations). In figures showing the results in graph form, the confidence interval is represented by uncolored circles superimposed on each bar (Machlis, L., and J.G. Torrey. Plants In Action. W.H. Freeman and Company, San Francisco. 1956. pp. 111-145).

RESULTS AND OBSERVATIONS

Exposure of flower pedicels to 5µl/l of ethylene over a period of five hours, revealed that tissue homogenates of abscission zone tissue sections had an increase in peroxidase activity, starting at three hours, with the peak of activity occurring at the four hour period, followed by a continual decrease in activity between four and five hours (Figure 2). The peroxidase activity of control tissue has a profile very similar to that of ethylene-treated tissue between one and three hours (Figure 2). The control tissue shows a small increase in peroxidase activity between three and four hours, with a slight decrease between four and one-half and five hours. An examination of the control curve indicates that there is not a significant difference in peroxidase activity in control tissue between one and three hours (Figure 2).

A comparison of the levels of peroxidase activity in ethylene and non-ethylene-treated flower pedicel tissue (divided into abscission zone, distal, and proximal segments), indicates that the peak level of activity occurs in ethylene-treated abscission zone tissue at four hours, followed by a drop in peroxidase activity between three and five hours (Figure 3). The amounts of peroxidase activity in the

Fig. 2. Time course study of ethylene-treated and control flower pedicel tissue (including proximal (2 mm), distal (2 mm), and abscission zone (2 mm) segments). A comparison of control and ethylene-treated pedicel tissue (comprised of proximal (2 mm), abscission zone (2 mm), and distal (2 mm) segments) for peroxidase activity in the presence of 5 µl/l of exogeneously applied ethylene gas over a five hour time course period.

FIGURE 2

TIME COURSE STUDY OF ETHYLENE–TREATED AND CONTROL PEDICEL TISSUE

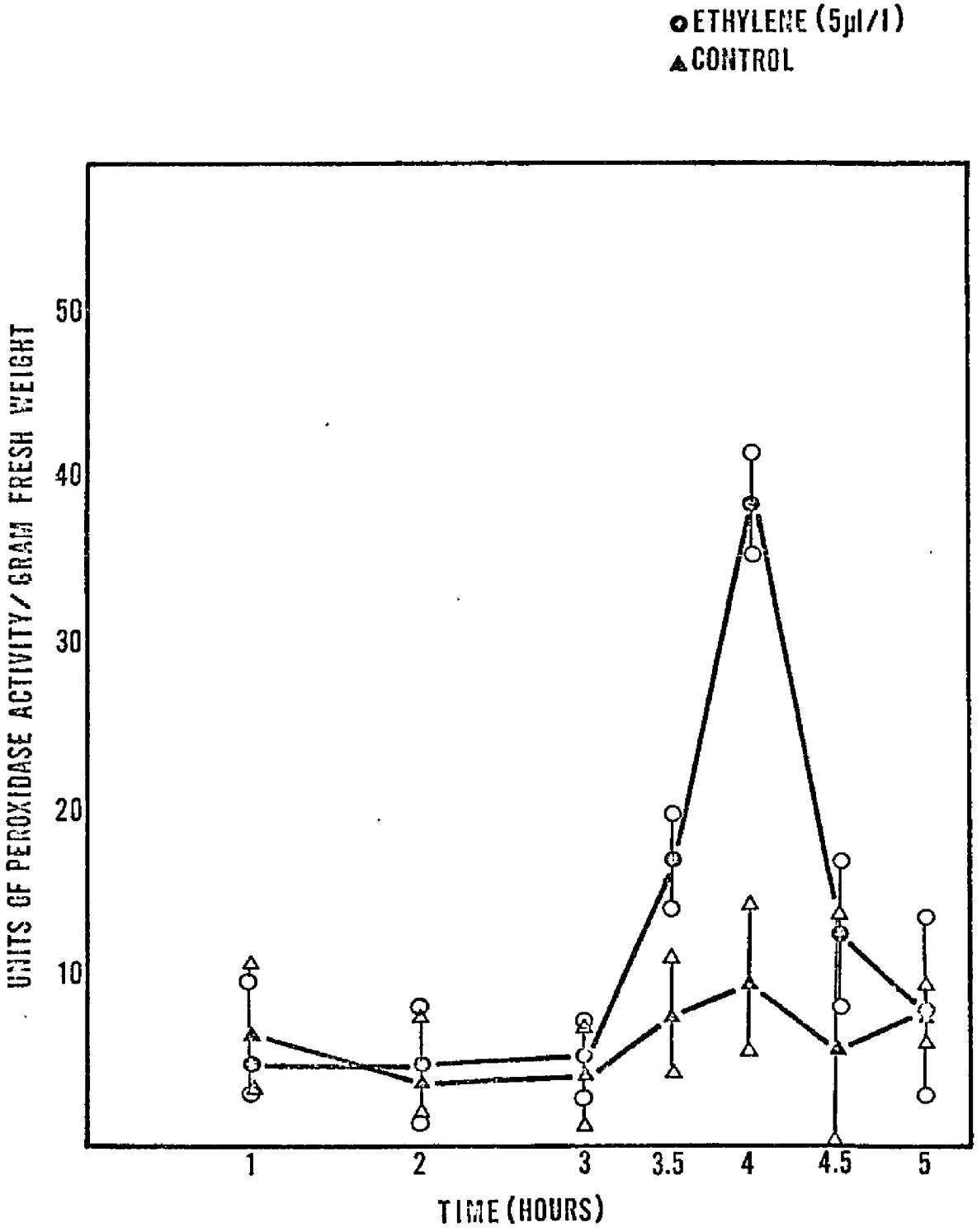
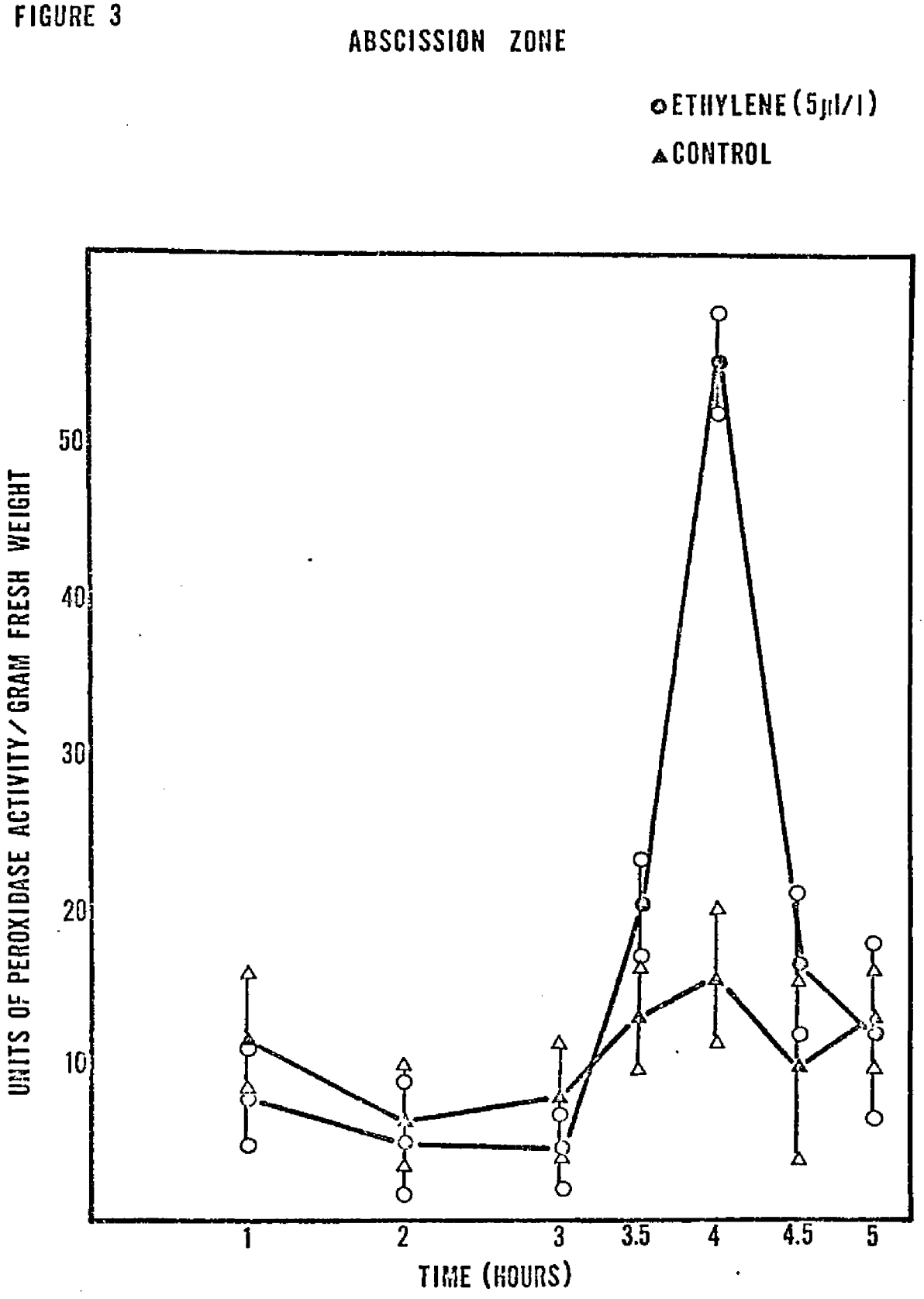


Fig. 3. Time course stydy of ethylene-treated and control flower pedicel tissue (2 mm segments of abscission zone tissue illustrated in Figure 1). A comparison of control and ethylene-treated pedicel tissue (2 mm segments of abscission zone tissue illustrated in Figure 1) for peroxidase activity in the presence of 5 µl/l of exogeneously applied ethylene gas over a five hour time course period.

FIGURE 3


ABSCISSION ZONE
ETHYLENE (5µl/l)
CONTROL
UNITS OF PEROXIDASE ACTIVITY/GRAM FRESH WEIGHT
50
40
30
20
10
1
2
3
3.5
4
4.5
5
TIME (HOURS)

abscission zone is appreciably larger than the amount of activity in the distal portion of the pedicel (tissue adjacent to the abscission zone, between the abscission zone and the flower pedicel). The profile of the ethylene-treated distal tissue is quite similar to that of the abscission zone tissue except that the levels of peroxidase activity in the distal tissues are much lower at all time intervals of the same time course period (Figure 4). The distal ethylene-treated tissue also shows a decrease in peroxidase activity between three and five hours (Figure 4). A comparison of the profiles of the ethylene-treated distal tissue (Figure 4), reveals a similarity in profiles between the two respective samples, with the amount of peroxidase activity being appreciably greater in the abscission zone tissue over the time course of the experiment. The ethylene-treated proximal tissue has a very low level of peroxidase activity, with only a relatively small peak observable at the four hour stage (Figure 4). If the profile of the ethylene-treated proximal tissue is compared to the profiles of the ethylene-treated distal (Figure 5) and abscission zone tissue (Figure 3), it is apparent that the proximal tissue has a much lower amount of peroxidase activity than the other two tissue sections of the pedicel.

Fig. 4. Time course study of ethylene-treated and control flower pedicel tissue (2 mm distal segments as illustrated in Figure 1). A comparison of control and distal pedicel tissue (2 mm distal segments as illustrated in Figure 1) for peroxidase activity in the presence of 5 µl/l of exogeneously applied ethylene gas over a five hour time course period.

FIGURE 4

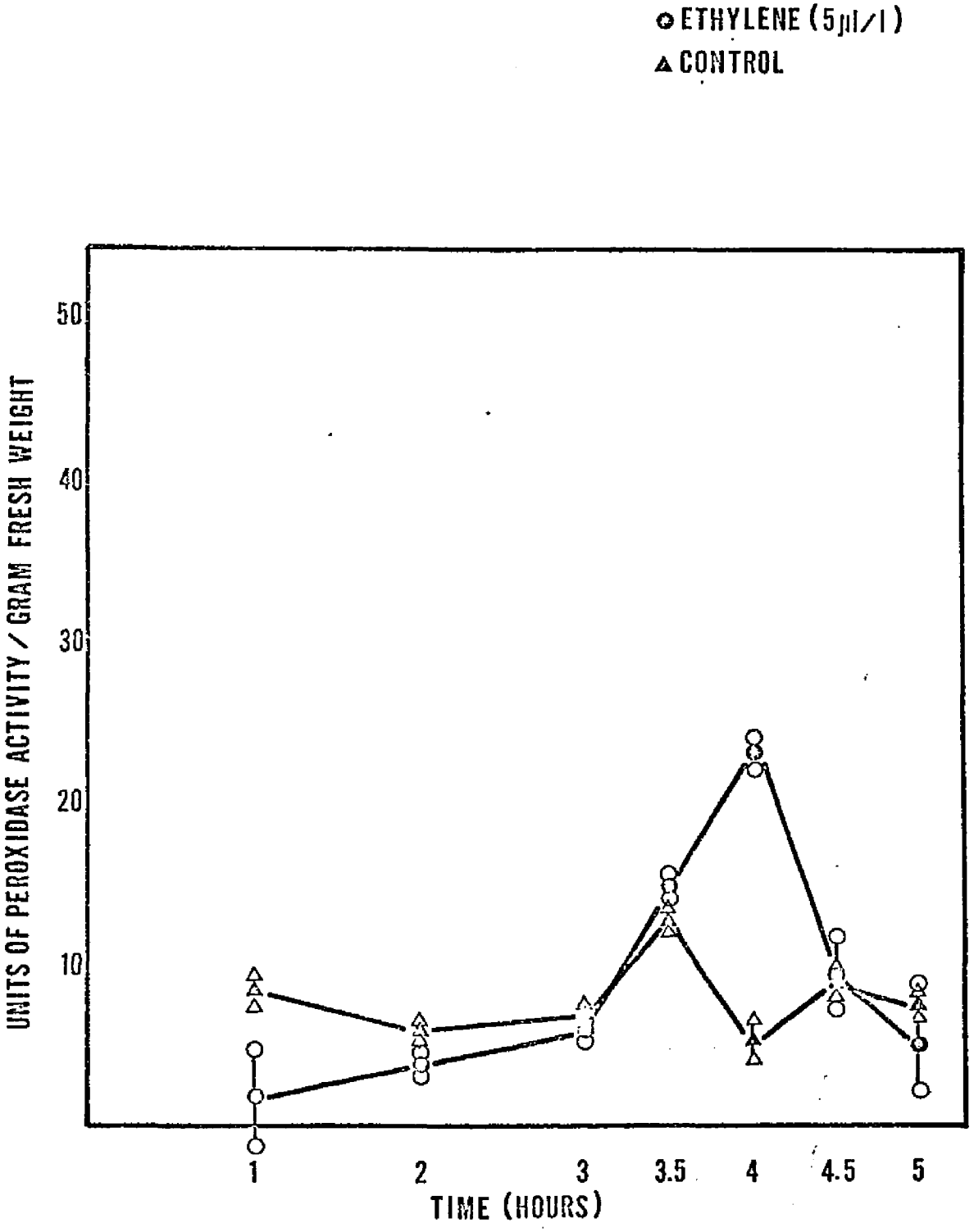
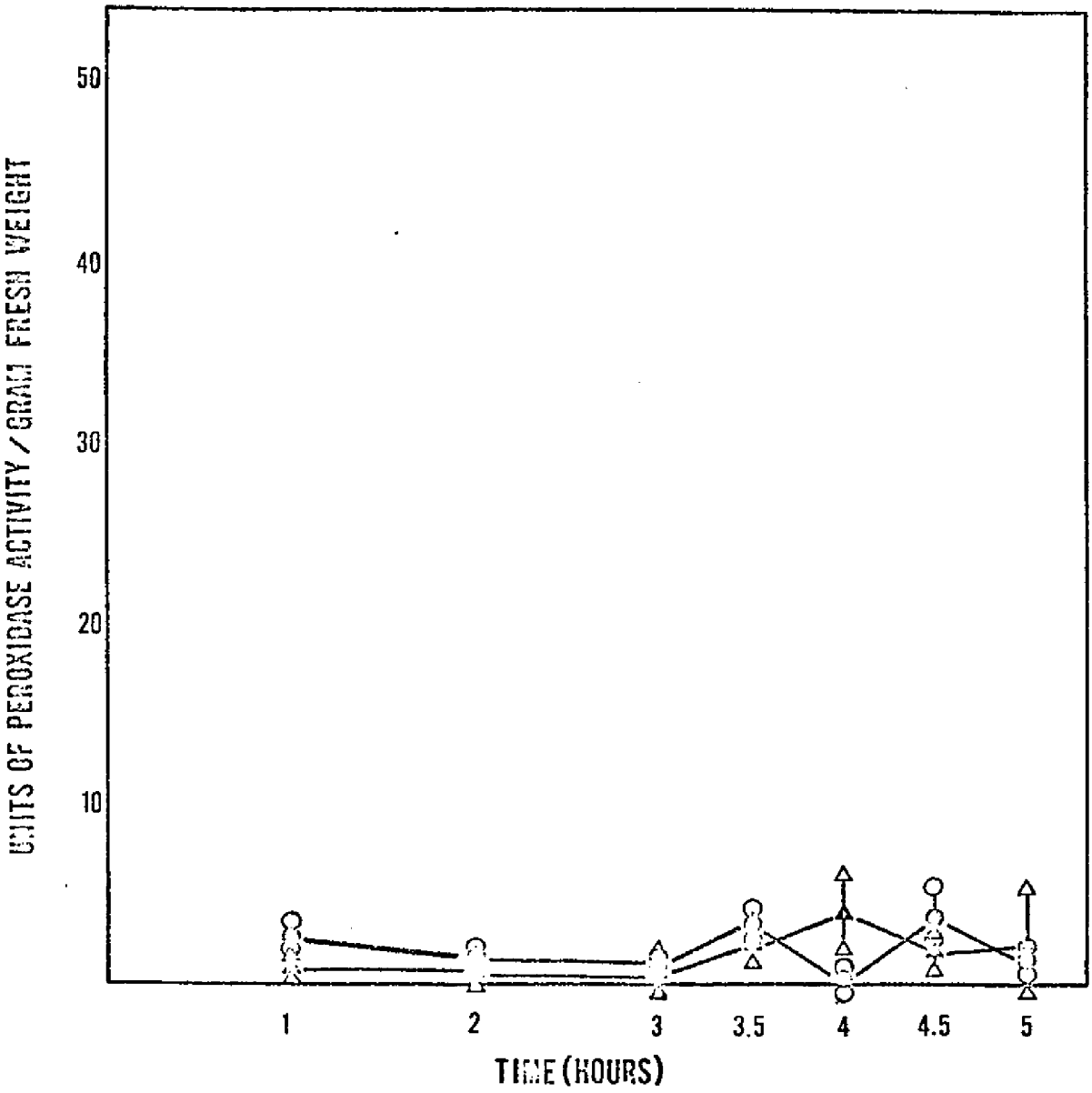

DISTAL
ETHYLENE (5µl/l)
CONTROL
UNITS OF PEROXIDASE ACTIVITY / GRAM FRESH WEIGHT
50
40
30
20
10
1
2
3
3.5
4
4.5
5
TIME (HOURS)

Fig. 5. Time course study of ethylene-treated and control flower pedicel tissue (2 mm proximal segments as illustrated in Figure 1). A comparison of control and proximal tissue (2 mm segments as illustrated in Figure 1) for peroxidase activity in the presence of 5 µl/l of exogeneously applied ethylene gas over a five hour time course period.

FIGURE 5

PROXIMAL

● ETHYLENE (5µl/l)
▲ CONTROL



Electrophoresis

Enzyme samples (the supernatant portion) were collected from ethylene-treated and control tissue, at two, three, four, and five hour intervals. The electrophoretic gels of each respective enzyme sample revealed the presence of two well-defined bands of peroxidase activity (Figure 10). There was no change in the banding pattern between the ethylene-treated and control tissue which indicates that there is not an increase in the total number of peroxidase isozymes over the five-hour ethylene exposure period.

Soluble Protein

When the amount of tissue protein, expressed as milligrams per gram fresh weight, is compared in ethylene-treated versus control abscission zone tissue, there is a relatively close parallel correlation between the two respective samples over the five hour time course period (Figure 6). There is a decrease in protein content between four and four and one-half hours, in both the control and ethylene-treated abscission zone samples (Figure 6). The amount of tissue protein is highest at five hours, for both control and ethylene-treated abscission zone tissue (Figure 6). There is a very close similarity between the profiles of the control and ethylene-treated abscission zone

Fig. 10. Electrophoretic gels of ethylene-treated abscission zone tissue. A comparison of control and abscission zone tissue with respect to banding patterns of peroxidase activity. The tissue was treated with 5 µl/l of ethylene gas over a five hour time course period.

FIGURE 10

ELECTROPHORETIC GELS FROM ETHYLENE-TREATED TOBACCO (NICOTIANA TABACUM L.) ABSCISSION ZONE TISSUE

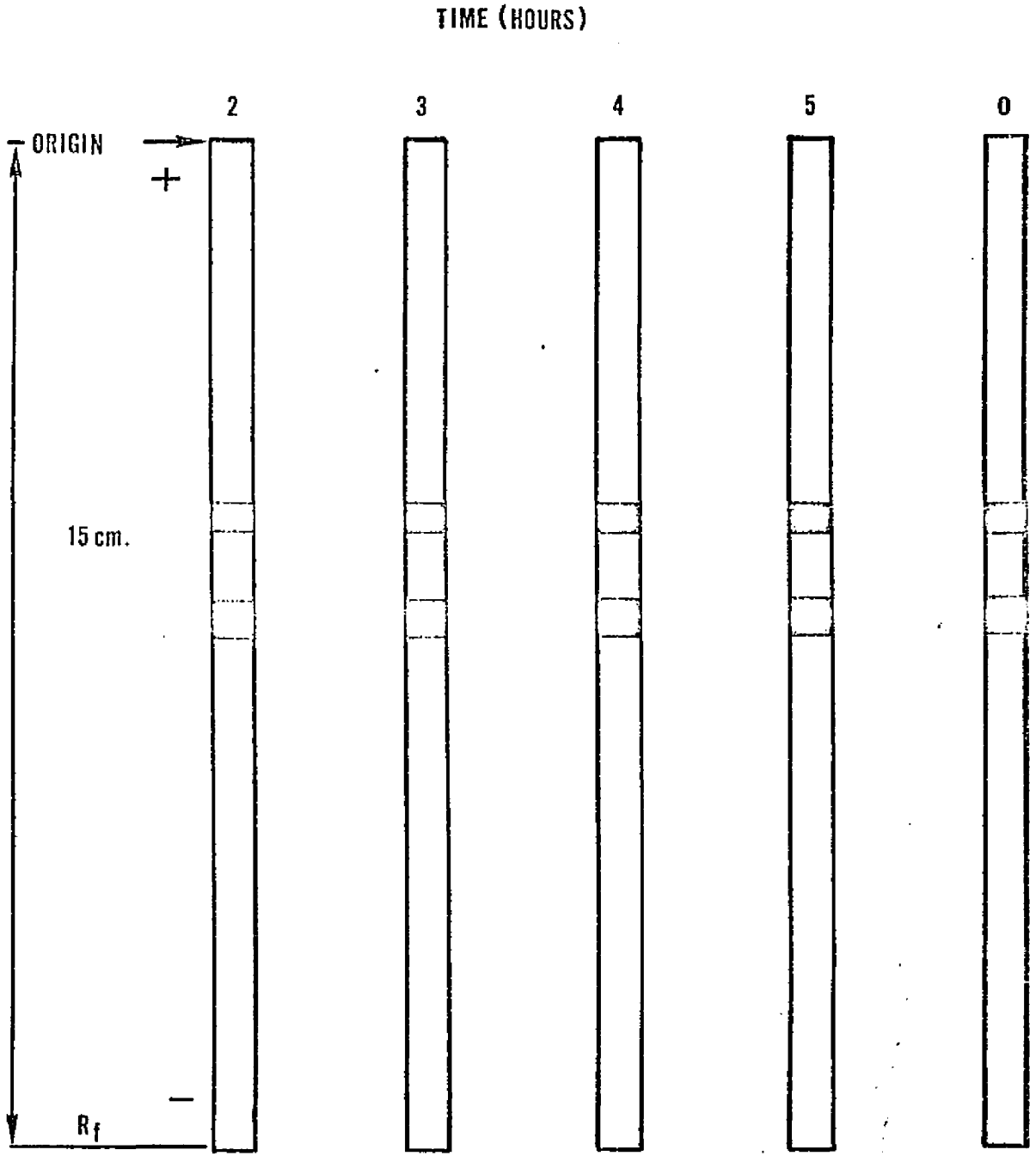
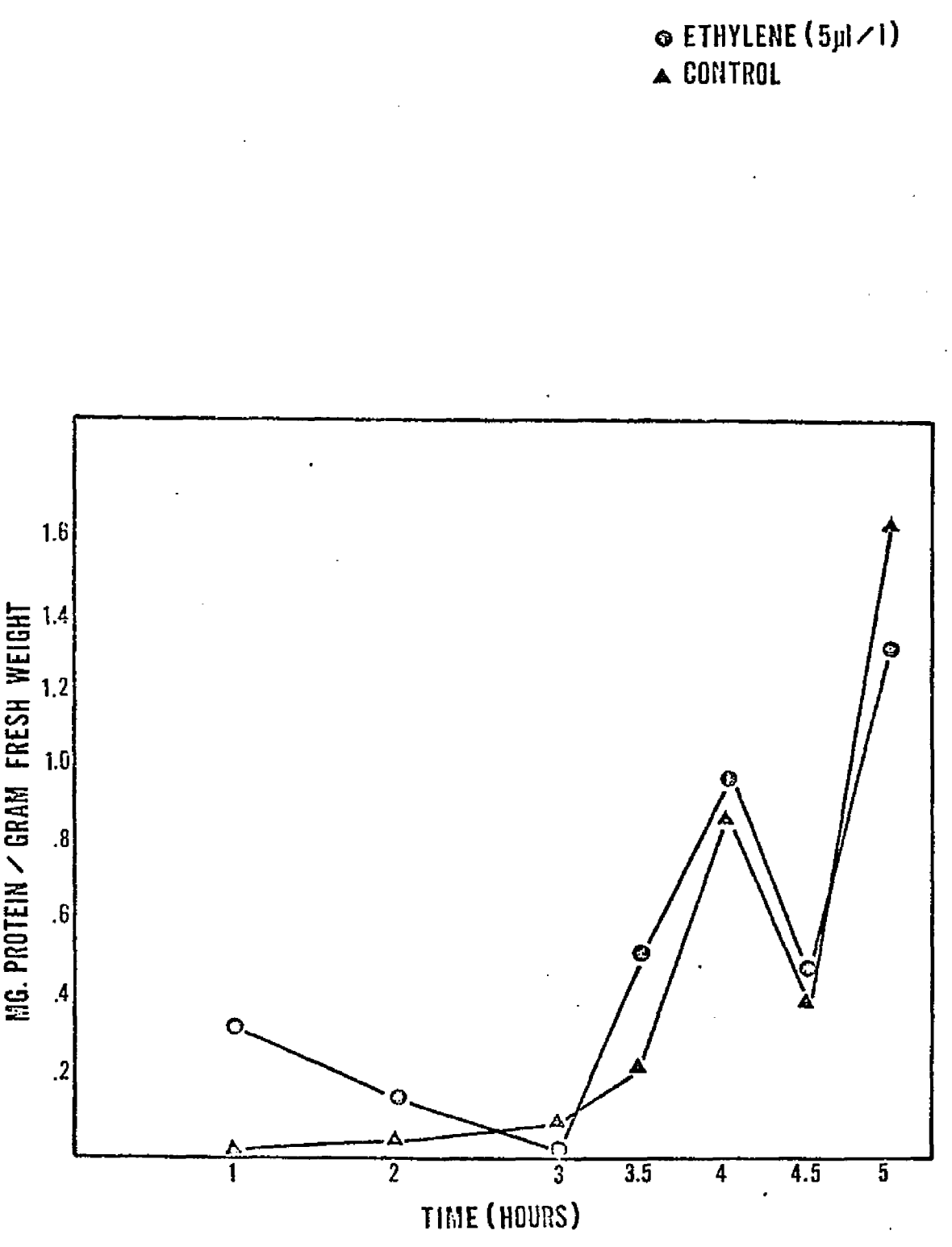


Fig. 6. Time course study of ethylene-treated and control flower pedicel tissue (2 mm segments of abscission zone tissue as illustrated in Figure 1). A comparison of control and abscission zone tissue (2 mm segments as illustrated in Figure 1) with respect to soluble protein content of tissue treated with 5 µl/l of ethylene gas over a five hour time course period.

FIGURE 6

PROTEIN–ABSCISSION ZONE



curves from three to five hours (Figure 6). Between one and three hours, the ethylene-treated abscission zone tissue shows a slight rise in protein between one and two hours, followed by a perceptible drop in protein between one and three hours (Figure 7). The ethylene-treated distal tissue has a very low amount of protein between three and four hours; however, there is a significant increase in distal tissue protein between four and four and one-half hours, with the peak being reached at four and one-half hours (Figure 7). There is a falling-off in the amount of distal tissue protein between four and one-half and five hours, in ethylene-treated tissue (Figure 7). The control distal tissue has a very low amount of tissue protein between one and three hours, followed by an increase between three and four hours (Figure 7). There is a decrease of control distal tissue protein between four and four and one-half hours, followed by an increase between four and one-half and five hours (Figure 7). The amount of distal tissue protein at four and one-half hours is considerably higher than that of the control (Figure 7).

The profile of the ethylene-treated proximal tissue indicates that there is a sharp rise in the amount of tissue protein between three and one-half and four hours (Figure 8). The amount of detectable protein at four hours, in the ethylene-treated proximal tissue, is markedly higher than the protein content of either ethylene-treated abscission zone (Figure 6) or distal tissue (Figure 7). There is a

Fig. 7. Time course study of ethylene-treated and control flower pedicel tissue (2 mm segments of distal tissue as illustrated in Figure 1). A comparison of control and distal tissue (2 mm segments of distal tissue as illustrated in Figure 1) with respect to soluble protein content of tissue treated with 5 µl/l of ethylene gas over a five hour time course period.

FIGURE 7

PROTEIN – DISTAL

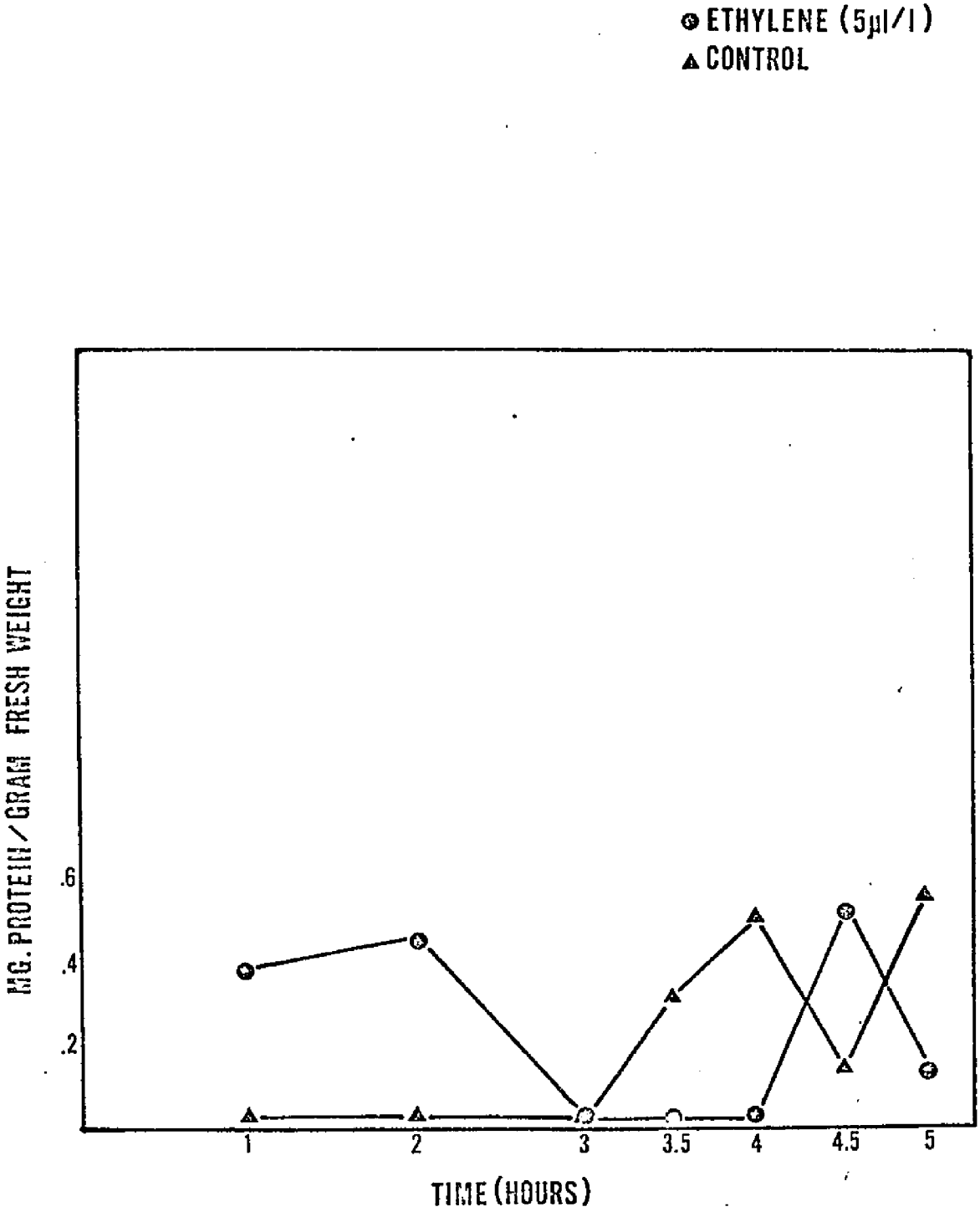
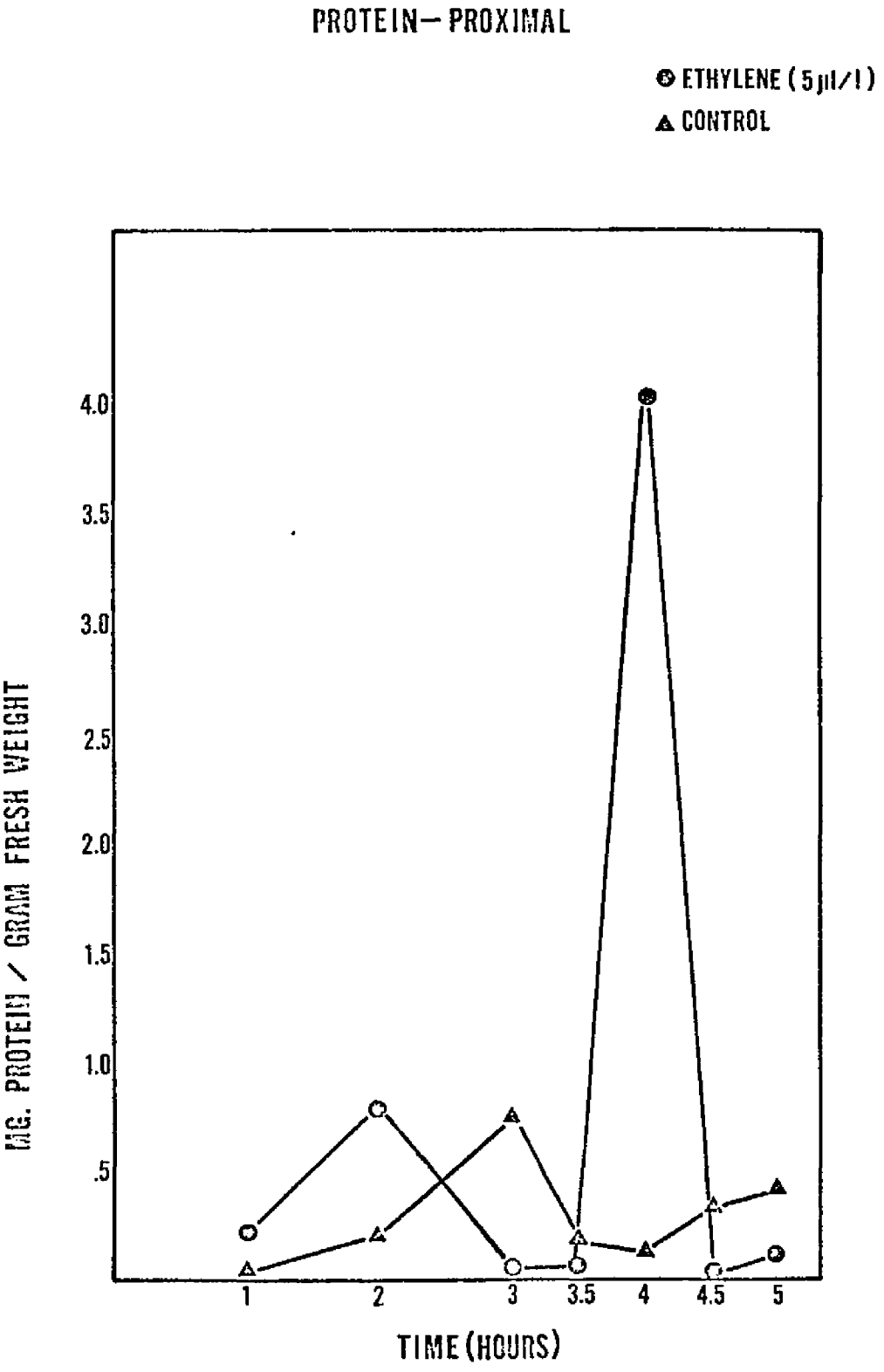


Fig. 8. Time course study of ethylene-treated and control flower pedicel tissue (2 mm segments of proximal tissue). A comparison of control and proximal tissue (2 mm segments of proximal tissue as illustrated in Figure 1) with respect to soluble protein content of tissue treated with 5 μl/l of ethylene gas over a five hour time course period.

FIGURE 8


PROTEIN—PROXIMAL
ETHYLENE (5 µl/l)
CONTROL
MG. PROTEIN / GRAM FRESH WEIGHT
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
1
2
3
3.5
4
4.5
5
TIME (HOURS)

decrease in protein content in ethylene-treated tissue between four and four and one-half hours, followed by a small rise between four and one-half and five hours (Figure 8). The control proximal tissue demonstrates a relatively small increase in protein content between one and three hours, followed by a decrease from three through four hours (Figure 8). There is a slight increase in the amount of control proximal tissue protein between four and five hours (Figure 8).

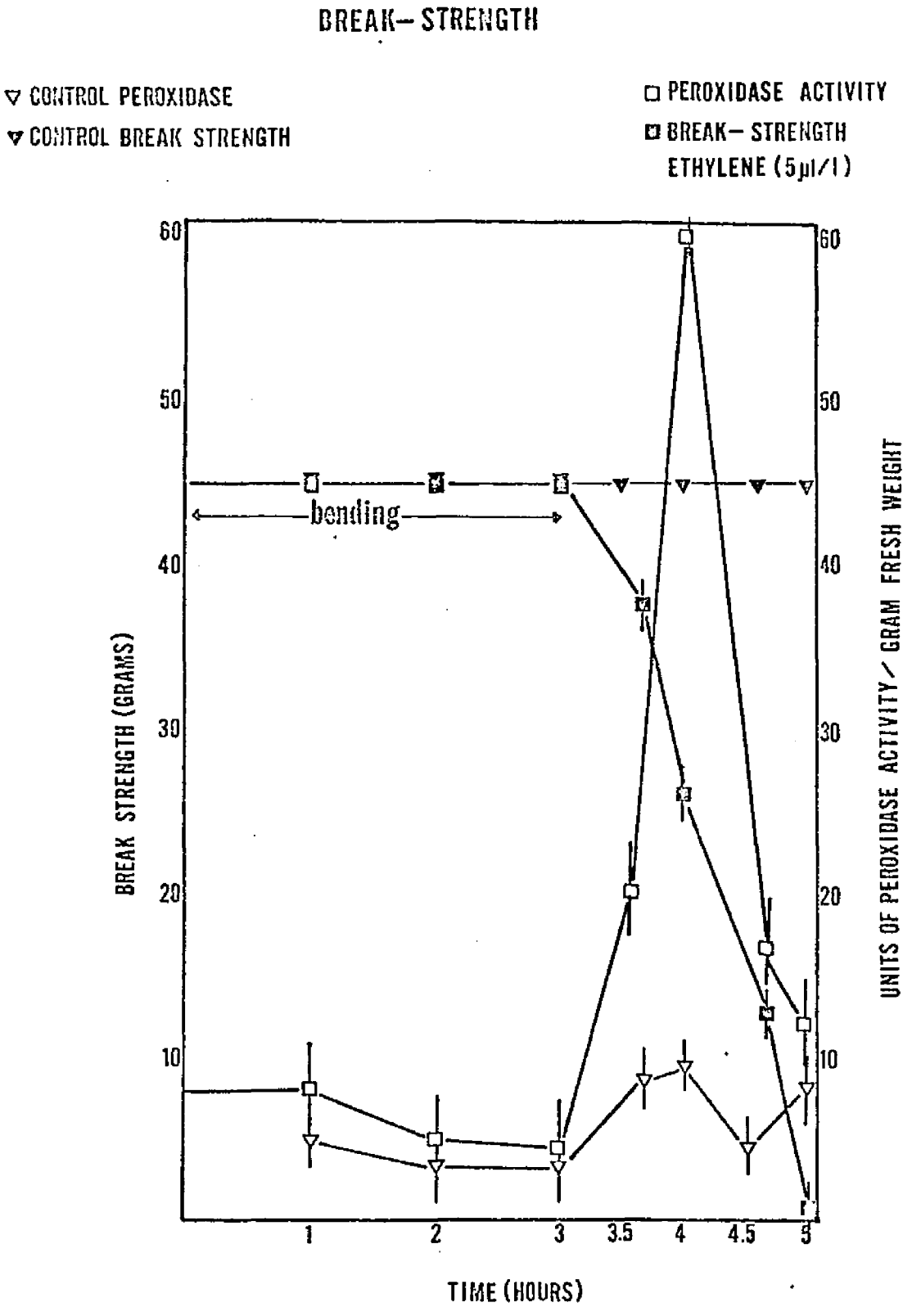
Break-Strength Determinations

A measure of the breaking force required to rupture the abscission zone, for each determination of break-strength, represents the testing of 10 separate pedicels. The standard error of the mean for each plot is represented by a vertical line through each point. The flower pedicels were exposed to 5µl/l of ethylene over the five hour time course period of the experiment.

The purpose of this assay is to determine when there is a perceptible change in the break-strength during the time course of ethylene treatment (Figure 9). Flower pedicels able to withstand a break force of 45 grams will not break across the abscission zone (Figure 9). A drop of 2 grams in the break-strength from 45 grams to less than 5 grams results in breakage across the abscission layer, in flower pedicels exposed to ethylene for three hours (Figure

Fig. 9. Time course study of break-strength and peroxidase activity in ethylene-treated and control flower pedicel tissue (including proximal (2 mm), distal (2 mm), and abscission zone (2 mm) segments as illustrated in Figure 1). A comparison of the break-strength and peroxidase activity in tissue treated with 5 µl/l of ethylene gas over a 5 hour time course period indicates that the pedicels only bend and do not break during the first 3 hours of ethylene treatment. From 3½ to 5 hours of ethylene treatment, the pedicels break with the amount of break-force necessary to cause breakage (in gr) decreasing between 3½ and 5 hours. The control pedicels never break and only bend over the entire 5 hour time course (45 gr). The portion of the pedicel used for the peroxidase assay included proximal (2 mm), distal (2 mm), and abscission zone (2 mm) segments of tissue (Figure 1; Figure 2). The ethylene-treated pedicels begin to break (between 3 and 3½ hr) at a similar time (after 3 hrs.) when peroxidase activity begins to show an increase. It appears that the decrease in break-strength occurs concomitantly or very close to the start in the rise of peroxidase activity (both occurring after 3 hrs. of ethylene treatement).

FIGURE 9


BREAK–STRENGTH
▽ CONTROL PEROXIDASE
▼ CONTROL BREAK STRENGTH
□ PEROXIDASE ACTIVITY
■ BREAK–STRENGTH
ETHYLENE (5µl/l)
bending
BREAK STRENGTH (GRAMS)
UNITS OF PEROXIDASE ACTIVITY / GRAM FRESH WEIGHT
TIME (HOURS)
60
50
40
30
20
10
1
2
3
3.5
4
4.5
5

9). Between three and five hours, there is a uniform decrease in breaking force required to rupture the abscission zone, representing a decrease from 43 grams at three hours, to one gram at five hours (Figure 9). A comparison of break-strength with levels of peroxidase activity over the five-hour time course period shows that the break-strength begins to decrease at three hours and the peroxidase activity also starts to rise at three hours (Figure 9).

Cytochemical Localization of Peroxidases

Figure 12 is a picture, at the light microscope level, of the cells comprising the abscission zone of Nicotiana tabacum L. This view of abscission zone control tissue shows the actual indentation of groove with the opening making up the groove, extending down through an area comprising several tiers of cortical cells (Figure 12).

A light microscope view of the actual separation that is occurring in three hour ethylene-treated flower pedicel tissue is indicated (arrows) in Figure 13. As the abscission process continues, the number of tiers of cells involved in the zone of separation increases.

Cell Walls

Non-abscising cortical tissue demonstrates peroxidase reactive sites throughout the cell wall areas, with particu-

larly intense electron dense deposits in the intercellular spaces of the cell wall areas between adjacent cells (Figure 14). There are peroxidase reactive sites in the cell walls of all ethylene-treated tissue, with staining in the intercellular spaces of the walls in ethylene-treated tissue (Figure 15); however, in abscising cells, the peroxidase activity is primarily concentrated in the more central areas of the cell wall (Figure 16). There is diaminobenzidine reaction product in the intercellular spaces of cell walls of non-ethylene-treated tissue but the reaction product is much less intense than in ethylene-treated tissue. The concentration of peroxidase reactive sites is most intense in the middle lamellar area of abscising cells in five hour ethylene-treated tissue (Figure 17). All of the cortical cells of ethylene-treated tissue do not show the same degree of peroxidase localization in the middle lamellar region. Some of the cortical cells have more peroxidase staining in the intercellular spaces of the cell walls where the cells are undergoing separation, while other cells have the enzyme reaction product concentrated more in the middle lamellar region of the cell walls. The detection of either of these types of cell staining is, in some measure, dependent upon the random occurrence of cortical cells as they are obtained in the sectioning of a particular tissue sample.

Potassium cyanide, at a concentration of 0.02M, partially inhibited cell wall staining in diaminobenzi-

dine-treated flower pedicel tissue that was non-ethylene-treated (Figure 18). The darkening in the cell wall area may be due to uranyl acetate post-staining. Potassium cyanide does not appreciably inhibit cell wall staining in three and five hour ethylene-treated tissue (Figure 19A; Figure 19B).

Microbodies

The single membrane-bound microbodies have peroxidase reaction product in the presence of diaminobenzidine (Figure 20). However, the enzyme localized in the microbody is catalase and not a peroxidase, since the diaminobenzidine staining reaction is inhibited in the presence of amino-triazole (Figure 21A; Figure 21B). The microbodies do not stain positively for peroxidase or catalase in the presence of diaminobenzidine and potassium cyanide (Figure 19B). The typical microbody of the abscission tissue has a crystalloid core composed of parallel sheets of osmiophilic material and does not have cores with a cross-hatched arrangement. Ethylene-treatment of cortical tissue does not alter the appearance of the crystalloid cores of the microbodies. There is no detectable change in the intensity of the catalase reaction product in microbodies of abscission tissue treated for five hours with ehtylene (Figure 25A). The microbodies of the five-hour ethylene-treated tissue appear to exhibit less continuity in the

in the membrane encircling the microbody matrix (Figure 20). This observation is in agreement with those made by Valdovinos, Jensen, and Sicko (1971).

Endoplasmic Reticulum

Smooth endoplasmic reticulum is found in control tissue in the vicinity of the cell walls (Figure 23A). The segments are relatively short and do not stain when incubated in diaminobenzidine (Figure 23B). However, the endoplasmic reticulum of control tissue is weakly stained for peroxidase activity and has not proliferated or spread throughout the cytoplasm of the cell (Figure 23A; Figure 23B). The three and five hour ethylene-treated tissue exhibits more rough endoplasmic reticulum near the cell wall areas and the peroxidase reaction product is more intense on the endoplasmic reticulum (Figure 24). It is not possible to ascertain if there is a greater degree of proliferation and staining in the rough endoplasmic reticulum of the two and three hour ethylene-treated tissue compared to the five hour ethylene-treated tissue (Figure 25B). However, control tissue rough endoplasmic reticulum (Figure 25A) does not stain for peroxidase whereas the five hour ethylene-treated rough endoplasmic reticulum (Figure 25B) is intensely stained for peroxidase reaction product.

Mitochondria

In the tobacco flower pedicel tissue incubated in diaminobenzidine at the alkaline pH of 9.0, mitochondria do not stain positively for peroxidase or cytochrome oxidase activity (Figure 15). However, tissue incubated at an acidic pH of 6.0 demonstrates positive cytochrome oxidase staining on the inner mitochondrial and intracristae membranes (Figure 26A; Figure 26B). According to Novikoff and Goldfischer (1968), mitochondria (animal) stain for cytochrome oxidase activity only when incubated at lower pH of 6.0.

Chloroplasts

Many of the chloroplasts observed in tobacco (Nicotiana tabacum L.) abscission zone tissue contain a single membrane-bound granular component (Figure 27; Figure 33). Diaminobenzidine stained tissue reveals that this granular component stains for peroxidase with about the same electron density as do the microbodies (compare Figure 20). The peroxidase staining reaction within the granular component is slightly inhibited by aminotriazole (Figure 28). The single membrane-bound granular component of chloroplasts was partially inhibited by potassium cyanide (Figure 18; Figure 22) since there is still a small amount of density present.

This indicates that peroxidase is the major enzyme contained within this body (Figure 20). The single membrane-

bound granular component of chloroplasts was partially inhibited by potassium cyanide (Figure 18). The chloroplast thylakoids do not appear to be more electron dense in ethylene-treated tissue than in controls (Figure 15; Figure 23B). Moreover, neither aminotriazole nor potassium cyanide affects the electron density of the diaminobenzidine-treated tissue (Figure 29). There were no significant structural changes observed within the chloroplasts nor was there variation in the electron density of the peroxidase reaction product between ethylene-treated and control tissue (Figure 15). Cortical tissue incubated in diaminobenzidine at an acidic pH of 6.0 also shows a weak degree of peroxidase staining in the thylakoids (Figure 26B). A great number of chloroplasts, both in control and ethylene-treated pedicel tissue, contain aggregations of phytoferritin-like particles within the cytoplasmic matrix (Figure 21B).

Golgi Apparatus

The Golgi apparatus of control abscission zone cortical tissue does not stain positively for peroxidase in the presence of diaminobenzidine (Figure 30). The Golgi apparatus of abscission tissue of flower pedicels treated for two hours with ethylene stain more intensely for peroxidase than do those of controls (Figure 31A). The five hour ethylene-treated tissue reveals that the Golgi apparatus is made up of smaller vesicles (Figure 31B). The Golgi vesicles appear to stain for

peroxidase when they are located in close approximation to the membraneous cristae of the Golgi apparatus. Five hour ethylene-treated cortical tissue shows positive diaminobenzidine staining in the presence of 0.02M amino-triazole (Figure 32A; Figure 32B; Figure 32C; Figure 32D).

DISCUSSION

The Significance of Diaminobenzidine Staining In The Cortical Cells of The Abscission Layer of Tobacco Flower Pedicels

Chloroplasts

Diaminobenzidine-stained control tissue has many chloroplasts with a deeply stained single membrane-bound granular component (Figure 14; Figure 20; Figure 27; Figure 33). The electron density of the granular component (Figure 20) is of the same order as that of the microbodies. Potassium cyanide inhibits the staining of the granular component (Figure 18) whereas aminotriazole only causes partial inhibition of the peroxidase reaction product (Figure 28). According to Novikoff and Goldfischer (1969) and Fahimi (1969), complete inhibition of the diaminobenzidine staining reaction by the specific catalase inhibitor, 3-amino-1,2,4-triazole, is positive confirmation that the enzyme being localized is catalase rather than a peroxidase.

In a study of several plant species for peroxidase enzyme content, Tolbert et al., (1968) found tobacco leaf chloroplast fractions to be devoid of catalase activity. Any detectable catalase in the whole chloroplast fraction is attributed to contamination by the broken microbody fraction

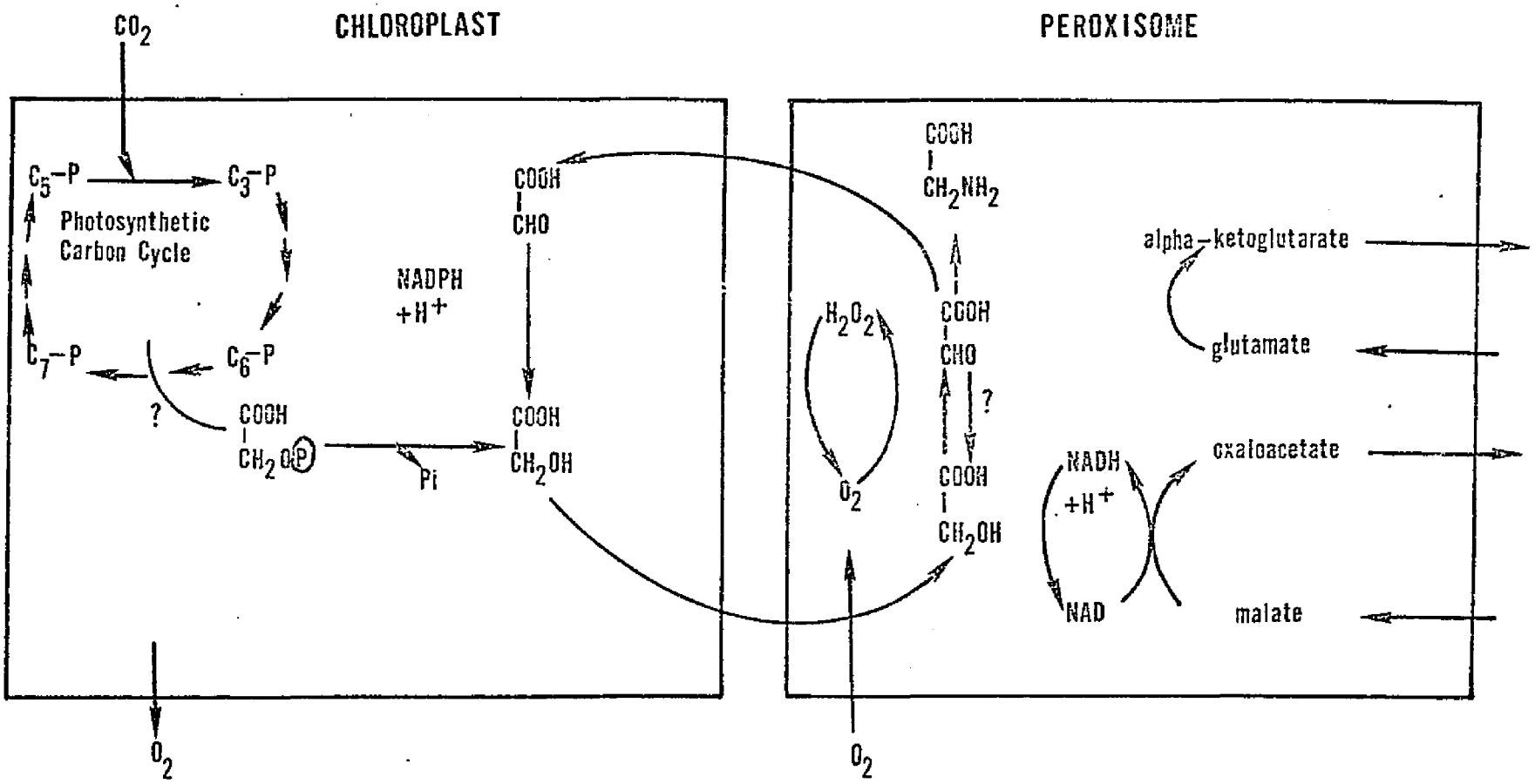
(Tolbert et al., 1968). Deisseroth and Dounce (1967) suggest that catalase may be expected to be present where there is an electron transport system; however, they cite no evidence for this phenomenon in plant tissue. The glycolate-glyoxylate pathway proposed by Tolbert et al., (1968) is viewed as occurring in all photorespiring plants (Figure 11). In this scheme, glyoxylate is reduced to glycolate by the chloroplasts, in the presence of light (Kearney and Tolbert, 1962). After the glycolate passes from the chloroplasts out into the cytoplasm, a specific cytoplasmic glycolic acid oxidase oxidizes glycolate back into glyoxylate (Kearney and Tolbert, 1962). The two carbon glyoxylate can now re-enter the chloroplast to be reduced to glycolate by glyoxylic acid reductase (Kearney and Tolbert, 1962). Thus the presence of peroxidase within the granular component of the chloroplast may possibly involve peroxidase in this cycle. However, as most chloroplasts lack a granular component with a single membrane this suggests that such involvement may not necessarily be the case. It could also be that the single membrane-bound granular component of the chloroplast is involved in thylakoid formation. The possession of the single membrane-bound organelle by the chloroplast, containing a supply of peroxidase, could help to make the chloroplast a more self-sufficient organelle capable of controlling the formation of thylakoids. Another possible explanation for the staining of the dense granular component is that cytochrome oxidase may be reacting with the

Fig. 11. Relationship between photosynthesis and peroxisomal respiration.*

(*Tolbert, N.E., and R.K. Yamazaki, 1969)

FIGURE 11

RELATIONSHIP BETWEEN PHOTOSYNTHESIS AND PEROXISOMAL RESPIRATION *



* TOLBERT, N.E., and R.K. YAMAZAKI, 1969

diaminobenzidine. Novikoff and Goldfischer (1969) indicate that rat liver mitochondria do not show positive staining for cytochrome oxidase at a pH of 9.0 and have decided that cytochrome oxidase is detectable with the diaminobenzidine staining technique only at a pH of 6.0. However, it is possible that in tobacco tissue cytochrome oxidase in the thylakoids may be detected at the higher pH of 9.0.

The particularly heavy deposits of peroxidase reaction product, present in the middle lamellar area of abscising cells, may have its genesis from two possible areas: (1) movement from intercellular spaces of the cell walls; and (2) cytoplasmic synthesis and subsequent movement of the enzymes into the middle lamellar area. The intercellular spaces of the cell walls between adjacent cells are consistently stained for peroxidases, with the staining being more intense in the three and five hour ethylene-treated tissue. It is transported out into the middle lamellar region as abscission proceeds during the five hour time course period. The second consideration takes into account the possibility of cytoplasmic synthesis of new enzyme protein, which subsequently is transported out of the cytoplasm into the cell wall where it would then participate in the cell wall dissolution that occurs as abscission takes place.

Endoplasmic Reticulum

Diaminobenzidine-stained control tissue does not show deep staining in the endoplasmic reticulum (Figure 23A; Figure 23B). The strands are not unusually long (Figure 23A). This would indicate that in non-abscising control tissue there is little synthesis of new proteins occurring in association with the rough endoplasmic reticulum. However, in the three and five hour ethylene-treated flower pedicel tissue, there is more intense diaminobenzidine staining of the rough endoplasmic reticulum, particularly at five hours (Figure 24; Figure 25B). The rough endoplasmic reticulum of the five hour tissue also is composed of unusually long strands in close proximity to the cell walls (Figure 25B). The rough endoplasmic reticulum staining is inhibited by 0.02M potassium cyanide (Figure 29) but not by aminotriazole (Figure 28). Due to the deep staining for peroxidase in the rough endoplasmic reticulum of the three and five hour ethylene-exposed tissue, it is suggested that increased peroxidase and perhaps other protein synthesis are occurring during this period. These findings in regard to rough endoplasmic reticulum, are in agreement with those of Valdovinos et al., (1971) wherein a marked increase in rough endoplasmic reticulum was observed in the three and five hour ethylene-treated flower pedicels.

Golgi Apparatus

The Golgi apparatus of diaminobenzidine-treated control tissue are not stained deeply for peroxidase (Figure 30). The intensity of the diaminobenzidine reaction within the Golgi apparatus is not appreciably altered by potassium cyanide or aminotriazole (Figure 31A; Figure 32A; Figure 32B; Figure 32C; Figure 32D). There is no indication that the control tissue have Golgi undergoing increased synthetic activity. The Golgi apparatus of the five hour ethylene-treated pedicels are more intensely stained for peroxidase than are the controls (Figure 31B). However, the Golgi vesicles are more compact and the cisternae appear to be uniformly stained (Figure 31B).

The diaminobenzidine staining is especially intense in the Golgi cisternae with some staining occurring in the vesicles (Figure 31B). There appears to be a heightened need for peroxidase synthesis and/or mobilization occurring in the Golgi areas during the three to five hour interval of applied ethylene. The diaminobenzidine staining of the Golgi indicates that they are involved in the movement of peroxidases from the cytoplasm of the cell out into the cell walls.

The significance of Peroxidases in Abscission Processes

Spectrophotometric measurements indicate that applied ethylene causes a change in the levels of peroxidase activity in abscission zone tissue over a five hour time

couse period (Figure 2). The rise in peroxidase activity begins after three hours, reaching a peak at four hours, with an increase in activity between four and five hours (Figure 2). The present experiments show peroxidase activity to peak after the cortical tissue has been exposed to ethylene for four hours (Figure 2; Figure 3). It is interesting to note that there is a gradual increase in peroxidase activity between three and four hours corresponding to the time period where the cells across the abscission zone begin to require less break-strength (Figure 9). The increase in peroxidase activity and the concomitant decrease in break-strength, both at three hours, imply that peroxidase participates in the chemical events involved in the rupturing of the cells in the abscission zone. It is difficult to ascertain whether the actual buildup of peroxidase activity orecedes the start of the decrease in break-strength, as there may be enzymatic synthesis which occurs prior to three hours, but becomes detectable only in the three hour tissue samples. If the rise in peroxidase activity actually precedes the fall in break-strength, then peroxidase may participate in triggering the onset of abscission, according to Lewis and Varner (1970). However, if the rise in peroxidase activity parallels the beginning of the decrease in break-strength, as appears to be the case in the present stydies, peroxidase may participate in the cell wall dissolution phenomena without having been responsible for actually triggering the start of abscission. The marked increase in peroxidase activity, observable after four hours of ethylene treatment, may be due to: (1) de novo

synthesis of new peroxidase isozymes; (2) an increase in the mobilization of previously inactive peroxidases that are activated by the applied ethylene; or (3) a translation of existing messenger RNA. With respect to de novo peroxidase synthesis, it is possible that it may be occurring at the rough endoplasmic reticulum areas with subsequent packaging at the Golgi apparatus, followed possibly by translocation of the enzymes into the regions of the cell walls. Experiments showing an increase in the number of peroxidase isozymes, after exposure to ethylene (Gahagan et al., 1968; Imaseki et al., 1968; Ridge and Osborne, 1970), indicated the probability of de novo synthesis of new peroxidase isozymes. Also, de novo synthesis is further indicated as the increase in peroxidase activity was inhibited by both actinomycin D and Cycloheximide (Gahagan et al., 1968; Imaseki et al., 1968; Ridge and Osborne, 1970). Gahagan et al., (1968) found that Cycloheximide was more effective than actinomycin D in inhibiting peroxidase activity.

In the present studies, when the flower pedicel was divided into proximal, distal, and abscsission zone segments, the abscission zone tissue still possessed the greatest amount of peroxidase activity (Figure 3). The distal tissue had the next highest amount of peroxidase activity (Figure 4), with the proximal segments showing the lowest amount of peroxidase activity (Figure 5). The peak of peroxidase activity in the abscission zone and distal segments occurs after four hours of exposure of the tissue to ethylene.

This indicates that there is an increase in peroxidase activity in the abscission zone tissue and a possible correlation between this peak level of peroxidase and the decreased requirement of break-strength (26.4 grams).

A group of compounds known as phenolics (caffeic acid, chlorogenic acid, and 2,4-dichlorophenol) may be involved in the abscission process by serving as inhibitors or promotors of the cell dissolution that is an active part of abscission (Schwertner and Morgan, 1966). The phenolics may also be involved in the genesis of peroxidases and their substrates and therefore may be considered as possible precursors of peroxidase (Tomaszewska, 1964; Schwertner and Morgan, 1966).

Tomaszewska (1964) reported that phenolic compounds regulate abscission in Leutzia wilsonii and Schwertner and Morgan (1966) indicate a role for phenolics in cotton (Gossypium hirsutum) abscission. However, in an examination of bean (Phaseolus vulgaris) and Coleus blumei explants, Gahagan et al., (1968) found no evidence which indicated phenolics to have an effect on abscission. There are conflicting reports on the effects of phenolic compounds on cotton abscission. Greenblatt (1965) studied the effects of dichlorophenol, caffeic acid, and catechol, on cotton abscission. He found that abscission was accelerated by caffeic acid and catechol but was not affected by manganese ion and dichlorophenol. Schwertner and Morgan (1966) found that caffeic acid and catechol did not accelerate cotton

abscission. The precise mechanism of phenolics in cotton abscission remains to be clarified. Gahagan et al., (1968) added indoleacetic acid to Coleus blumei explants, pre-treated with phenolic compounds (either caffeic acid, p-coumaric acid, or dichlorophenol). The found that the presence of indoleacetic acid oxidase cofactors, dichloro-phenol and p-coumaric acid, did not accelerate abscission of indoleacetic acid-treated Coleus blumei explants (Gahagan et al., 1968). If added indoleacetic acid causes an increase in ethylene production within Coleus blumei explants, Gahagan et al., (1968) reasoned that any compound that would decrease the level of indoleacetic acid oxidase would inhibit abscission and cause an increase in ethylene evolution (Gahagan et al., 1968). The results were conflicting as it was observed that caffeic acid inhibited abscission but did not promote the evolution of ethylene (Gahagan et al., 1968). Gahagan et al., (1968) rule out a role for indoleacetic acid oxidase in abscission of Coleus blumei explants.

The role of ethylene in affecting the mode of auxin action may be through a direct effect of applied ethylene on diffusable auxin levels (Burg and Burg, 1968; Hall and Morgan, 1964; Morgan et al., 1968; Osborne and Mullins, 1969; Valdovinos et al., 1967). It is also a possibility that applied ethylene may influence the levels of auxin conjugation (Cracker et al., 1970; Ernest and Valdovinos, 1971). In the experiment of Cracker et al., (1970) indoleacetic acid was applied for one hour to bean (Phaseolus vulgaris) explants

and abscission was inhibited during an eight hour aging period. When indoleacetic acid--$_{14}C$ was used, it was suggested that the applied indoleacetic acid became conjugated, which presumably accounts for the decreased ability of indoleacetic acid to inhibit abscission longer than eight hours (Cracker et al., 1970). Application of indoleacetic acid inhibited abscission for the entire eight hour period of the experiment after a one hour application (Cracker et al., 1970). It was suggested that indoleacetic acid can inhibit the loss of break-strength if it is available to the plant at any time before the processes of delayed aging are completed in the explant tissue (Cracker et al., 1970). These authors suggest that abscission is not due to auxin translocation or auxin destruction, but to increased auxin conjugation (Cracker et al., 1970). Auxin could simply be unavailable to act to prevent abscission (Cracker et al., 1970). In this view, indoleacetic acid destruction is regarded as merely one of the participating factors in aging (Cracker et al., 1970). Galston and Baker (1951) suggest that indoleacetic acid oxidase is made up of a combination of enzymes: a light-activated flavoprotein oxygenase capable of producing hydrogen peroxide from some unknown substrate, plus a peroxidase which can use oxygen from the peroxide for the oxidation of indoleacetic acid. Siegel and Galston (1955) suggest that indoleacetic acid itself may be the actual peroxidase substrate and thereby provide a peroxidase source for its own destruction. Pilet and Galston (1957) studied

indoleacetic acid oxidase activity in meristematic tissue of lentil roots and found that the enzyme activity was limited by a phenolic cofactor. The authors suggest that the cofactor may control tissue response to auxin and thus qualify as a true "aging factor" (Pilet and Galston, 1957). In this view, the older tissues have a higher cofactor concentration, causing increased indoleacetic acid oxidase activity, thereby preventing any response to indoleacetic acid, and these factors lead to increased aging of the cells (Pilet and Galston, 1957). Since young cells do not possess the cofactor and are unable to produce peroxide, indoleacetic acid can cause an increase in the genesis of peroxide, which means that it may be giving rise to the peroxide that is involved in its own destruction (Pilet and Galston, 1957). This interpretation of indoleacetic acid oxidase action does not take into consideration the possible effect of applied ethylene on the mode of action of the enzyme (Gahagan et al., 1968). Galston et al., (1950) indicate that indoleacetic acid does give rise to the peroxide required for its own destruction; they suggest that scopoletin, by its own auxin-sparing action, stimulates auxin, thereby inhibiting growth at low levels and promoting growth at high concentrations (Galston et al., 1950). However, the mode of action of indoleacetic acid oxidase and its relationship to peroxidase may not necessarily be at the transcription or translational level.

The present studies indicate that there is a marked

increase in soluble protein in the proximal abscission zone tissue with four hours of ethylene (5 µl/l) treatment (Figure 8). The levels of soluble protein in abscission zone tissue (Figure 6) and in distal tissue (Figure 7) under the same conditions, are much lower. There appears to be a translocation of soluble protein out of the abscission zone with movement into the proximal tissue as the processes of abscission occur. Scott and Leopold (1966) suggest that, in excised bean (Phaseolus vulgaris) petioles, the mobilization of protein from the distal to the proximal portion of the petiole may trigger the developmental processes of abscission. At the present time, the reason for the appearance of no new peroxidase isozymes in abscission cells has not been fully clarified and warrants further study.

SUMMARY

The present studies indicate that peroxidase is localized in certain areas of the abscising and non-abscising cortical cells of tobacco (intercellular spaces and middle lamellar areas of the cell walls, rough endoplasmic reticulum, and Golgi apparatus). The localization of peroxidase in these particular cellular areas suggests the possibility that de novo peroxidase synthesis is occurring at the rough endoplasmic reticulum sites with subsequent packaging at the Golgi apparatus, followed possibly by movement of the enzymes into the areas of the cell walls. The peroxidase may then serve in some manner to begin the processes of cell degradation and dissolution that are to occur in the abscising cells.

There does not appear to be a significant alteration in the structure of mitochondria, chloroplasts, and nuclei during the five hours of ethylene treatment--in terms of peroxidase activity--in comparison to untreated controls. The single membrane-bound granular component of the chloroplasts, the rough endoplasmic reticulum, the Golgi, and the middle lamellar region of the cell walls, show positive peroxidase staining in the presence of diaminobenzidine. However, the proliferation of peroxidase

staining in rough endoplasmic reticulum is particularly intense in the three and five hour ethylene-treated tissue. The cell wall middle lamellar area and intercellular spaces of the cell wall stain more intensely for peroxidase in three and five hour ethylene-treated tissue than in untreated controls. Examination of the microtubules and plasmodesmata does not reveal a difference between ethylene-treated and untreated controls (Table 1).

When a comparison is made between tissue stained in diaminobenzidine at the usual pH of 9.0 and the acidic one of 6.0, the only observable change at the lower pH is the positive cytochrome oxidase staining of the intracristae mitochondrial membranes. In addition, the lower pH mitochondria show structural alterations in abnormal swelling and distrubances in the cristae arrangement. While the electrophoretic data suggest that ethylene treatment does not necessarily cause the induction of new peroxidase isozymes, ethylene may cause de novo synthesis and enhance the amount of peroxidase isozymes originally present within the tissue.

Spectrophotometric studies indicate an increase in peroxidase activity between three and five hours after treatment of pedicels with ethylene. A comparison of the break-strength data and peroxidase levels in ethylene-treated cortical tissue suggests that the initial increase in peroxidase activity may not significantly precede the onset of the decline in break-strength. Thus a triggering role cannot be definitely ascribed to peroxidase, but the increase in peroxidase activity between three and five hours indicates

TABLE 1.

Control and ethylene-treated cortical tissue was stained in five different types of media: (1) DAB; (2) DAB w/o H_2O_2; (3) domplete medium w/o DAB; (4) DAB plus KCN; and (5) DAB plus aminotriazole. The respective tissue samples were examined for staining intensity at 2, 3, and 5 hours. Incubations were at pH 9.0 and 6.0 respectively.

Legend for figures used in Table 1:

Mb----------microbody

Ch. Mem.----------chloroplast membrane

Ch. Gr. Comp.----------single membrane-bound granular component

Cw----------cell wall

RER----------rough endoplasmic reticulum

Go----------Golgi

Mi----------mitochondria

Pl----------plasmodesmata

AT----------aminotriazole

C----------controle tissue

2,3, and 5 hours----- time tissue was exposed to 5 µl/l of ethylene

TABLE 1. DIAMINOBENZIDINE STAINING INTENSITY OF CELLULAR COMPONENTS IN CORTICAL TISSUE OF NICOTIANA TABACUM L.

pH 9.0	Mb.				Ch. Mem.				Ch:Gr.Comp.				CW				RER				Go.				Mi.				Pl.			
Time (hrs.)	C	2	3	5	C	2	3	5	C	2	3	5	C	2	3	5	C	2	3	5	C	2	3	5	C	2	3	5	C	2	3	5
DAB	4	4	4	4	1	1	2	3	4	4	4	4	2	2	3	3	0	1	3	4	0	1	2	3	0	0	0	0	2	2	2	2
DAB w/o H_2O_2	4	4	4	4	1	1	2	3	4	4	4	4	2	2	3	4	0	1	3	4	0	1	2	3	0	0	0	0	2	2	2	2
Complete Medium w/o DAB	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DAB plus KCN	1	1	1	1	2	1	1	2	1	0	1	1	1	1	1	1	0	0	1	1	0	0	1	1	0	0	0	0	1	1	1	1
DAB plus AT	0	0	0	0	1	1	2	3	4	4	4	4	2	2	3	3	0	1	3	4	0	1	2	3	0	0	0	0	2	2	2	2
ph 6.0																																
DAB	3	3	3	3	1	1	1	1	1	1	1	1	1	2	2	3	0	0	1	1	0	0	0	0	3	3	3	3	0	0	0	0
DAB w/o H_2O_2	3	3	3	3	1	1	1	1	1	1	1	1	1	2	2	2	0	0	1	1	0	0	0	0	2	2	2	2	0	0	0	0
Complete Medium w/o DAB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DAB plus KCN	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
DAB plus AT	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	0	0	1	1	0	0	0	0	3	3	3	3	0	0	0	0

that peroxidase isozymes may in some way participate in the cell wall dissolution processes that accompany abscission. Ethylene induced abscission in tobacco may involve alteration of the movement of auxin (increased conjugation and mobilization), concomitant with the increased synthesis and release from inhibition, of the peroxidase isozymes that are thought to participate in the rupture of cells comprising the abscission zone.

The present research suggests several further areas of investigation. It is important to examine the possible relationship between lignin and peroxidase. Previous research has implied that peroxidase-like phenolics are involved in lignin biosynthesis (Siegel, 1953); however, in lignin breakdown and cell wall dissolution, the role of peroxidases has yet to be clarified. It is worthwhile to examine the role of ethylene in lignin biosynthesis since ethylene can cause an increase in peroxidase activity and may thereby exert an indirect effect on lignification by enhancing the levels of phenolics and peroxidases. Lastly, further study is needed to investigate the role of peroxidases such as indoleacetic acid oxidase in terms of the ability of indoleacetic acid oxidase to regulate the levels of endogeneous auxin. It is possible that some of the metabolic effects attributed to peroxidase may indeed involve alteration of the levels of auxin through auxin destruction, conjugation, or sparing mechanisms. Inherent in all of the suggested avenues for further research is the need

to attempt to clarify the manner in which ethylene interacts within the tissue. The exact role of ethylene interaction in relation to auxin levels, protein synthesis, lignin biosynthesis, and peroxidase enzymatic activity is still unclear. Further clarification of the aforementioned suggested areas for future research will help to explain the physiological and fine structural alterations that occur during the course of abscission.

BIBLIOGRAPHY

Abeles, F.B. 1967. Mechanism of action of abscission accelerators. Physiologia Plantarum. 20: 442-454.

Abeles, F.B. 1969. Abscission: role of cellulase. Plant Physiology. 43: 1577-86.

Addicott, F.T. 1969. Aging, senescnece, and abscission in plants: phytogerontology. Horticultural Science. 4: 14-16.

Addicott, F.T. 1970. Plant hormones in the control of abscission. Biological Review. 45: 485-524.

Agrawal, B.B.L., and E. Margoliash. Modification of the active histidine in catalase-H_2O_2 complex. In: 158th American Chemical Society Meeting, Division of Biological Chemistry, New York, 1969, Abstract No. 246.

Arnon, D.I., and D.R. Hoagland. 1940. Crop production in artificial solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Science. 50: 463.

Battelli, F., and L. Stern. 1908. Activation of tissue respiration by muscle extracts. Journal of General Physiology and Pathology. 9: 737.

Beard, M.E., and A.B. Novikoff. 1968. A comparison of microbody and lysosome distribution in the rat nephron. Journal of Histochemistry and Cytochemistry. 16: 512 (abstract).

Beers, R.F., Jr., and I.W. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. Journal of Biological Chemistry. 195: 133.

Blanchard, M., Green, D.E., Nocito-Carroll, V., and S. Ratner. 1946. L-Hydroxy acid oxidase. Journal of Biological Chemistry. 163: 137.

Bonner, T., and J.E. Varner. Plant Biochemistry. Academic Press, New York. 1965.

Bornman, C.H. Spurr, A.R., and F.T. Addicott. 1967. Abscission, auxin, and gibberellin effects on the developmental aspects of abscission in cotton (Gossypium hirsumtum). American Journal of Botany. 54: 125-35.

Burg, S.P., and E.A. Burg. 1968. Ethylene, plant senescence and abscission. Plant Physiology. 43: 1503-11.

Burstone, M.S. 1959. New histochemical techniques for the demonstration of tissue oxidase (cytochrome oxidase). Journal of Histochemistry and Cytochemistry. 7: 112-122.

Chance, B. 1952. The effect of pH upon the equilibria of catalase compounds. Journal of Biological Chemistry. 194: 483-496.

Chambach, A., and D. Rodbard. 1971. Polyacrylamide gel electrophoresis. Science. 172: 440-451.

Cracker, L.E., and Abeles, F.B. 1969. Abscission: role of abscisic acid. Plant Physiology. 44: 1144-49.

Cracker, L.E., Chadwick, A.V., and G.R. Leather. 1970. Abscission. Movement and conjugation of auxin. Plant Physiology. 45: 790-793.

Deisseroth, A., and A.L. Dounce. 1967. Nature of the change produced in catalase by lyophilization. Archives of Biochemistry and Biophysics. 120: 671-692.

De Jong, D.W., Olson, A.C., Hawker, K.V., and E.F. Jansen. 1968. Effect of cultivation temperature on peroxidase isozymes of plant cells grown in suspension. Plant Physiology. 43: 841-844.

Dela Fuente, R.K., and A.C. Leopold. 1968. Kinetics of abscission in the bean petiole explant. Plant Physiology. 44: 251-254.

Desborough, S.L., and S.T. Peloquin. 1966. Disc electrophoresis of tuber proteins from Solanum species and interspecific hybrids. Phytochemistry. 5: 727-733.

Dixon, M., and K. Kleppe. 1955. D-amino acid oxidase. III effect of pH. Biochemistry and Biophysics Acta. 96: 383.

Dounce, A.L., and V. Frampton. 1930. Crystalline horse liver catalase. Science. 89: 300.

Dowben, R.M. 1969. General Physiology, A Molecular Approach. Harper and Row, London.

Ernest, L.C., and J.G. Valdovinos. 1971. Regulation of auxin levels in Coleus blumei by ethylene. Plant Physiology. 48: 402-406.

Fahimi, H.D. 1968. Cytochemical localization of peroxidase activity in rat hepatic microbodies (peroxisomes). The Journal of Histochemistry and Cytochemistry. 16: 547.

Fahimi, H.D. 1969. Cytochemical localization of peroxidatic activity of catalase in rat hepatic microbodies (peroxisomes). The Journal of Cell Biology. 43: 275.

Fahimi, H.D. 1970. The fine structural localization of endogenous and exogenous peroxidase activity in Kupffer cells of rat liver. The Journal of Cell Biology. 47: 247-262.

Fox, D.J., Thurman, D.A., and O. Butler. 1964. Studies of the proteins of seeds of the Leguminoseae I. Albumins. Phytochemistry. 3: 417-419.

Frederick, S.E., and E.H. Newcomb. 1969. Cytochemical localization of catalase in leaf microbodies (peroxisomes). The Journal of Cell Biology. 43: 343-353.

Fruton, J.S., and S. Simmonds. General Biochemistry. 2nd ed. John Wiley and Sons, Inc., New York (1958).

Gahagan, H.E., Holm, R.E., and F.B. Abeles. 1968. Effects of ethylene on peroxidase activity. Physiologia Plantarum. 21: 1270-1279.

Galston, A.W., and R.S. Baker. 1951. Studies on the physiology of light action III. Light activation of a flavoprotein enzyme by reversal of a naturally occurring inhibition. American Journal of Botany. 38: 190-195.

Galston, A.W., and W.S. Hillman. 1961. The degradation of auxin. In: Encyclopedia of Plant Physiology. 14: 647-670.

Galston, A.W., Lavee, S., and B.Z. Siegel. 1968. The induction and repression of peroxidase isozymes by 3-indoleacetic acid. In: F. Wightman and G. Setterfield, eds. Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa. pp. 455-472.

Gerola, F.M., and E.G. Dassu. 1960. L'evoluzione dei cholroplasti durante l'inverdimento sperimentale di frammenti di frammenti di tuberi di topenambour (Helianthus tuberosus). Nuovo Giornale Botanico Botanico Italiano. 67: 63-78.

Goodman, J.E., and T.H. Tephly. 1968. The role of hepatic microbody and soluble oxidases in the peroxidation of methanol in the rat and monkey. Molecular Pharmacology. 4: 492.

Gordon, A.R. 1968. Histochemical localization by differential inactivation of peroxidase isozymes and changes in isozyme distribution during development and wound healing in tomato stem. Ph.D. dissertation. Case Western Reserve University, Cleveland, Ohio.

Graham, R.C., Jr., and M.J. Karnovsky. 1965. The histochemical demonstration of uricase activity. The Journal of Histochemistry and Cytochemistry. 13: 448.

Greenblatt, G.A. 1965. Interaction of hormones and growth retardants in abscission. Ph.D. dissertation, University of California, Davis.

Gueskens, M., et W. Bernard. 1966. Cytochimie ultrastructurale du nucleole. III. Action de l'actinomycin D sur le metalbolisme du RNA nucleolaire. Experimental Cell Research. 44: 579-598.

Hall, W.C., and Morgan, P.W. 1964. Auxin-ethylene interrelationships. In: Regulateurs naturels de la croissance vegeta le, pp. 727-745. Ed. J.P. Nitsch.

Hanker, F., Kasler, F., Bloom, M.G., Copeland, J.S., and A.M. Seligman. 1967. Coordination polymers of osmium: the nature of osmium black. Science. 156: 1737.

Hare, R.C. 1964. Indole acid oxidase. The Botanical Review: 129-265.

Hart, G.E., and C.R. Bhatia. 1967. Acrylamide gel electrophoresis of soluble leaf proteins and enzymes from Nicotiana species. Canadian Journal of Genetics and Cytology. 9: 367-374.

Hirai, K-I. 1969. Light microscopic study of the peroxidase activity of catalase in formaldehyde-fixed rat liver. The Journal of Histochemistry and Cytochemistry. 17: 585-590.

Hirai, K-I. 1968. Specific affinity of oxidized amine dye (radical intermediate) for heme enzymes: study in light and electron microscopy and spectrophotometry. Acta Histochemistry and Cytochemistry. 1: 54.

Horton, R.F., and D.J. Osborne. 1967. Senescence, abscission, and cellulase activity in Phaseolus vulgaris. Nature, London. 214: 1086-88.

Imaseki, H., Uchiyama, M., and I. Uritani. 1968. Effect of ethylene on the inductive increase in metabolic activities in sliced sweet potato roots. Agricultural Biological Chemistry, Vol. 32: 387-389.

Israel, H.W., and F.C. Steward. 1967. The fine structure and development of plastids in cultured cells of Daucus carota. Annals of Botany. 31: 1-18.

Jackson, M.B., and D.J. Osborne. 1970. Ethylene, the natural regulator of leaf abscission. Nature, London. 225: 1019-1022.

Jansen, E.F., Jang, R., Alberseim, P., and J. Bonner. 1960. Binding of enzymes to Avena coleoptile cell walls. Plant Physiology. 35: 87.

Jensen, T.E., and J.G. Valdovinos. 1967. Fine structure of abscission zones. I. Abscission zones of the pedicels of tobacco and tomato flowers at anthesis. Planta (Berl.). 77: 2980318.

Jensen, T.E., and J.G. Valdovinos. 1968. Fine structure of abscission zones. Cytoplasmic changes in abscising pedicels of tobacco and tomato flowers. Planta (Berl.). 83: 303-313.

Johnson, B.L., and O. Hall. 1965. Analysis of phylogenic affinities in the Triticinae by protein electrophoresis. American Journal of Botany. 52: 506-513.

Karnovsky, M.J. 1961. Simple methods for "staining" with lead at high pH in electron microscopy. Journal of Biophysical and Biochemical Cytology. 11: 729-732.

Kearney, P.C., and N.E. Tolbert. 1962. Appearance of glycolate

and related products of photosynthesis outside of chloroplasts. Archives of Biochemistry and Biophysics. 98: 164-171.

Keilin, D., and E.F. Hartree. 1954. Reactions of methaemoglobin and catalase with peroxides and hydrogen donors. Nature. 173: 720-723.

Kenten, R.H. 1955. The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase systems. Biochemical Journal. 59: 110-121.

Kiseler, N.A., Shpitzberg, C.L., and B.K. Vainshtein. 1967. Crystallization of catalase in the form of tubes with macromolecular walls. Journal of Molecular Biology. 25: 433-441.

Kun, E., Dechary, J.M., and H.C. Pitot. 1954. The oxidation of glycolic acid by a liver enzyme. Journal of Biological Chemistry. 210-269.

Larsen, P. 1940. Uber Henimung des Strechkungswachstums durch naturlich vorkommende, atherlosliche Stoffe. Planta (Berl.). 30: 160-167.

Lee, E. 1911. The morphology of leaf fall. Annals of Botany. 25: 51-107.

Leinweber, C.L., and W.C. Hall. 1959. Foliar abscission in cotton. 1. Effect of age and defoliants on the respiratory rate of blade, petiole, and tissue of the abscission zone. Botanical Gazette. 120: 144-151.

Lewis, L.N., Palmer, R.L., and H.A. Hield. 1968. Interactions of zuxins, abscission accelerators, and

ethylene in the abscission of citrus fruit. In: Biochemistry and Physiology of Plant Growth Substances, pp. 1303-13. Ed. F. Wightman and G. Setterfield. Ottawa: Runge Press.

Lewis, L.N., and Varner, J.E. 1970. Synthesis of cellulase during abscission of Phaseolus vulgaris leaf explants. Plant Physiology. 46: 194-199.

Lipetz, J., and Garro, A.J. 1965. Ionic effects on lignification and peroxidase in tissue cultures. The Journal of Cell Biology. 25: 109-16.

Lison, L. 1956. Peroxydases. In: Histochimie et Cytochimie Animales. Gauthier-Villars, Paris, 1956: 589.

Loew, O. 1901. In: A. White, P. Handler, and E.L. Smith, eds.; Principles of Biochemistry. 3rd ed. McGraw-Hill, New York (1964). pp. 357.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and R.J. Randall. 1951. Protein measurement with the Folin-phenol reagent. Journal of Biological Chemistry. 193: 265-275.

Luft, J.H. 1961. Improvements in epoxy resin embedding methods. Journal of Biophysical and Biochemical Cytology. 9: 409-414.

Machlis, L., and J.G. Torrey. Plants In Action. W.H. Freeman and Co., San Francisco (1956).

Maehly, A.C., and B. Chance. 1954. The assay of catalase and peroxidases. Methods of Biochemical Analysis, Vol. I, (Glick, D., ed.). Interscience Publishers, Inc., New York (1954). pp. 357.

Maehly, A.C. 1955. Plant Peroxidase. Methods in Enzymology. Vol. 11 (Colowick, S.P., and Kaplan, N.O., eds.) pg. 807, Academic Press, Inc., New York. (1955).

Mahler, H.R., Hubscher, G., and H. Baum. 1955. Studies of uricase. I. Preparation, purification, and properties of a cupro-protein. Journal of Biological Chemistry. 216: 625.

Margoliash, E., and B. Agrawal. 1969. Active site of catalase protein (abstract). Federation Proceedings. 28: 828.

Marinos, N.G. 1967. Multifunctional plastids in the meristematic region of potato tuber buds. Journal of Ultrastructural Research. 17: 91-113.

Millonig, G. 1961. A modified procedure for lead staining of thin sections. Journal of Biophysical and Biochemical Cytology. 11: 736-739.

Mollenhauer, H.H., and C. Totten. 1970. Studies on seeds. V. Microbodies, glyoxysomes, and ricinosomes of castor bean endosperm. Plant Physiology. 46: 794-799.

Morgan, P.W., Beyer, E., Jr., and H.W. Gausman. 1968. Ethylene effects on auxin physiology. In: Biochemistry and Physiology of Plant Growth Substances, pp. 1255-73. Ed. F. Wightman and G. Setterfield. Ottawa: Runge Press.

Morre, D.J. 1968. Cell wall dissolution and enzyme secretion during leaf abscission. Plant Physiology. 43: 1545-1559.

Morikawa, S., and T. Harada. 1968. Immunohistochemical localization of catalase in mammalian tissues. Journal of Histochemistry and Cytochemistry. 17: 30.

Newcomb, E.H. 1967. Fine structure of protein-storing plastids in bean root tips. The Journal of Cell Biology. 33: 143-163.

Novikoff, A.B., and S. Goldfischer. 1968. Visualization of microbodies for light and electron microscopy. Journal of Histochemistry and Cytochemistry. 16: 507.

Novikoff, A.B., and S. Goldfischer. 1969. Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. Journal of Histochemistry and Cytochemistry. 17: 675.

Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Annals of the New York Academy of Science. 121: 321-349.

Osborne, D.J., and M.G. Mullins. 1969. Auxin, ethylene and kinetin in a carrier-protein model system for the polar transport of auxins in petiole segments of Phaseolus vulgaris. New Phytology. 68: 977-91.

Pellegrini, S., and F.M. Gerola. 1969. Chloroplasts with an "opaque body" in basil (Ocymum basilicum L.). Journal of Submicroscopic Cytology. 1: 53-68.

Pilet, P.E., and A.W. Galston. 1957. Auxin destruction, peroxidase activity, and peroxide genesis in the roots of Lens culinaris. Plant Physiology. 8: 888-898.

Ponting, J.D., and M.A. Joslyn. 1948. Ascorbic acid oxidation and browning in apple tissue extracts. Archives of Biochemistry. 19: 47-63.

Poole, B. 1969. Biogenesis and turnover of rat liver peroxisomes. Annals of The New York Academy of Sciences. 168: 229-243.

Ray, R.M., and K.V. Thimann. 1955. Steps in the oxidation of indoleacetic acid. Science. 122: 187-188.

Ridge, I., and D.J. Osborne. 1970. Regulation of peroxidase activity by ethylene in Pisum sativum: requirements for protein and RNA synthesis. Journal of Experimental Botany. 21: 720-734.

Rossman, M.G., and L.W. Labaw. 1967. A comparison of electron microscopy and X-ray diffraction results for ox liver catalase crystals. Journal of Molecular Biology. 29:315.

Rubinstein, B., and F.B. Abeles. 1965. Relationship between ethylene evolution and leaf abscission. Botanical Gazette. 126: 255-259.

Sabatini, D.C., Bensch, D., and R.J. Barrnett. 1963. Cytochemistry and electron microscopy: the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. The Journal of Cell Biology. 17: 19-58.

Schonbein, C.F. 1855. In: A. White, P. Handler, and E.L. Smith, eds. Principles of Biochemistry. 3rd ed. McGraw-Hill, New York (1964). pp. 357.

Schwertner, H.A., and P.W. Morgan. 1966. Role of IAA-oxidase in abscission control in cotton. Plant Physiology. 41: 1513-1519.

Scott, P.C., and A.C. Leopold. 1966. Abscission as a mobilization phenomenon. Plant Physiology. 26: 189-91.

Seligman, A.M., Plapinger, R.E., Wasserkrug, H.L., and J.S. Hanker. 1967. Ultrastructural demonstration of cytochrome oxidase activity by the Nadi reaction with osmiophilic reagents. Journal of Cell Biology. 34: 787.

Seligman, A.M., Karnovsky, M.J., Wasserkrug, H.L., and J.S. Hanker. 1968. Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reabent, diaminobenzidine (DAB). The Journal of Cell Biology. 38: 1-14.

Shannon, L.N., Kay, E., and J.Y. Lew. 1966. Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. Journal of Biological Chemistry. 241: 2166.

Sheen, S.J. 1970. Peroxidases in the genus Nicotiana. Theoretical Applied Genetics. 40: 18-25.

Sheen, S.J., and G.R. Rebagay. 1970. On the localization and tissue difference of peroxidases in Nicotiana tabacum and its progenitor species. The Botanical Gazette. 131: 297-304.

Sherwood, S.B., Evans, J.O., and C. Ross. 1971. Gel electrophoresis studies of proteins from leaves of photoperiod-

ically induced and begetative cocklebur plants. Plant and Cell Physiology. 12: 111-116.

Siegel, S.M. 1953. On the biosynthesis of lignin. Physiologia Plantarum. 6: 134-39.

Siegel, S.M., and A.W. Galston. 1955. Peroxide genesis in plant tissues and its relation to indoleacetic acid destruction. Archives of Biochemistry and Biophysics. 54: 102-113.

Smith, H.H., Hamill, D.E., Weaver, E.A., and K.H. Thompson. 1970. Multiple molecular forms of peroxidases and esterases among Nicotiana species and amphiploids. Journal of Heredity. 61: 203-212.

Srivastava, L.M. 1966. On the fine structure of the cambium of Fraxinux americana L. The Journal of Cell Biology. 31: 79-93.

Stafford, H.A., and A.W. Galston. 1970. Ontongeny and hormonal control of polyphenoloxidase isozymes in tobacco pith. Plant Physiology. 46: 7630767.

Stempack, J.G., and R.T. Ward. 1964. An improved staining method for electron microscopy. Journal of Cell Biology. 22: 697-701.

Stetler, D.A., and W.M. Laetsch. 1969. Chloroplast development in Nicotiana tabacum "Maryland Mammoth." American Journal of Botany. 56: 260-270.

Stutz, R.E. 1957. The indole-3-acetic acid oxidase of Lupinus albus L. Plant Physiology. 32: 31-39.

Sumner, J.B., and N. Gralen. 1938. The molecular weight of crystalline catalase. Journal of Biological Chemistry. 125: 33-36.

Sund, H., Weber, K., and E. Tolbert. 1967. Dissoziation der Rinderleber-Katalase in iher Untereinheiten. European Journal of Biochemistry. 1: 400-410.

Tanford, D., and R. Lovrien. 1962. Dissociation of catalase into subunits. Journal of the American Chemical Society. 84: 1892-1896.

Tang, Y.W., and J. Bonner. 1947. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Archives of Biochemistry. 13: 11-25.

Thenard, L.J. 1818. In: A. White, P. Handler, and E.L. Smith, eds., Principles of Biochemistry. 3rd ed. McGraw-Hill, New York (1964). pp. 357.

Thimann, K.V. 1934. Studies on the growth hormone in plants. VI. The dissociation of the growth substance in plant tissues. Journal of General Chysiology. 18: 23-34.

Thornton, R.M., and K.V. Thimann. 1964. On a crystal-containing body in cells of the oat coleoptile. Journal of Cell Biology. 20: 345-350.

Tolbert, N.E., Oeser, A., Kisake, T., Hageman, R.H., and R.K. Yamazaki. 1968. Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. Journal of Biological Chemistry. 243: 5179-5184.

Tolbert, N.E., and R.K. Yamazaki. 1969. Leaf peroxisomes and their relation to photorespiration and photosynthesis. Annals of The New York Academy of Sciences. 168: 244-264.

Tomaszewska, E. 1964. Phenols and auxin as internal factors controlling leaf abscission. Bulletin of The Academy of Political Science, Ser. Science Biology. 12: 541-45.

Valdovinos, J.G., Ernest, L.C., and E.W. Henry. 1967. Effect of ethylene and gibberellic acid on auxin synthesis in plant tissue. Plant Physiology. 42: 1803-1806.

Valdovinos, J.G., and T.E. Jensen. 1968. Fine structure of abscission zones. II. Cell-wall changes in abscising pedicels of tobacco and tomato flowers. Planta (Berl.). 83: 295-302.

Valdovinos, J.G., Jensen, T.E., and L.M. Sicko. 1971. Ethylene-induced rough endoplasmic reticulum in abscission cells. Plant Physiology. 47: 162-163.

Valdovinos, J.G., Jensen, T.E., and L.M. Sicko. 1972. Fine Structure of Abscission Zones IV. Effects of ethylene on the ultrastructure of abscission cells of tobacco flower pedicels. Planta (Berl.). 102: 324-333.

Van Fleet, D.S. 1942. The significance of oxidation in the endodermis. American Journal of Botany. 29: 747-755.

van Loon, L.C. 1970. Tobacco polyphenoloxidases: a specific staining method indicating non-identity with peroxidases. Phytochemistry. 10: 503-507.

Vaughn, J.G., Waite, A., Boulter, D., and S. Waiters. 1966. Comparative studies of the seed proteins of Brassica campestris, Brassica oleracea, and Brassica nigra. Journal of Experimental Botany. 17: 332-343.

Vigil, E.L. 1969. Intracellular localization of catalase (peroxidative) activity in plant microbodies. Journal of Histochemistry and Cytochemistry. 17: 425-428.

Vigil, E.L. 1970. Cytochemical and developmental changes in microbodies (glyoxysomes) and related organelles of castor bean endosperm. Journal of Cell Biology. 46: 435.

Wagenknecht, A.C., and R.H. Burris. 1950. Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. Archives of Biochemistry. 25: 30-53.

Watson, M.L. 1958. Staining of tissue sections for electron microscopy with heavy metals. Journal of Biophysical and Biochemical Cytology. 4: 475-478.

Weiryb, I. 1966. The behavior of horseradish peroxidase at high hydrogen peroxide concentrations. Biochemistry. 5: 2003.

White, A., Handler, P., and E.L. Smith. Principles of Biochemistry. 3rd ed. McGraw-Hill, New York (1964).

Whitmore, F.W. 1971. Effect of indoleacetic acid and hydroxyproline on isozymes of peroxidase in wheat coleoptiles. Plant Physiology. 47: 169-171.

Wolff, J., and E. de Stoecklin. 1910. The peroxidase properties of oxyhemoglobin. Comptes Rendus Academy of

Science. Paris. Series D.

Yager, R.E. 1957. The mechanism of floral abscission in Nicotiana. Doctoral Dissertation.

Yamazaki, I., and H. Souzu. 1960. The mechanism of indole-acetic acid oxidase reaction catalyzed by turnip peroxidase. Archives of Biochemistry and Biophysics. 86: 294-301.

Yonetani, T. 1967. Studies on cytochrome c peroxidase. X. Crystalline apo-and reconstituted holoenzymes. Journal of Biological Chemistry. 242: 5008-5013.

APPENDIX A

ELECTRON MICROSCOPIC, ELECTROPHORETIC, AND SPECTROPHOTOMETRIC ASSAY PROCEDURES

Schedule I. Glutaraldehyde Fixation

1. Phosphate buffer, pH 7.2

   0.1M KH_2PO_4 13 ml.

   0.1M Na_2HPO_4 37 ml.

2. One and one-half ml. of 50% glutaraldehyde solution is added to 23.5 ml. of phosphate buffer, pH 7.2 to give a final solution of 3% glutaraldehyde.
3. Plant tissue was collected and immediately immersed in 3% glutaraldehyde solution for 1 hour at 4 degrees C.
4. Two mm tissue sections were sectioned into four smaller pieces and rinsed six separate times with 0.1M phosphate buffer, pH 7.2, in order to completely remove all of the glutaraldehyde from the tissue.

Schedule II. Osmium Fixation

1. Two percent osmium tetroxide was prepared by diluting a 4% osmium tetroxide solution one to one with 0.1M phosphate buffer, pH 7.2.

2. The tissue sections were incubated in this solution for 1 hour at 4 degrees C.

Schedule III. Dehydration

1. The tissue sections were removed from the 2% osmium tetroxide solution and put into 50% ethanol for 5 minutes.
2. The tissue sections were subsequently incubated as follows:

Time	Incubation Medium
5 minutes	70% ethanol
5 minutes	95% ethanol
5 minutes	100% ethanol
5 minutes	100% ethanol
5 minutes	100% ethanol
5 minutes	propylene oxide (Do not use near open flame--use when cold)
5 minutes	propylene oxide
5 minutes	propylene oxide

Schedule IV. Epon Embedding

1. Preparation of mixture A:

   62 cc of Epon 812

   100 cc of dodecanyl anhydride

2. Preparation of mixture B:

   100 cc of Epon 812

   89 cc of nadic methyl anhydride

3. Mix 3 parts A with 2 parts B and stir thoroughly.

4. Add 0.2 ml of dimethylaminophenol (DMP-30) per 10 ml of Epon mixture.

5. Mix thouroughly, preferably in a room with very low humidity and store at 4 degrees C, until ready for use.

Schedule V. Diaminobenzidine (DAB) Staining Procedure

1. Diaminobenzidine medium:

   10 mg of 3,3'-diaminobenzidine tetrachloride

   0.1 ml of 3% 2-amino-2-methyl-1,3-propandiol buffer, pH 9.0 or pH 6.0 for incubations at the lower pH.

2. The 0.1 ml of 3% hydrogen peroxide is added just before tissue sections are to be incubated.

3. Tissue sections are incubated at 37 degrees C for 1 hour.

4. Other tissue sections were incubated in the following mediums:

   a) Complete DAB medium without hydrogen peroxide

   b) Complete medium without DAB

   c) Complete DAB medium containing 0.02M 3-amino-1,2,4-triazole

   d) Complete DAB medium containing 0.02M potassium cyanide

5. After incubation, the tissue sections were carried through the osmium tetroxide fixation, dehydration,

and Epon embedding procedures.

Schedule VI. Spectrophotometric Assay for Peroxidase

1. Standard cuvette solution:

   1.3 ml of 0.1N hydrogen peroxide

   1.3 ml of 0.5% guaiacol

   0.8 ml of distilled water

2. Experimental cuvette:

   1.3 ml of 0.1N hydrogen peroxide

   1.3 ml of 0.5% guaiacol

   0.8 ml of enzyme supernatant solution

3. Hydrogen peroxide is added just before optical density readings are to be made.

4. Optical density readings are performed at 460 nm with color development measured at 5 second intervals for 3 minutes per individual enzyme sample.

Schedule VII. Protein Determination

1. Reagent A:

   2 percent Na_2CO_3 in 0.1N NaOH

2. Reagent B:

   0.5 percent $CuSO_4.5H_2O$ in 1 percent sodium or potassium tartrate.

3. Reagent C:

   Alkaline copper solution-mix 50 ml of reagent A with 1 ml of reagent B. Discard after 1 day.

4. Reagent E:

   1N Folin reagent

5. Standard Curve:

   0.2 ml of bovine serum albumin (prepared in dilutions from 10 to 350 gamma at 10 gamma intervals)

   1.0 ml of reagent C

   Mix and let stand 10 minutes at room temperature

6. Add 0.1 ml of reagent E (1N Folin) and mix rapidly in 2 seconds and let stand for 30 minutes

7. The standard curve for bovine serum albumin was made over a range from 10 to 350 gamma of protein.

Experimental Protein Determination

1. Cuvette contains 0.2 ml of enzyme supernatant solution plus 1.0 ml of reagent C. Mix and let stand for 10 minutes at room temperature.

2. Add 0.1 ml of reagent E (1N Folin), mix rapidly in 3 seconds and let stand for 30 minutes. Sample is ready to be read in spectrophotometer.

3. Blank cuvette contains 0.2 ml of distilled water instead of the enzyme supernatant solution.

4. Color change is read at 750 nm on a Beckman model DU spectrophotometer.

5. The optical density readings from the sample cuvette was compared to the standard bovine serum albumin curve to give protein concentrations in terms of mg of protein per gram fresh weight of tissue.

Schedule VIII. Electrophoresis

1. Solution A:

1N HCl	24 ml
TRIS	18.15 gm
TEMED	0.12 ml

Add distilled water to 50 ml.

2. Solution B:

1N HCl	24 ml
TRIS	2.99 gm
TEMED	0.23 ml

Add distilled water to 50 ml.

3. Solution C:

Acrylamide	5 gm
BIS	0.364 gm

Add distilled water to 50 ml.

4. Solution D:

Acrylamide	5 gm
BIS	1.25 gm

Add distilled water to 50 ml.

5. Solution E:

Riboflavin	2 mg

Add distilled water to 50 ml.

6. Solution F:

Sucrose	8 gm

Add distilled water to 20 ml.

7. Solution of ammonium persulfate:

0.14 gm of ammonium persulfate with additional distilled

water to 100 ml.

8. Separating Gel:

Solution A	1 part
Solution C	2 parts
Distilled water	1 part
Ammonium persulfate solution	4 parts

9. Spacing Gel:

Solution B	1 part
Solution D	2 parts
Solution E	1 part
Solution F	4 parts

10. Tracking Dye:

5 mg bromophenol plus distilled water to 100 ml

11. Gel Stains: O-dianisidine

Ortho-dianisidine (3,3'-dimethoxybenidine)	250 mg
95% ethyl alcohol	140 mg
Acetate buffer, pH 4.7 (0.88M sodium acetate and 0.62M glacial acetic acid)	20 ml

36 ml of distilled water

Add 5 ml of 3% hydrogen peroxide just before immersing gels in stain for 20 minutes. Transfer gels to distilled water.

12. Buffer, pH 8.5

TRIS	6.0 gm
Glycine	28.9 gm

Add distilled water to 2 liters. Adjust to pH 8.5.

Schedule IX. Spectrophotometric Peroxidase Assay

1. The reaction involves the oxidation of guaiacol in the presence of hydrogen peroxide.
2. The increase in intensity of the brown reaction product can be followed in a spectrophotometer at 460 nm.
3. 1.3 ml of 0.1N hydrogen peroxide is added to the cuvette containing the enzyme supernatant and optical density readings are taken at 5 second intervals.
4. Enzyme activity is expressed in terms of the increase in absorbance units per ten seconds over the linear portion of the reaction curve on a gram per fresh weight basis. The linear portion of the respective reaction curves was chosen between 70 and 150 seconds.

EXPLANATION OF FIGURES

CH-----Chloroplast

CW-----Cell wall

CY-----Cytoplasm

ER-----Endoplasmic reticulum

GC-----Golgi cisternae

GO-----Golgi apparatus

GR-----Granular component

GV-----Golgi vesicle

IN-----Intercellular space

MB-----Microbody

MI-----Mitochondrion

ML-----Middle lamella

MT-----Microtubule

NL-----Nucleolus

NM-----Nuclear membrane

NU-----Nucleus

PF-----Phyto-ferritin

PL-----Plasmodesma

PM-----Plasma membrane

RER----Rough endoplasmic reticulum

RI-----Ribosome

SB-----Starch body

TH-----Thylakoid

VA-----Vacuole

Fig. 12. A light microscopic view of tissues comprising the region of the pedicel where separation occurs during abscission. An indentation of epidermal tissue deliniates the abscission zone. The separation processes that take place during abscission occur between cells, 6-8 tiers in a distal direction with respect to the center of the abscission zone. The cortical cells of the abscission layer are smaller in size than cortical cells on either side of the layer. Non-ethylene and non-diaminobenzidine-treated control tissue, fixed in 3% glutaraldehyde in phosphate buffer, pH 7.2 and stained with osmium tetroxide. Approximately X3,000.

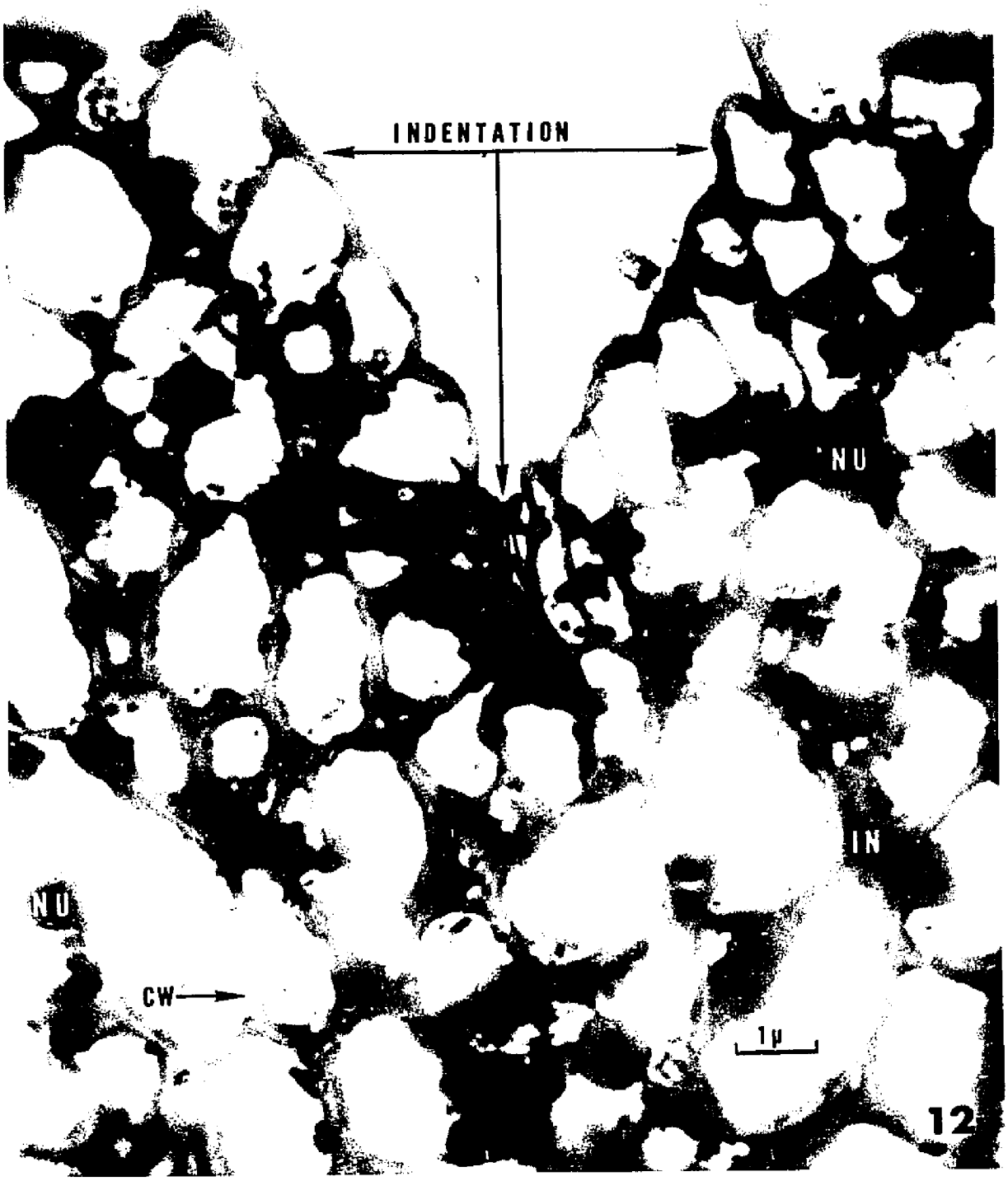

INDENTATION
NU
IN
NU
CW
1μ
12

Fig. 13. A light microscopic view of abscission zone cortical tissue showing the area of separation (arrows) with cortical cells on either side of the separation area. Three hour ethylene-treated pedicel tissue, fixed in 3% glutaraldehyde (phosphate buffer, pH 7.2) and stained with osmium. Approximately X2,500.

Fig. 14. Control cortical tissue fixed in paraformaldehyde-osmium tetroxide and incubated in diaminobenzidine. This non-abscising tissue shows peroxidase reactive sites throughout the cell wall (CW) area, with particularly dense concentrations in the intercellular spaces of the cell walls. The mitochondria (MI) do not show cytochrome oxidase staining sites. The single membrane-bound granular component (GR) of the chloroplast (CH) show positive diaminobenzidine staining for peroxidase. No post-staining. Approximately X25,000.

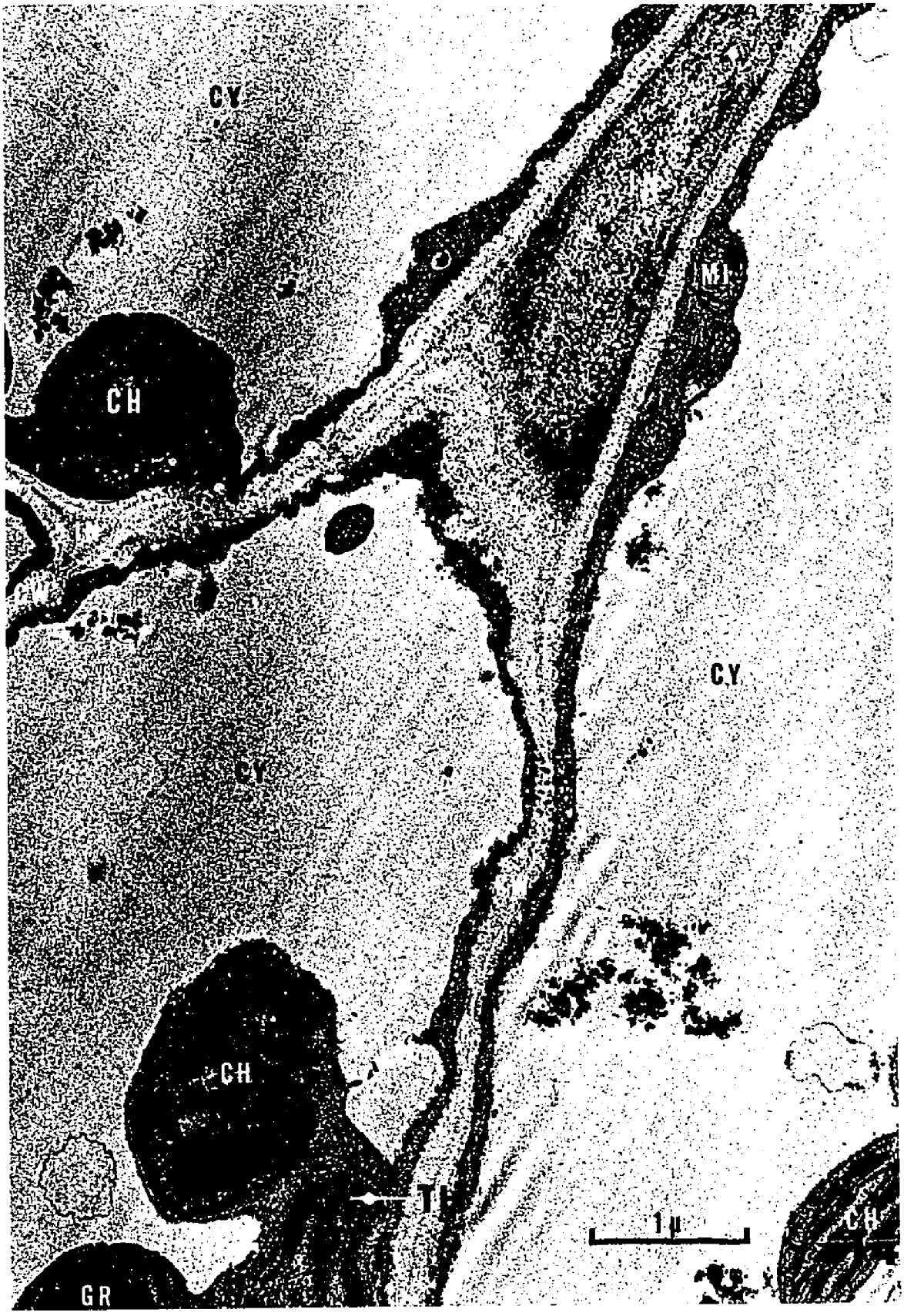

CY
CH
MI
CY
CY
CH
T
GR
1 μ
CH

Fig. 15. Diaminobenzidine stained cortical tissue pre-treated with 5 µl/l of ethylene for three hours. The peroxide reaction product is particularly dense in the intercellular spaces (IN) of the cell walls (CW). The mitochondrial membranes (MI) do not stain for cytochrome oxidase. The endoplasmic reticulum (ER) is not appreciably stained for peroxidase. Two dividing chloroplasts (CH) contain thylakoids (TH) that appear to be moderately dense. The cytoplasm (CY) does not stain for peroxidase. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X51,000.


CY
TH

Fig. 16. A portion of cortical tissue treated for three hours with 5µl/l of ethylene prior to being incubated in diaminobenzidine. The peroxidase reaction product is spread throughout the cell wall (CW) area with heavy granular concentrations in the middle lamellar (ML) region of the cell wall. Mitochondria (MI) are located in the cytoplasm (CY) near the cell wall but do not stain for cytochrome oxidase in either the inner or outer membranes as the tissue was incubated at the usual alkaline pH of 9.0 Osmium tetroxide fixation without uranyl acetate or lead citrate post-staining. Approximately X55,500.

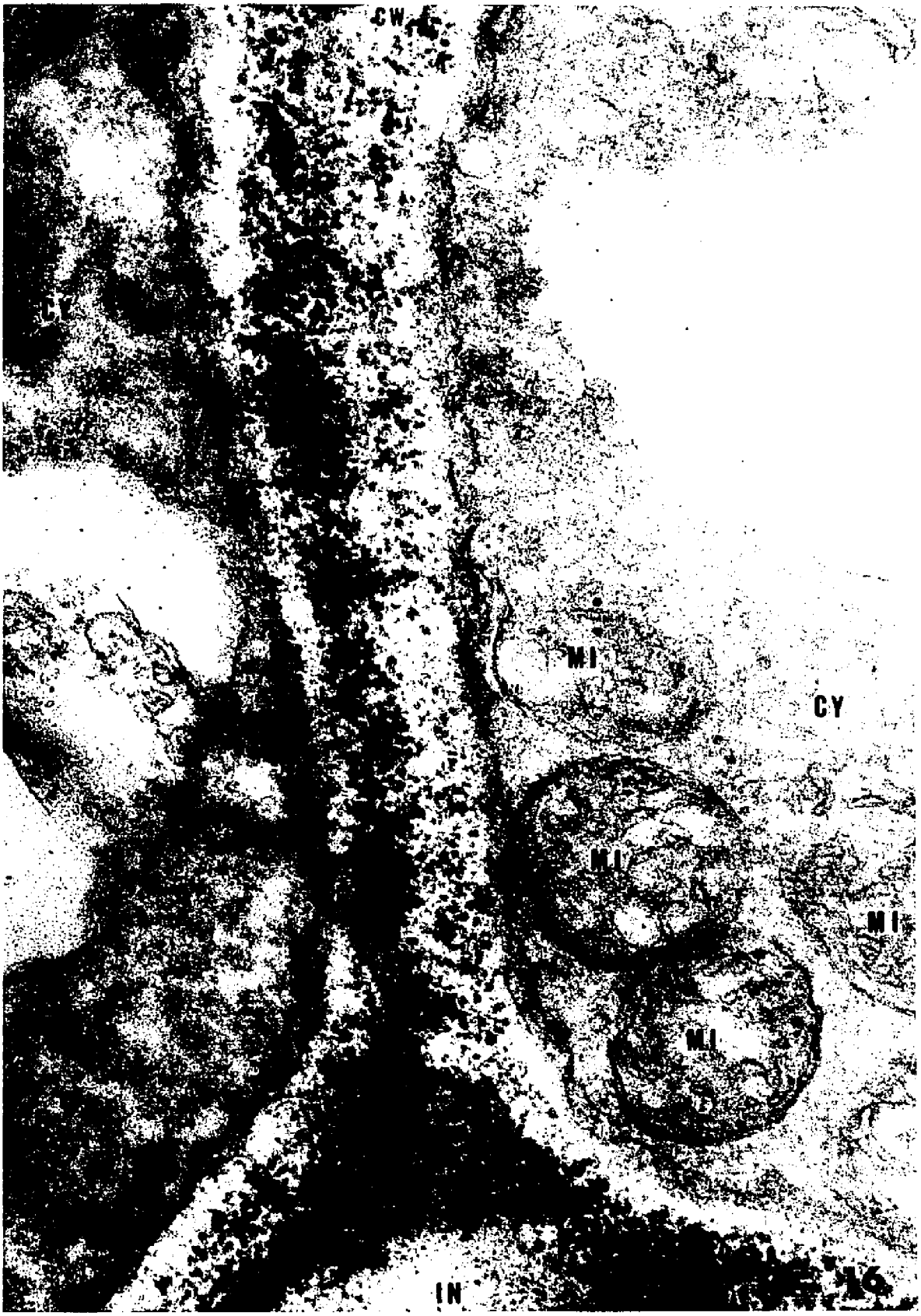

CW
MI
CY
MI
MI
MI
IN

Fig. 17. Five hour ethylene-treated cortical tissue shows intense peroxidase reaction product in the abscising cell. The peroxidase stain is concentrated in the middle lamellar (ML) area of the cell wall (CW) undergoing dissolution as abscission occurs. The plasma membrane (PM) is still intact and does not show peroxidase reaction sites. The mitochondrial membranes (MI) do not show positive reaction sites for cytochrome oxidase activity. The cytoplasm (CY) does not show evidense of peroxidase staining sites. Osmium tetroxide fixation without post-staining. Approximately X63,000.

Fig. 18. Control tissue incubated in diaminobenzidine with 0.02M potassium cyanide. The chloroplast (CH) contains a single membrane-bound granular component (GR) that is not appreciably inhibited by potassium cyanide. Several thylakoids (TH) of the chloroplast are situated close to the single membrane-bound granular component (GR). The thylakoid (TH) membranes appear to be continuous with the single membrane surrounding the granular component. Only a portion of the cell wall (CW) is visible due to section angle. The cytoplasm does not stain for peroxidase. Potassium cyanide does not completely inhibit the formation of peroxidase reaction product in the cell wall (CW). Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X71,500.


TH
GR
CH
PM

Fig. 19A. Cortical tissue of the abscission zone treated with 5µl/l of ethylene for three hours, followed by incubation in diaminobenzidine with 0.02M potassium cyanide. The cell wall (CW) area staining for peroxidase is not appreciably inhibited by potassium cyanide. The nucleus (NU) and nuclear membrane (NM) are visible in the cell. The mitochondria (MI) in the cytoplasm (CY) do not stain for cytochrome oxidase. A vacuole (VA) is also present in the cell. Osmium tetroxide fixation with no post-staining. Approximately X67,500.

Fig. 19B. Cortical tissue of the abscission zone treated with 5µl/l of ethylene for five hours, followed by incubation in diaminobenzidine with 0.02M potassium cyanide. Potassium cyanide does not appreciably inhibit cell wall (CW) staining for peroxidase. A chloroplast (CH), microbody (MB), and rough endoplasmic reticulum (RER) are present in the cytoplasm (CY). The rough endoplasmic reticulum is partially inhibited by potassium cyanide. The plasma membrane (PM) is visible along the cell wall (CW). The microbody (MB) does not show appreciable staining in the presence of potassium cyanide. Osmium tetroxide fixation. Uranyl acetate post-staining. Approximately X67,540.


CM
M1
VA

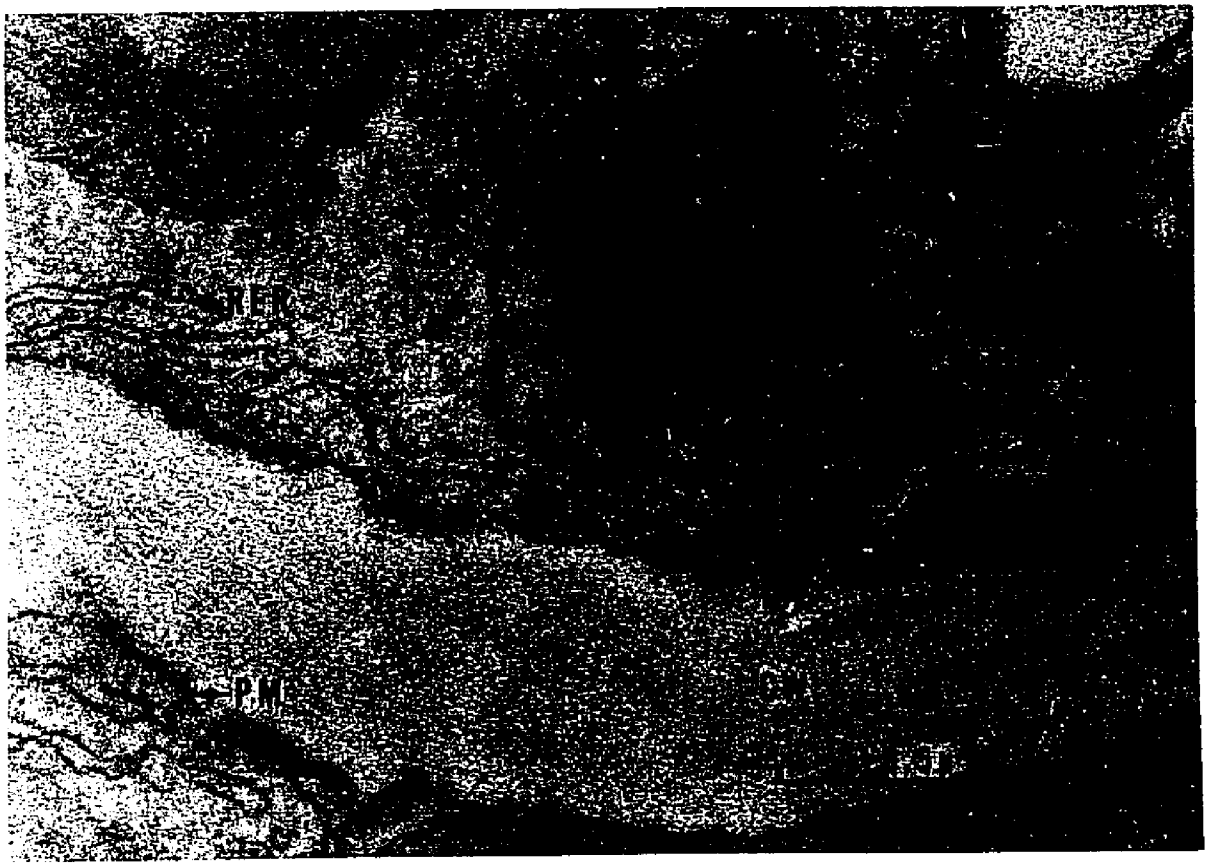
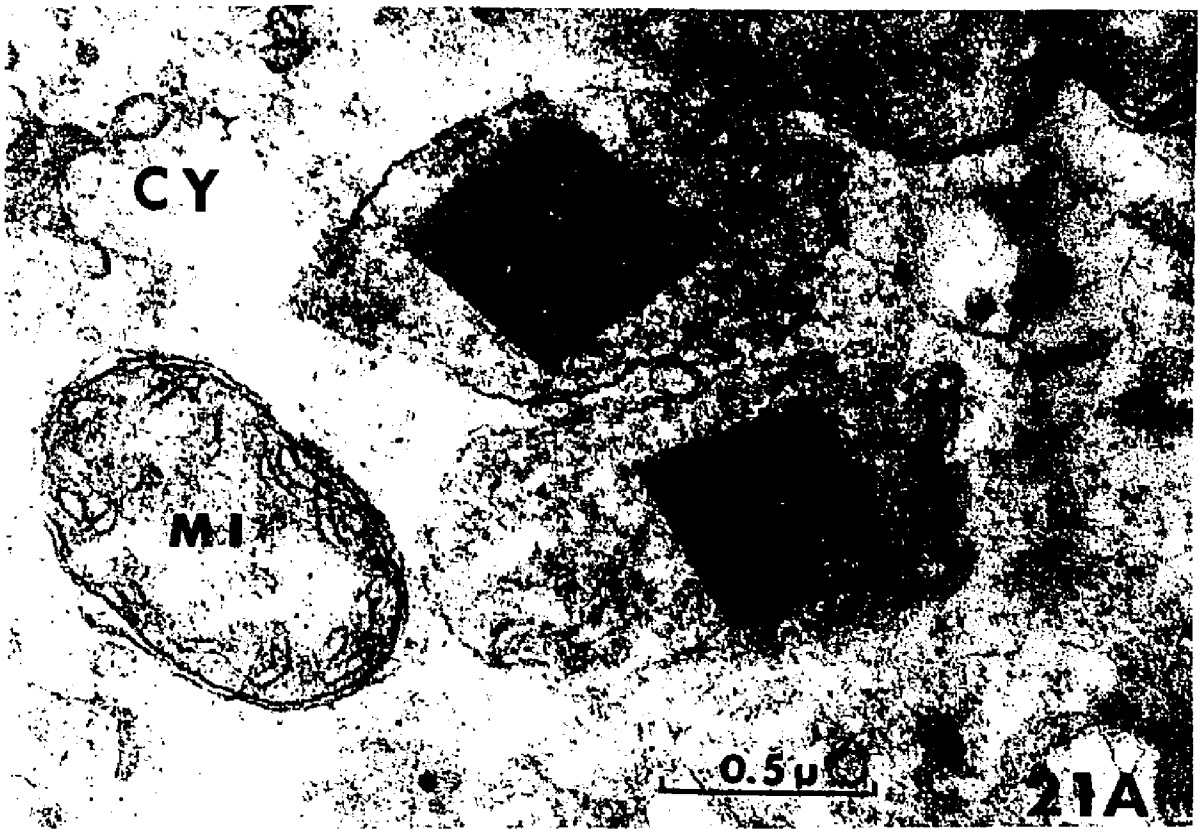

PM

Fig. 20. Diaminobenzidine-stained portion of non-abscising control cortical tissue demonstrating intense positive diaminobenzidine reaction product within the granular component (GR) of the chloroplast (CH) and within the microbodies (MB). The chloroplast (CH) contains thylakoids (TH) which are less dense than the granular component (GR). The microbody cores (MB) show granularity and the cytoplasm (CY) has a granular consistency. The mitochondrial membranes (MI) do not stain positive for cytochrome oxidase. The cell wall (CW) shows a small amount of peroxidase reaction product. Phyto-ferritin granules (PF) are situated within the matrix of the chloroplast (CH). Osmium tetroxide fixation without post-staining. Approximately X43,500.


TH
GR
PF
MB
GY
ML
CW
MB
MB
20

Fig. 21A. A portion of control cortical cell tissue incubated in diaminobenzidine medium contianing 0.02M 3-amino-1,2,4-triazole. The two closely oppressed microbodies (MB) demonstrate the lack of positive diaminobenzidine staining which indicates suppression of catalase activity. The microbody cores (MB) appear dark and the lack of granularity is indicative of the negative diaminobenzidine reaction. The membranes of the microbodies (MB) are incomplete and this condition is due to section angle. Mitochondria (MI) situated in the cytoplasm (CY) do not stain for cytochrome oxidase. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X61,000.

Fig. 21B. Control cortical tissue incubated in diaminobenzidine with 3-amino-1,2,4-triazole. The microbody (MB) within the cytoplasm (CY) is almost completely inhibited for catalase activity in the presence of aminotriazole. A chloroplast (CH) is visible with thylakoids (TH), a starch body (SB), and granules of phyto-ferritin (PF) within the matrix portion of the chloroplast. Osmium tetroxide fixation with no post-staining. Approximately X61,000.


CY
MI
0.5μ
21A

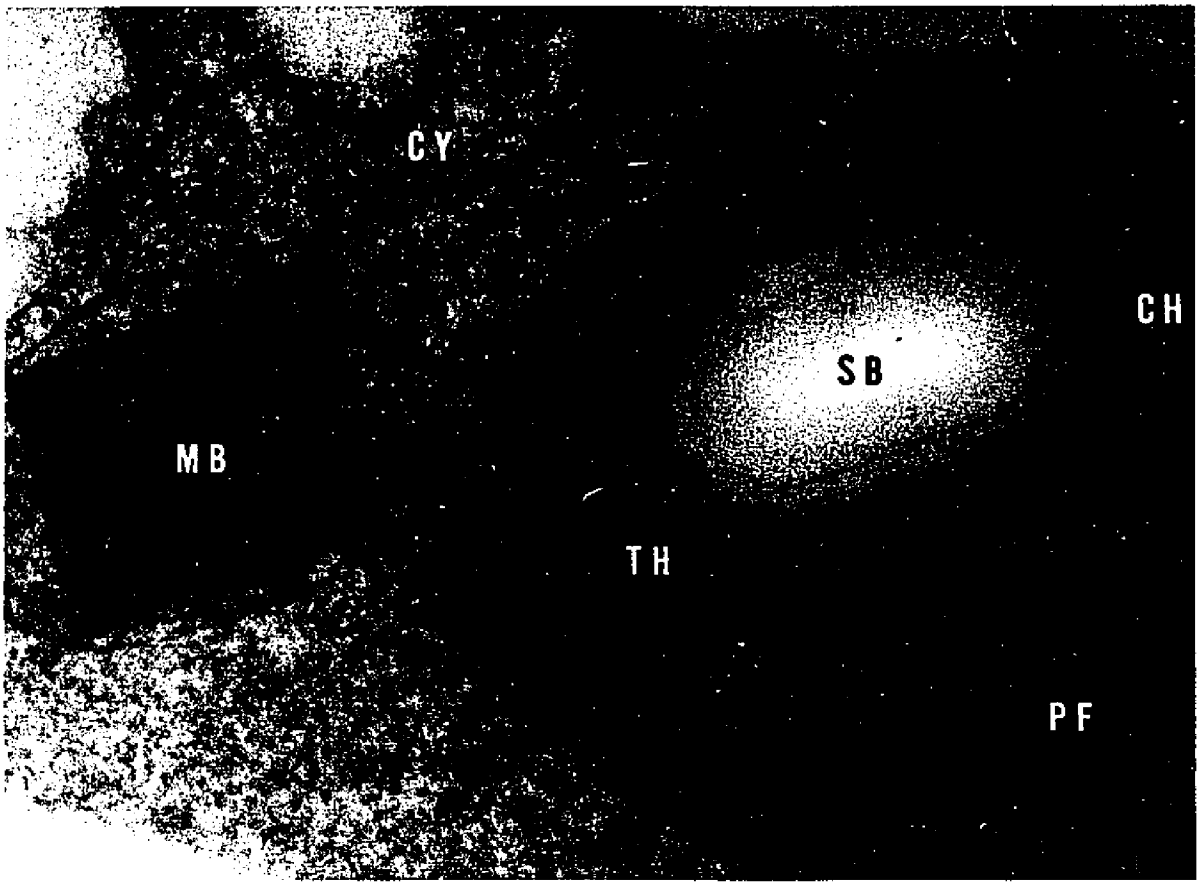
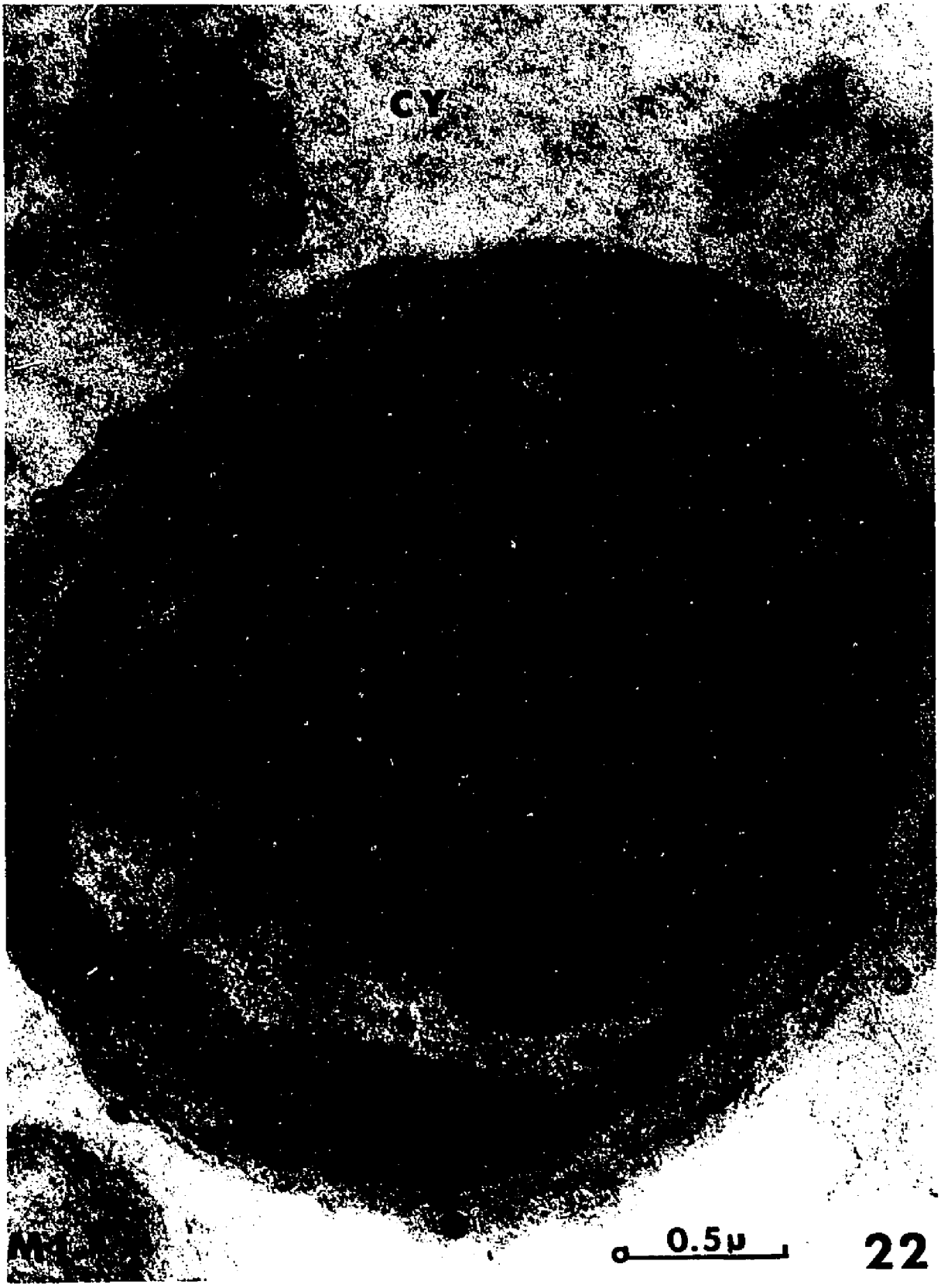

CY
CH
SB
MB
TH
PF

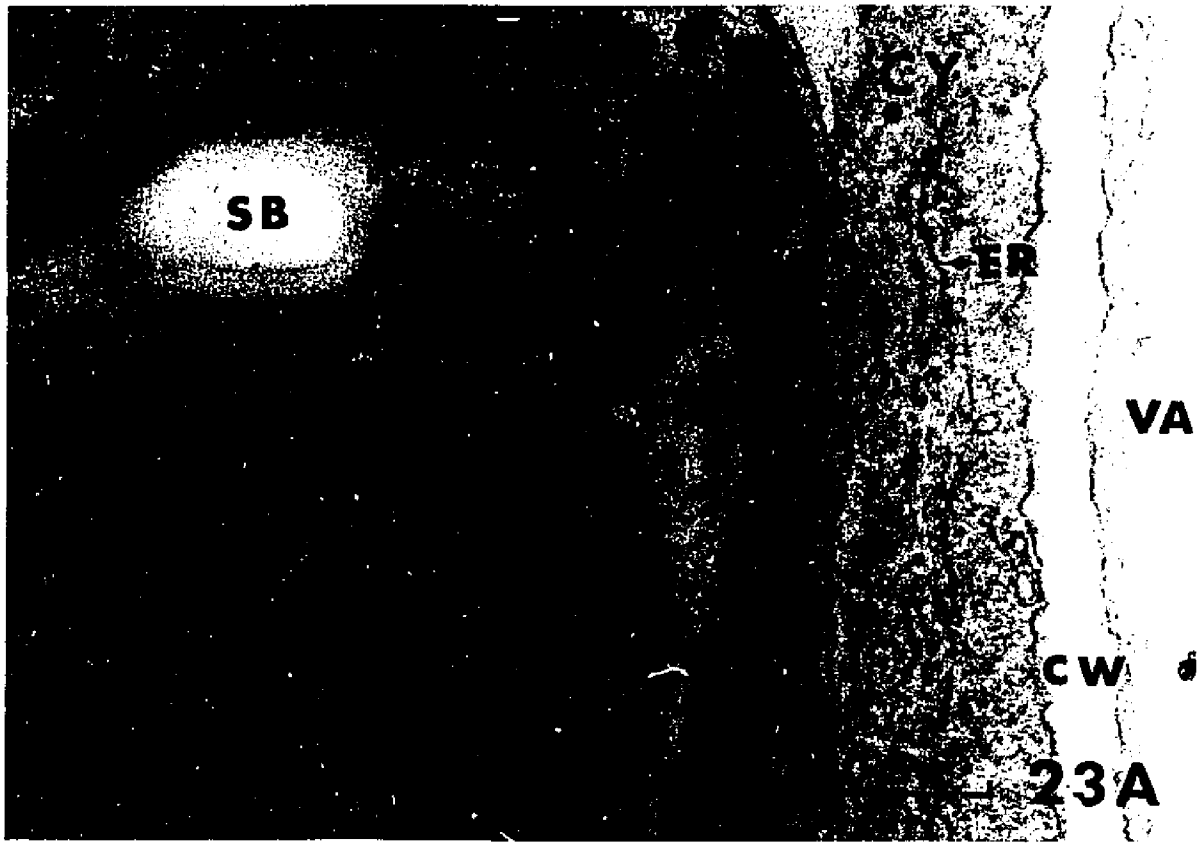
Fig. 22. Control cortical tissue incubated in diaminobenzidine with 0.02M potassium cyanide. The simgle membrane-bound granular component (GR) of the chloroplast (CH) is not appreciably inhibited for peroxidase staining reaction. Thylakoids (TH) and osmiophilic droplets (OD) are present in the matrix of the chloroplast. Mitochondria (MI) are present in the cytoplasm (CY) and do not stain positively for cytochrome oxidase. Osmium tetroxide fixation with no post-staining. Approximately X70,000.



22

Fig. 23A. A portion of control cortical tissue, incubated in complete medium minus diaminobenzidine, shows the absence of peroxidase staining reaction product within the single membrane-bound granular component (GR) of the chloroplast (CH). A starch body (SB), thylakoids (TH), and osmiophilic droplets (OD) are present in the matrix portion of the chloroplast. Segments of endoplasmic reticulum (ER) are present in the cytoplasm (CY) in the vicinity of the cell wall (CW). A vacuole (VA) is also present in the cytoplasm (CY) of the cell. Osmium tetroxide fixation with no post-staining. Approximately 61,000.

Fig. 23B. Control cortical tissue incubated in diamino-benzidine. The single membrane-bound granular component (GR) of the chloroplast (CH) stains weakly for peroxidase. The single membrane is not visible over the entire circum-ference of the granular component (GR) due to section angle. Granular deposits of phyto-ferritin (PF) and thylakoids (TH) are present in the matrix of the chloroplast. Moderate peroxidase staining occurs throughout the cell wall (CW) area. Mitochondria (MI) are present in the cytoplasm (CY). A small microbody (MB) is present near a chloroplast (CH) and stains positive for catalase. Osmium tetroxide fixation with uranyl acetate and lead citrate post-staining. Approx-imately X61,000.


SB
CY
ER
VA
CW
23A

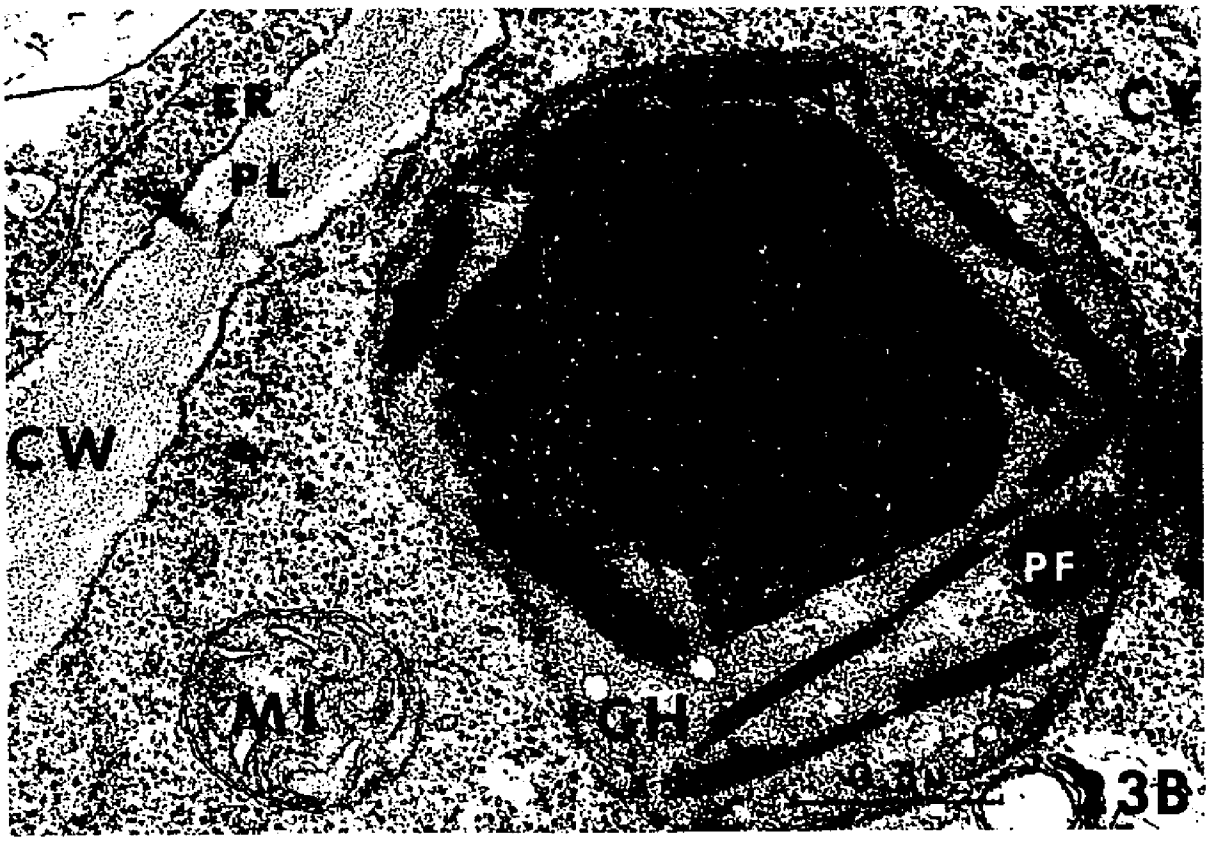

ER
PL
CW
CW
PF
MI
GH
3B

Fig. 24. Cortical tissue treated with 5µl/l of ethylene for five hours prior to incubation in diaminobenzidine. The rough endoplasmic reticulum (RER) stains positive for peroxidase activity along the length of the segments. Concentrations of peroxidase reaction product are present in the cell wall (CW) and intercellular spaces (IN) of the cell walls. The mitochondrial membranes (MI) do not stain positive for cytochrome oxidase. Rough endoplasmic reticulum (RER) and Golgi (GO) stain positive for peroxidase. A nucleus (NU), nucleolus (NL) and double nuclear membrane (NM) do not show positive peroxidase reaction product. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X27,000.

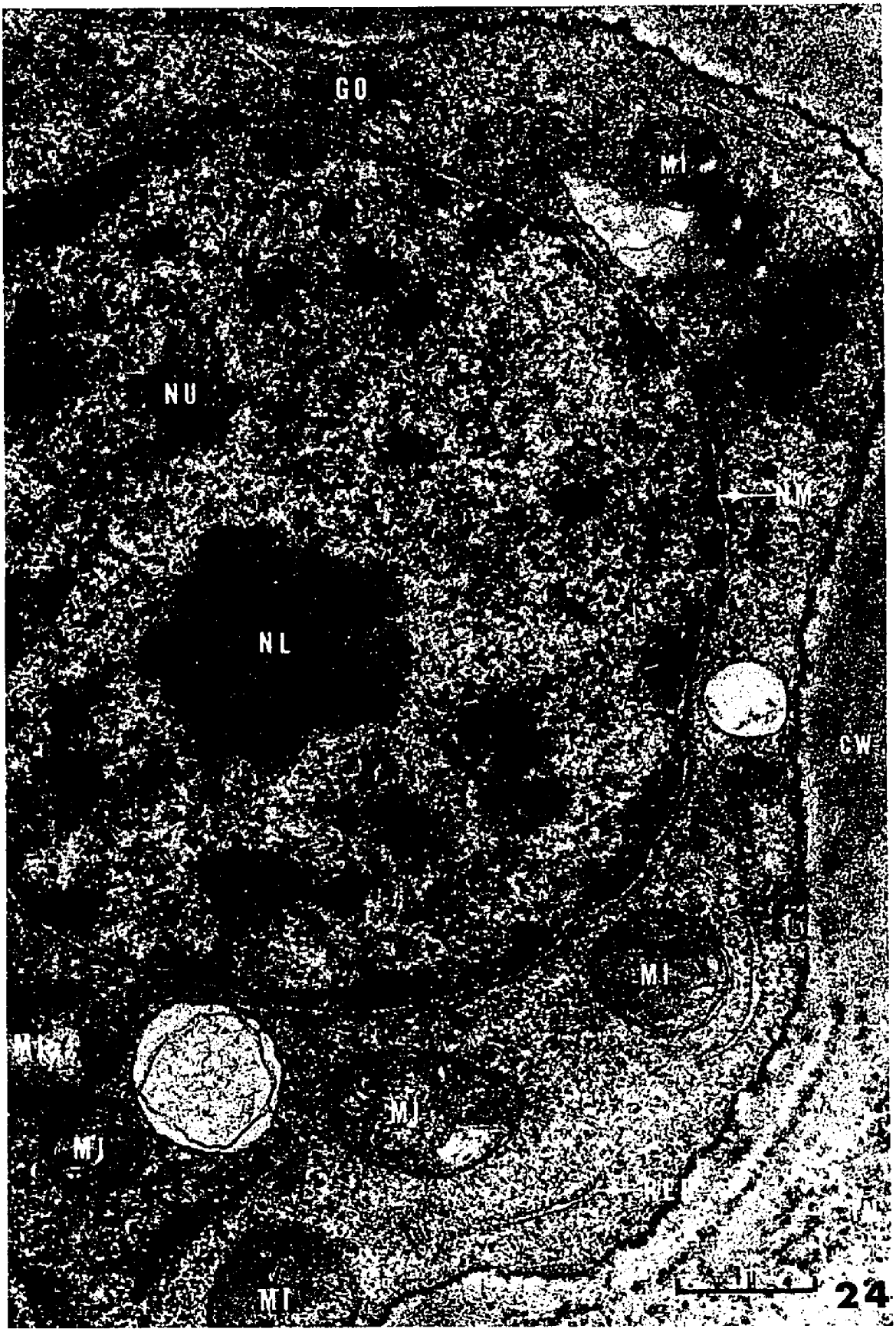
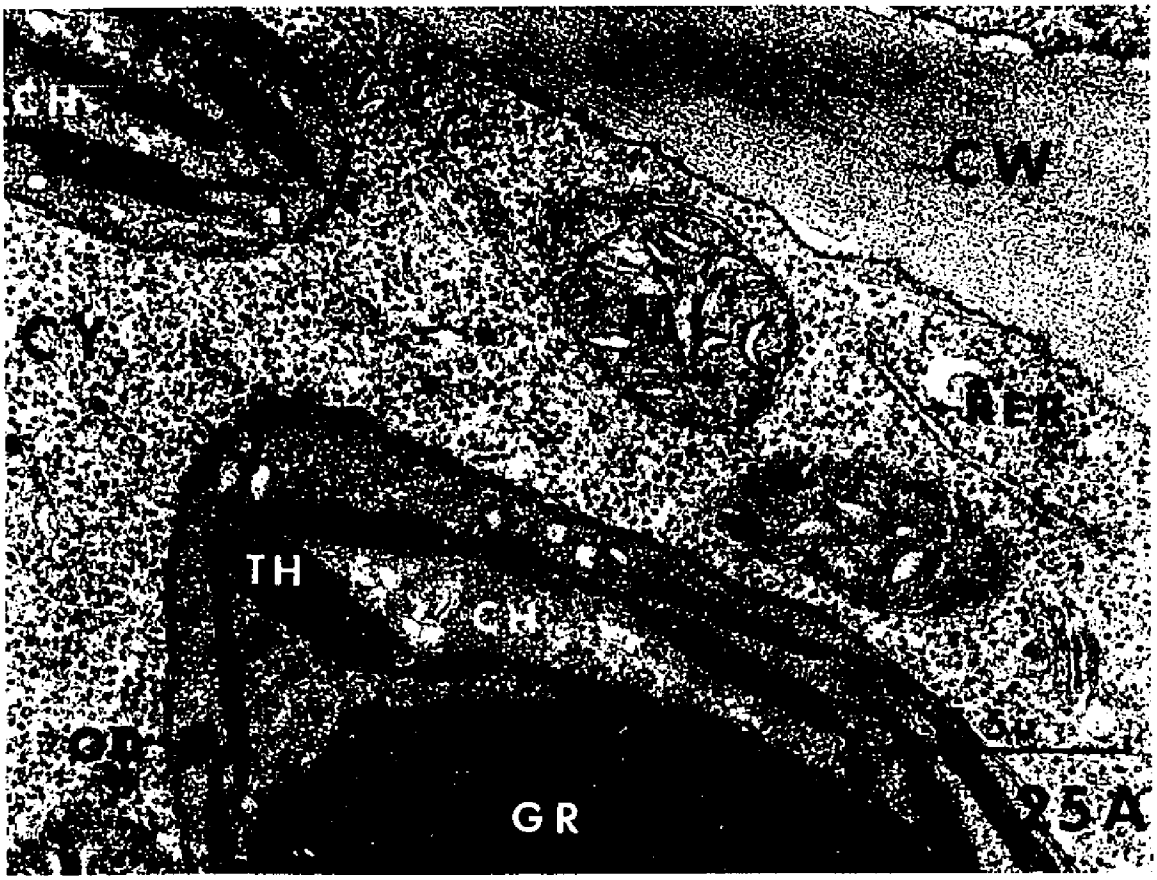

GO
MI
NU
NM
NL
CW
MI
MI
MJ
MJ
MI
24

Fig. 25A. Control cortical tissue incubated in diaminobenzidine. The single membrane-bound granular component (GR) of the chloroplast (CH) stains for peroxidase. Osmiophilic droplets (OD) and thylakoids (TH) are present in the matrix of the chloroplast. Rough endoplasmic reticulum (RER) and mitochondria (MI) are present in the cytoplasm (CY) of the cell. Several segments of rough endoplasmic reticulum (RER) are present in the cytoplasm (CY) near the cell wall (CW) and do not show appreciable peroxide staining reaction product. Osmium tetroxide fixation with uranyl acetate and lead citrate post-staining. Approximately X73,500.

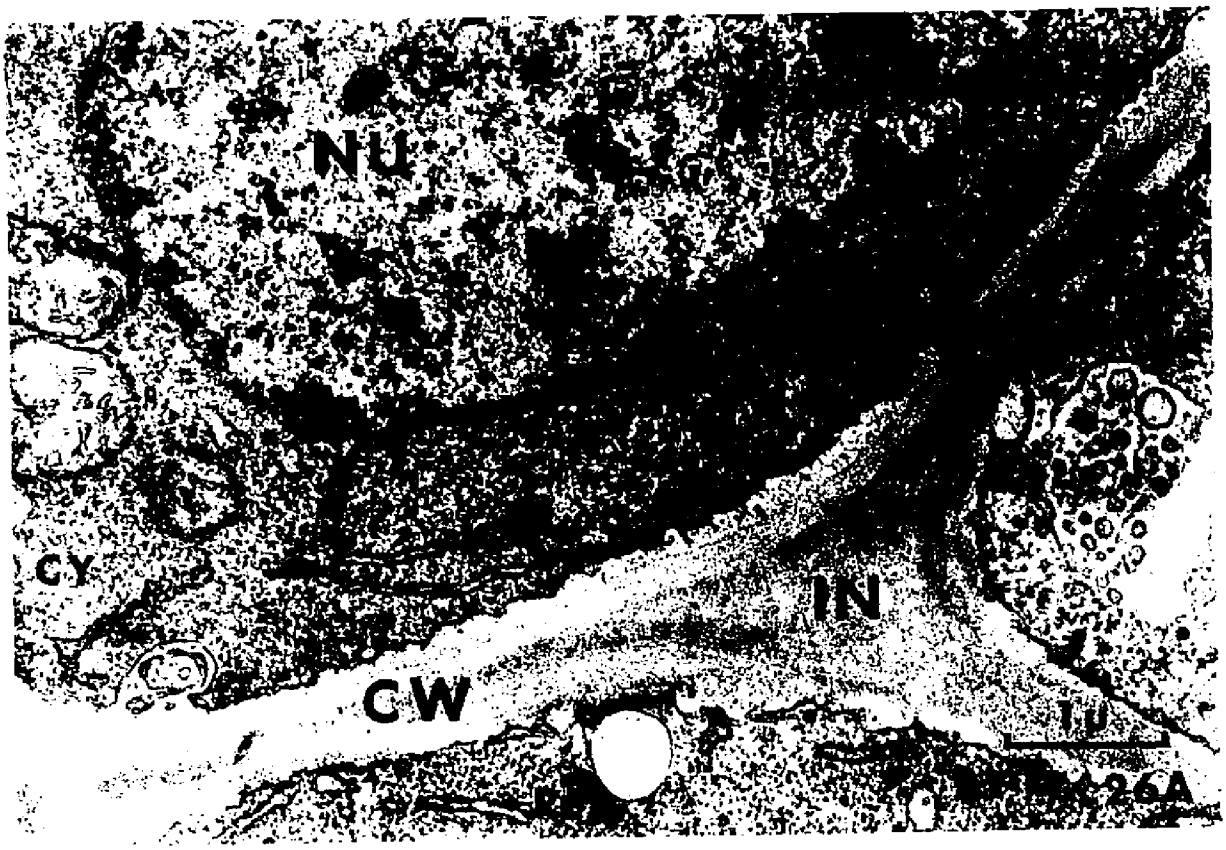
Fig. 25B. Cortical tissue treated with 5µl/l of ethylene prior to diaminobenzidine incubation. There is a proliferation of rough endoplasmic reticulum (RER) after five hours of ethylene exposure. Ribosomes (RI) are visible along the segments of rough endoplasmic reticulum (RER). The segments of rough endoplasmic reticulum (RER) appear to be longer in ethylene-treated than in non-ethylene-treated pedicel tissue. Mitochondria (MI) are present in the cytoplasm (CY) of the cell. A chloroplast (CH) with osmiophilic droplets (OD) in the matrix portion of the chloroplast is visible in the cell. Osmium tetroxide fixation with no post-staining. Approximately X73,500.


CH
CW
CY
RER
TH
CH
GR
25A


MI
RER
RI
GR

Fig. 26A. Control cortical tissue incubated in diaminobenzidine medium at pH 6.0. The mitochondrial membranes (MI) show positive reaction sites for cytochrome oxidase activity. There is also appreciable peroxidase stain in the intercellular spaces (IN). Segments of rough endoplasmic reticulum (RER) are present in the cytoplasm (CY). A portion of the nucleus (NU) and nuclear membrane (NM) are visible and do not show positive peroxidase reactive sites. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X44,000.

Fig. 26B. Control cortical tissue incubated in diaminobenzidine medium at an acidic ph of 6.0. There is deep cytochrome oxidase staining reaction product in the inner and intracristae membranes of the mitochondria (MI). The single membrane-bound granular component (GR) of the chloroplast (CH) does not stain positive for peroxidase activity. A deposit of phyto-ferritin (PF) is present in the cytoplasm of the chloroplast. The thylakoids (TH) of the chloroplasts do not stain positively for peroxidase. Osmium tetroxide fixation with no post-staining. Approximately X45,000.


NU
CY
IN
CW
TU
26A

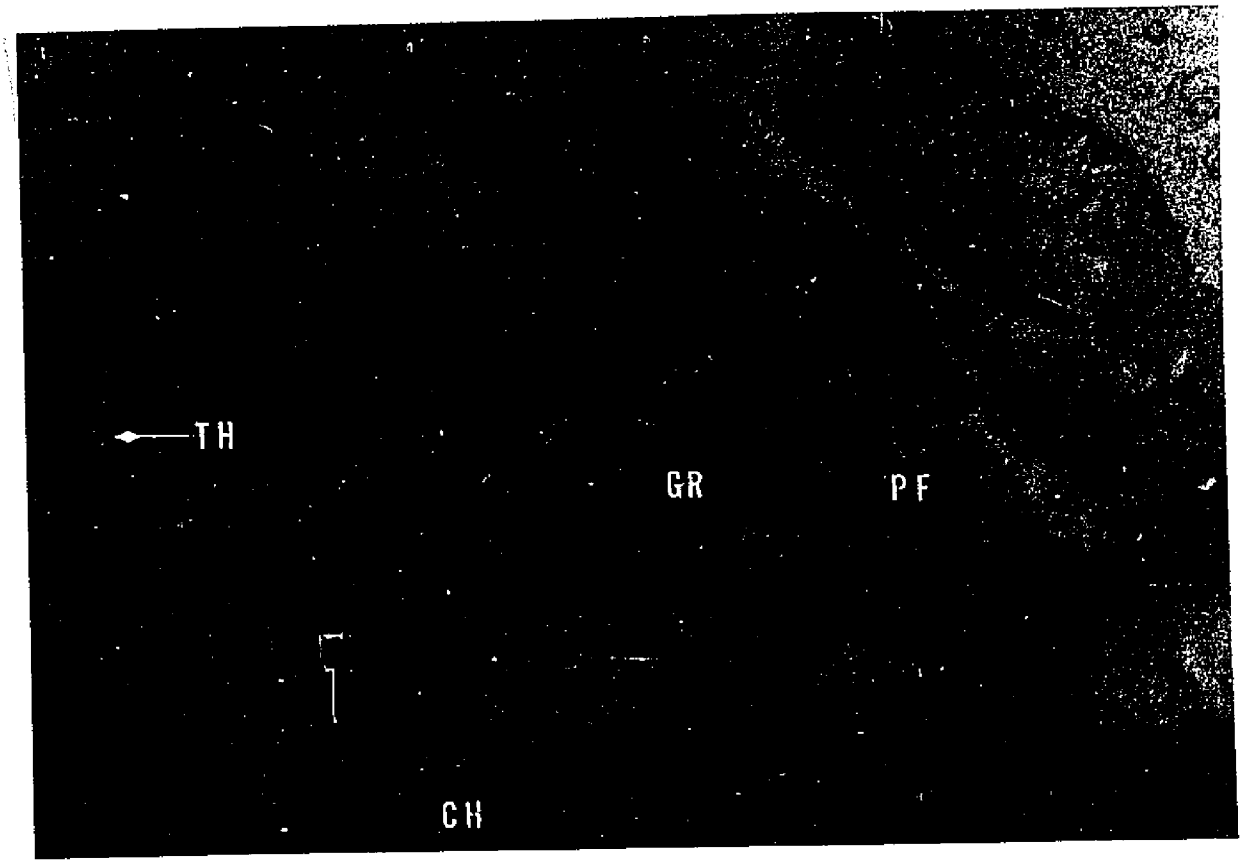

TH
GR
PF
CH

Fig. 27. Diaminobenzidine stained cortical tissue shows intense peroxidase reaction product in the single membrane-bound granular component (GR) of the chloroplast (CH). Segments of rough endoplasmic reticula (RER) are visible in the vicinity of the cell wall (CW). The mitochondrial membranes (MI) do not appear to stain positive for cytochrome oxidase; however, it is possible that the post-staining has rendered the membranes dark and the post-staining enhanced darkening could mask any staining due to diaminobenzidine. The chloroplast thylakoids (TH) appear to have an electron density similar to the granular component (GR). Osmium tetroxide fixation with uranyl acetate and lead citrate post-staining. Approximately X29,000.

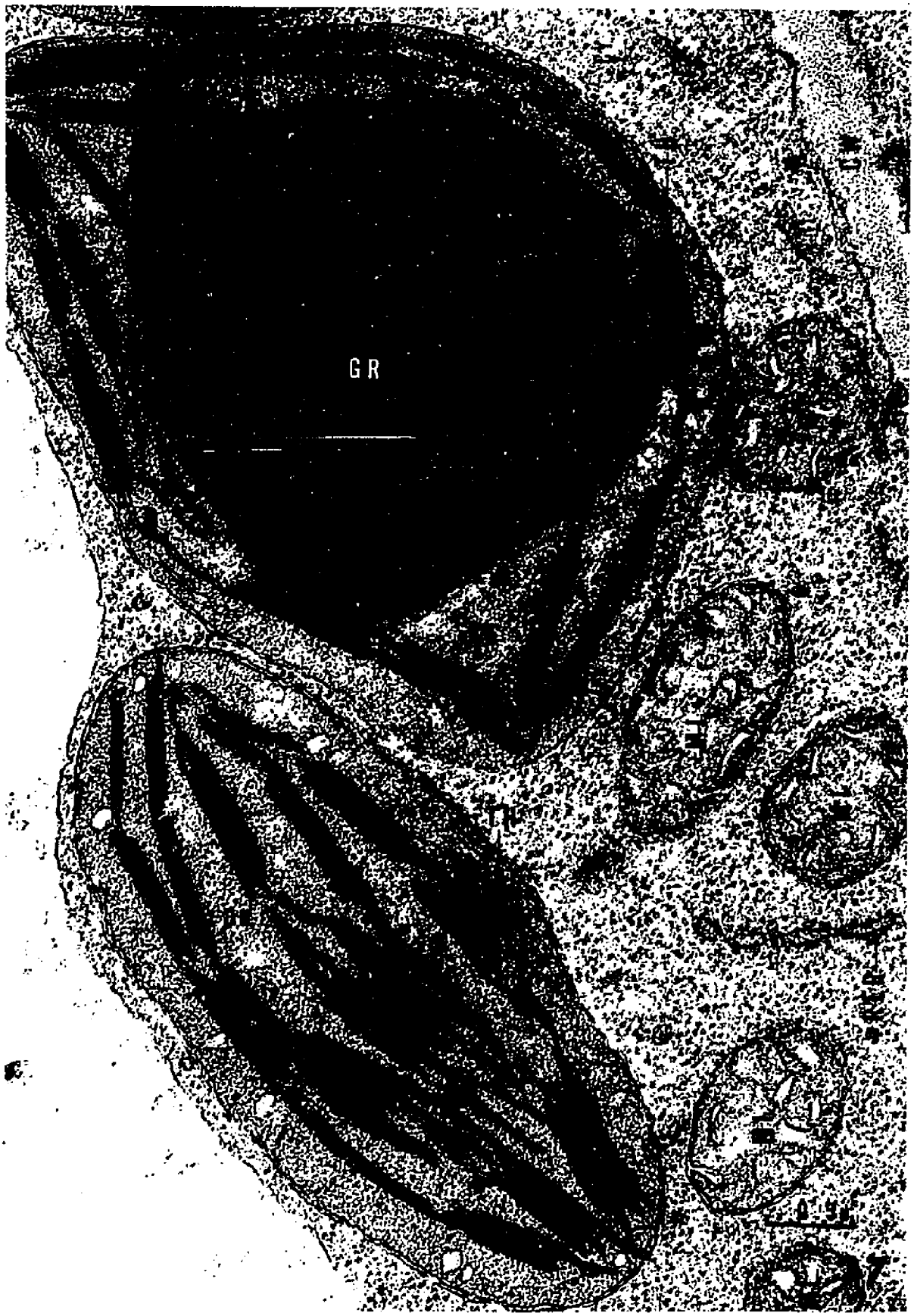

GR

Fig. 28. Control cortical tissue incubated in diaminobenzidine with 0.02M 3-amino-1,2,4-triazole. The developing granular component (GR) of the chloroplast (CH) is not completely inhibited by aminotriazole, indicating that peroxidase is the enzyme being localized. Granular deposits of phytoferritin (PF) and thylakoids (TH) are present in the matrix of the chloroplast. Microtubules (MT) are present in the cytoplasm (CY) near the cell wall. Starch bodies (SB) are present in the matrix of the chloroplast. The granular appearance of the starch bodies (SB) is due to the uranyl acetate post-stain. Portions of a nucleus (NU) with a double-stranded nuclear membrane (NM) are in close proximity to the chloroplast. Segments of rough endoplasmic reticulum (RER) are in the cytoplasm (CY) near the cell wall (CW) areas. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X63,000.

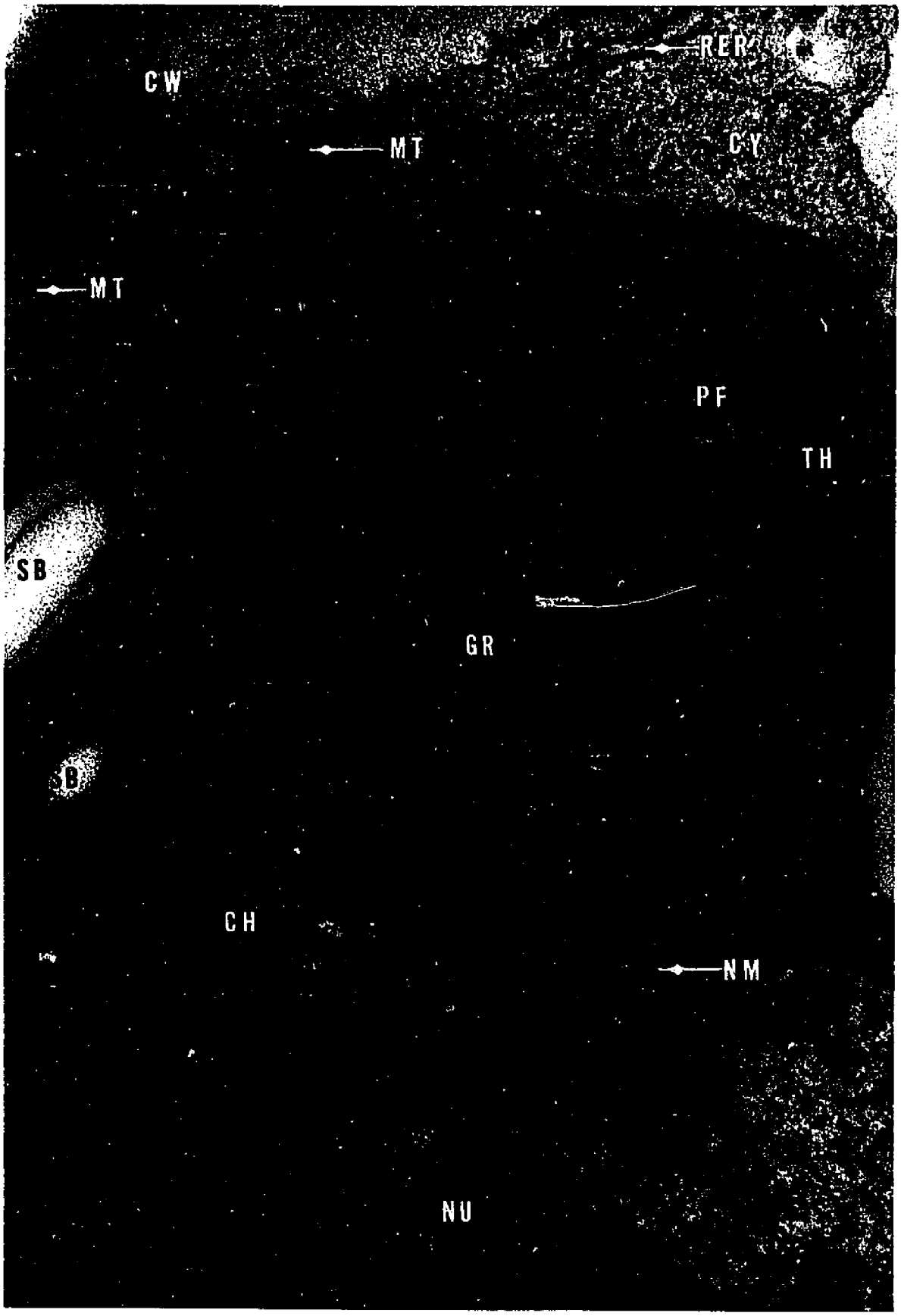

CW
RER
MT
CY
MT
PF
TH
SB
GR
B
CH
NM
NU

Fig. 29. Cortical tissue treated with 5µl/l of ethylene for five hours prior to incubation in diaminobenzidine with 0.02M potassium cyanide. Osmiophilic droplets (OD), thylakoids (TH), and a starch body (SB) are present in the matrix of the chloroplast (CH). The single membrane-bound granular component (GR) of the chloroplast is not inhibited by potassium cyanide and still stains positively for peroxidase reaction product. Segments of rough endoplasmic reticulum (RER) are present in the cytoplasm (CY). The cell wall (CW) has a moderate amount of peroxidase reaction product. A portion of a vacuole (VA) is also present. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X78,000.


VA
SB
CW
0.5μ
29

Fig. 30. Diaminobenzidine-stained cortical tissue showing the presence of peroxidase reaction product in the middle lamellar and adjacent portion of the cell wall (CW). The plasma membrane is present along the cell wall (CW). The Golgi (GO) is present in the cytoplasm (CY). The Golgi (GO), Golgi vesicles (GV), and Golgi cisternae (GC) do not stain for peroxidase. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X78,000.

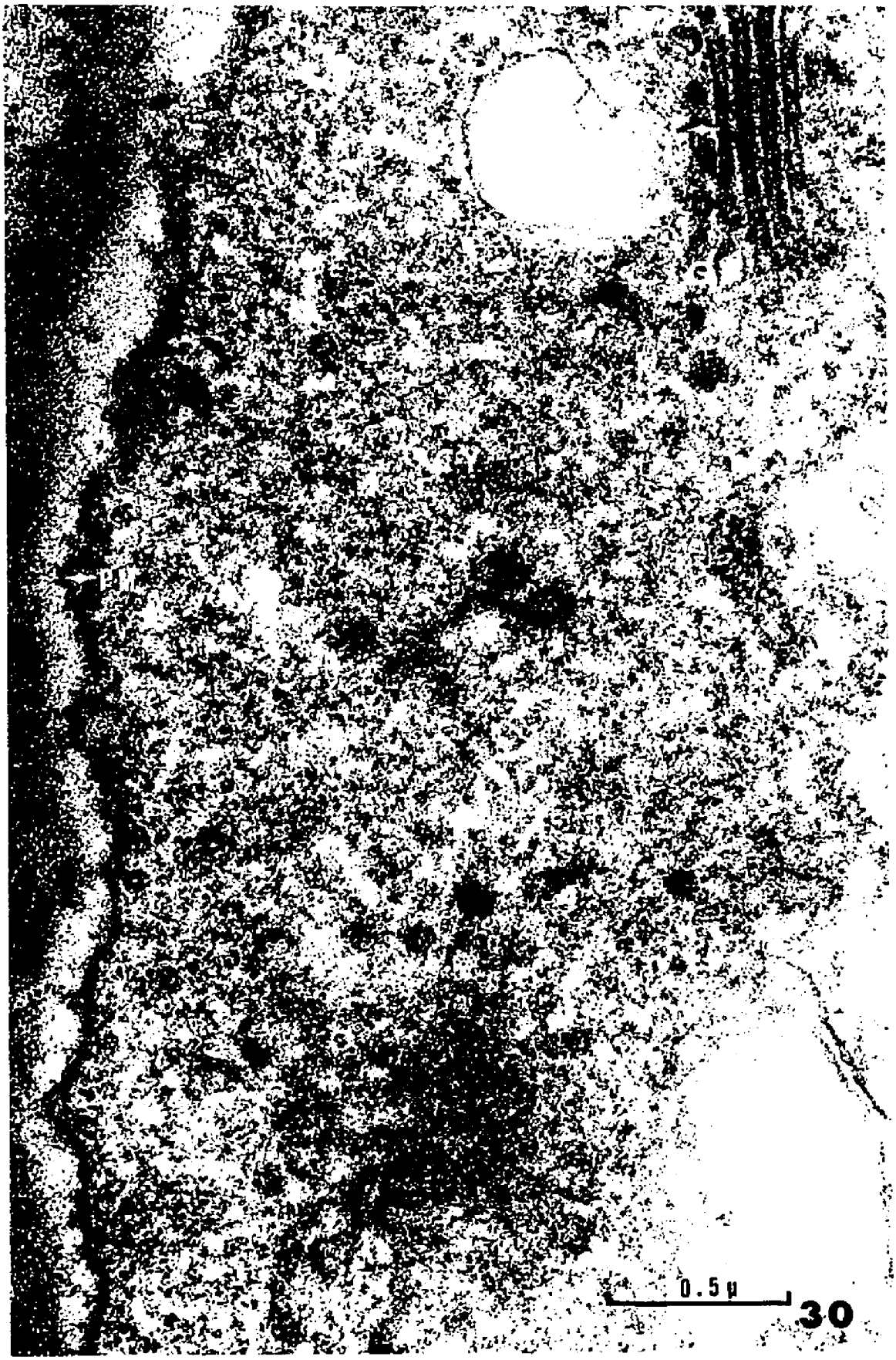
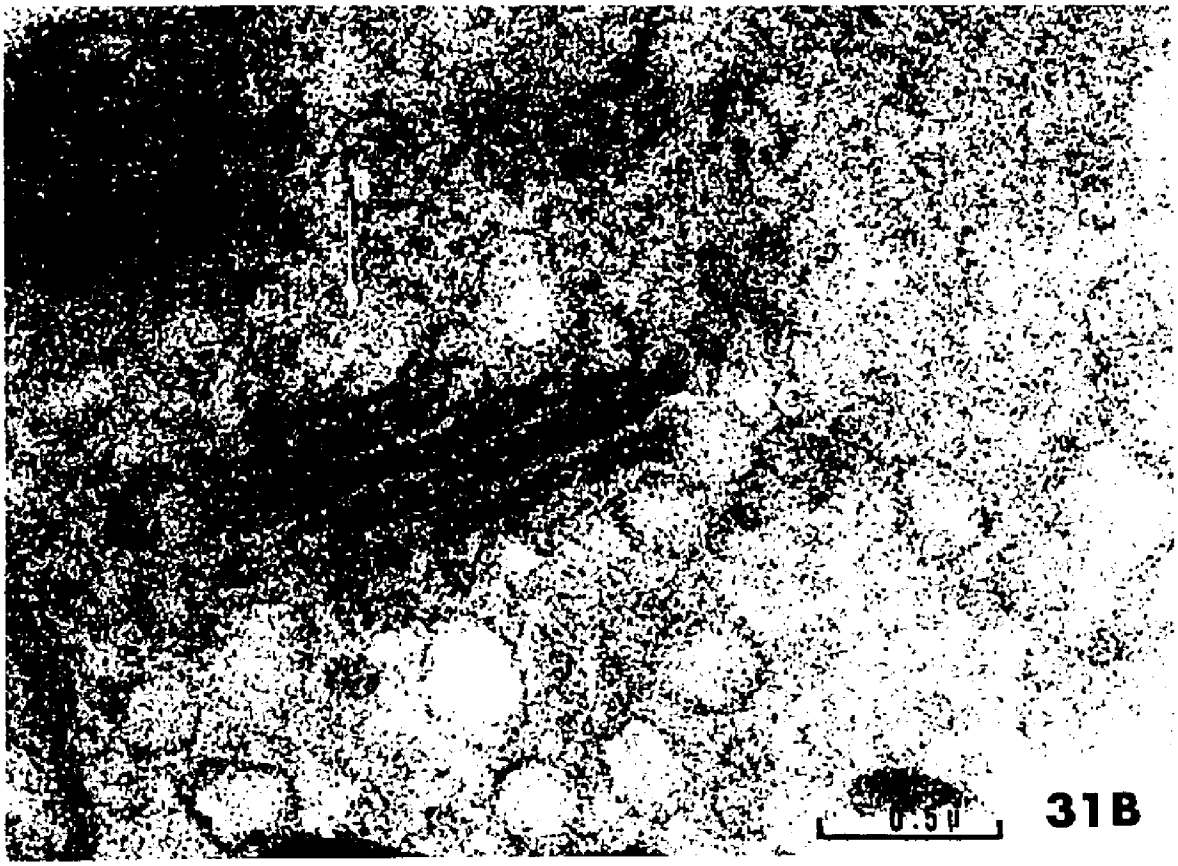

0.5 μ
30

Fig. 31A. Cortical tissue treated with 5µl/l of ethylene for two hours prior to incubation in diaminobenzidine with 0.02M potassium cyanide. The inhibitor does not completely mask the peroxidase reaction product in the Golgi vesicles (GV) and in the Golgi cisternae (GC). A microtuble (MT) is present in the cytoplasm (CY) near the plasma membrane (PM) of the cell wall (CW). Osmium tetroxide fixation with uranyl acetate post-staining. Approximately 67,500.

Fig. 31B. Cortical tissue treated with 5µl/l of ethylene for five hours prior to incubation in diaminobenzidine. The Golgi (GO) in the cytoplasm (CY) have peroxidase reaction product in both vesicular membranes (GV) and cisternae (GC). Osmium tetroxide fixation with no post-staining. Approximately X47,500.



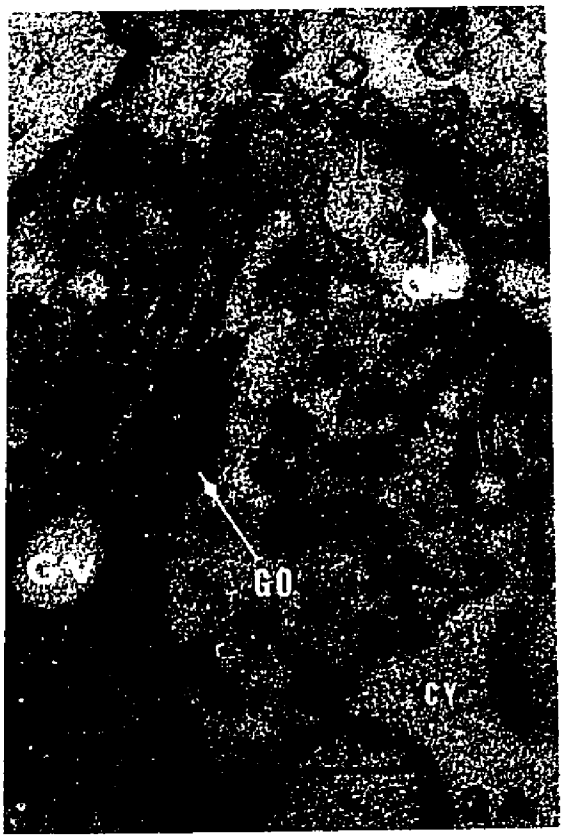
31B

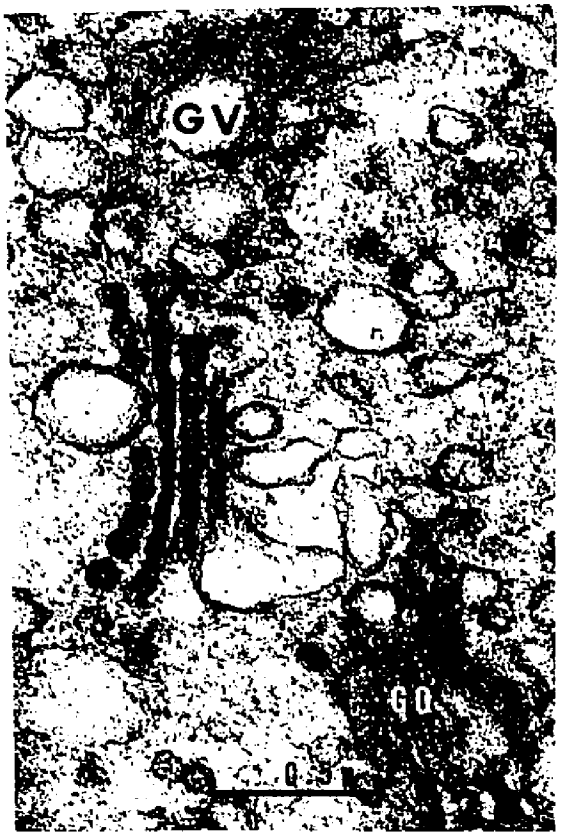
Fig. 32A. Cortical tissue treated for five hours with 5µl/l of ethylene prior to incubation in diaminobenzidine (DAB) with 0.02M aminotriazole (AT). Golgi (GO) composed of Golgi cisternae (GC) and Golgi vesicles (GV) are situated in the cytoplasm (CY). AT does not inhibit peroxidase staining. Approximately X69,500.

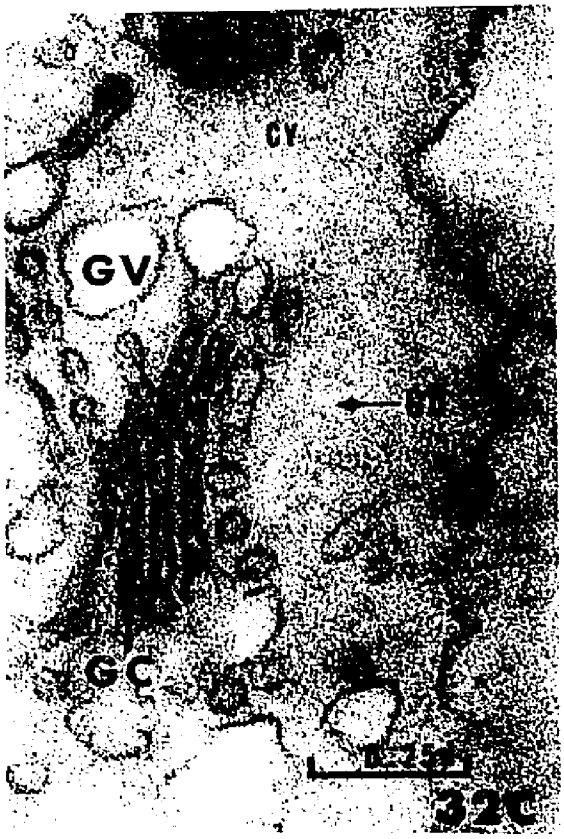
Fig. 32B. Cortical tissue treated with 5µl/l of ethylene for five hours prior to incubation in DAB with 0.02M AT. There is no inhibition of peroxidase staining by AT in the Golgi (GO), Golgi cisternae (GC), Golgi vesicles (GV), or cytoplasm (CY). Approximately X149,000.

Fig. 32C. Cortical tissue treated with 5 µl/l of ethylene for five hours prior to incubation in DAB plus 0.02M AT. AT does not inhibit Golgi (GO), Golgi cisternae (GC), Golgi vesicles (GV), or cytoplasm (CY). Approximately X149,000.

Fig. 32D. Cortical tissue treated with 5µl/l of ethylene for five hours prior to incubation in DAB with 0.02M AT. AT does not inhibit peroxidase staining in the Golgi (GO), Golgi cisternae (GC), Golgi vesicles (GV), or cytoplasm (CY). Osmium tetroxide fixation without post-staining in Figures 32A--32D. Approximately X149,000.


GO
CY


GV
GO
0.5μ


CY
GV
GO
GC
32C

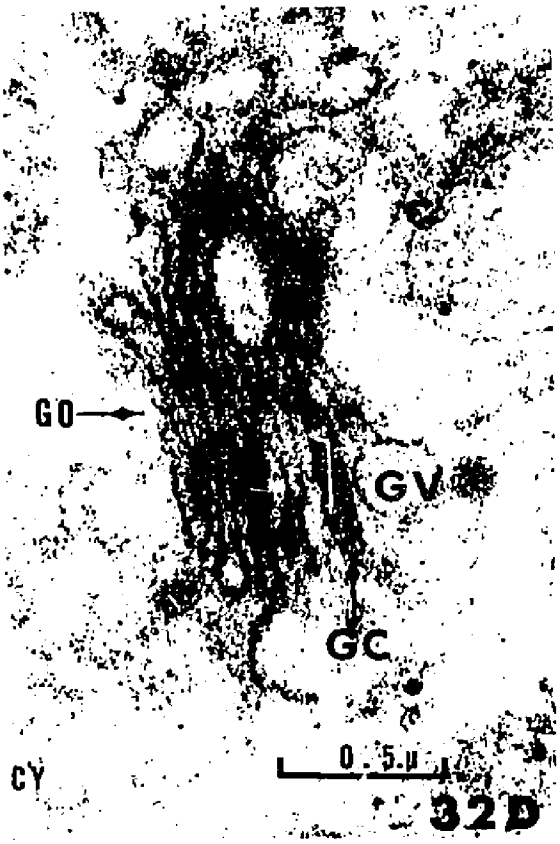

GO
GV
GC
CY
0.5μ
32D

Fig. 33. A portion of control cortical tissue incubated in diaminobenzidine. The single membrane-bound granular component (GR) of the chloroplast (CH) stains positive for peroxidase. There is positive peroxidase reaction product in the cell walls (CW) and in the intercellular spaces (IN). A nucleus (NU) is present with a nucleolus (NL). There are several mitochondria (MI) throughout the cytoplasm (CY). Plasmodesmata (PL) are visible at several places along the cell walls. Osmium tetroxide fixation without post-staining. Approximately X15,000.

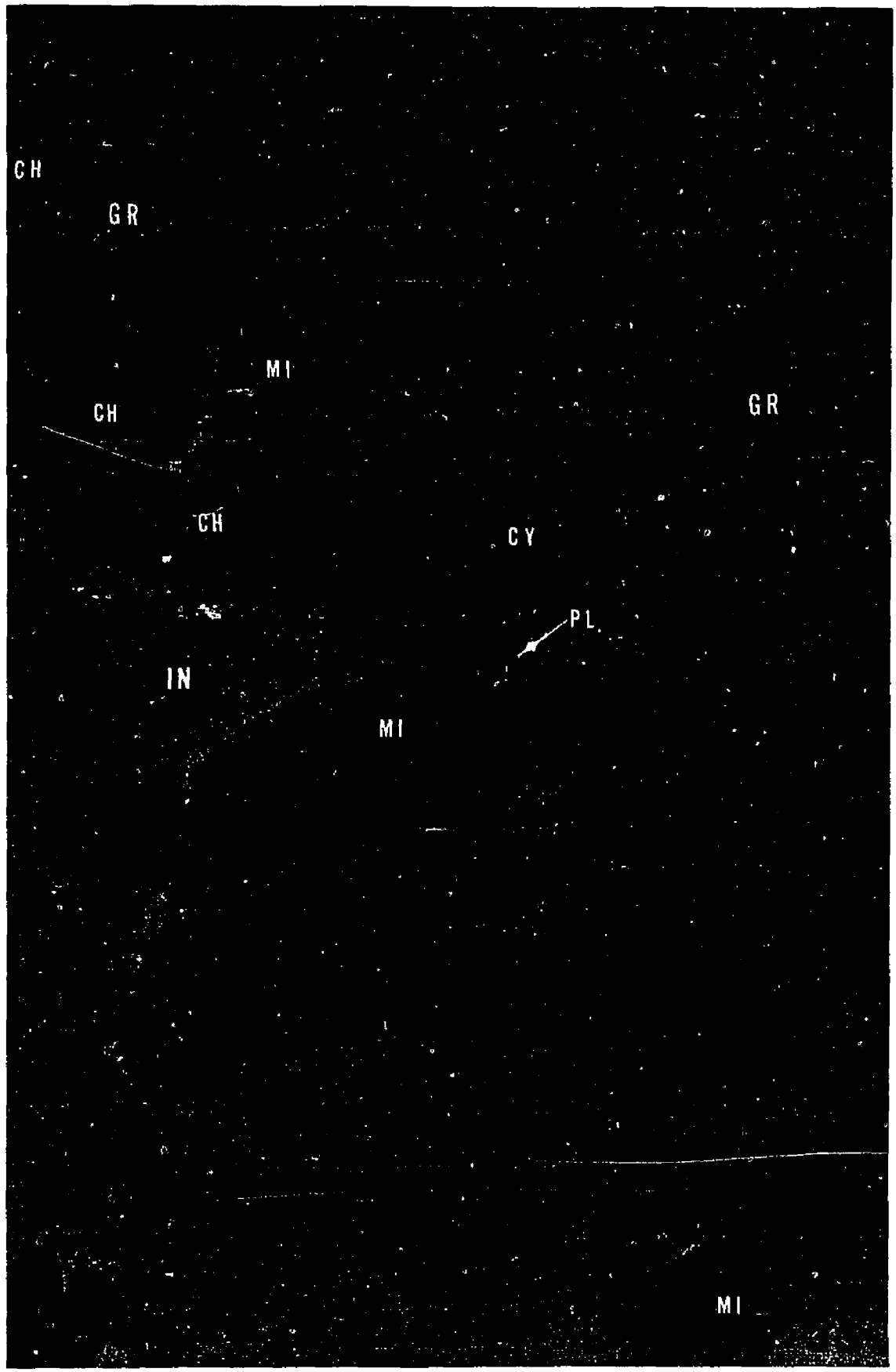
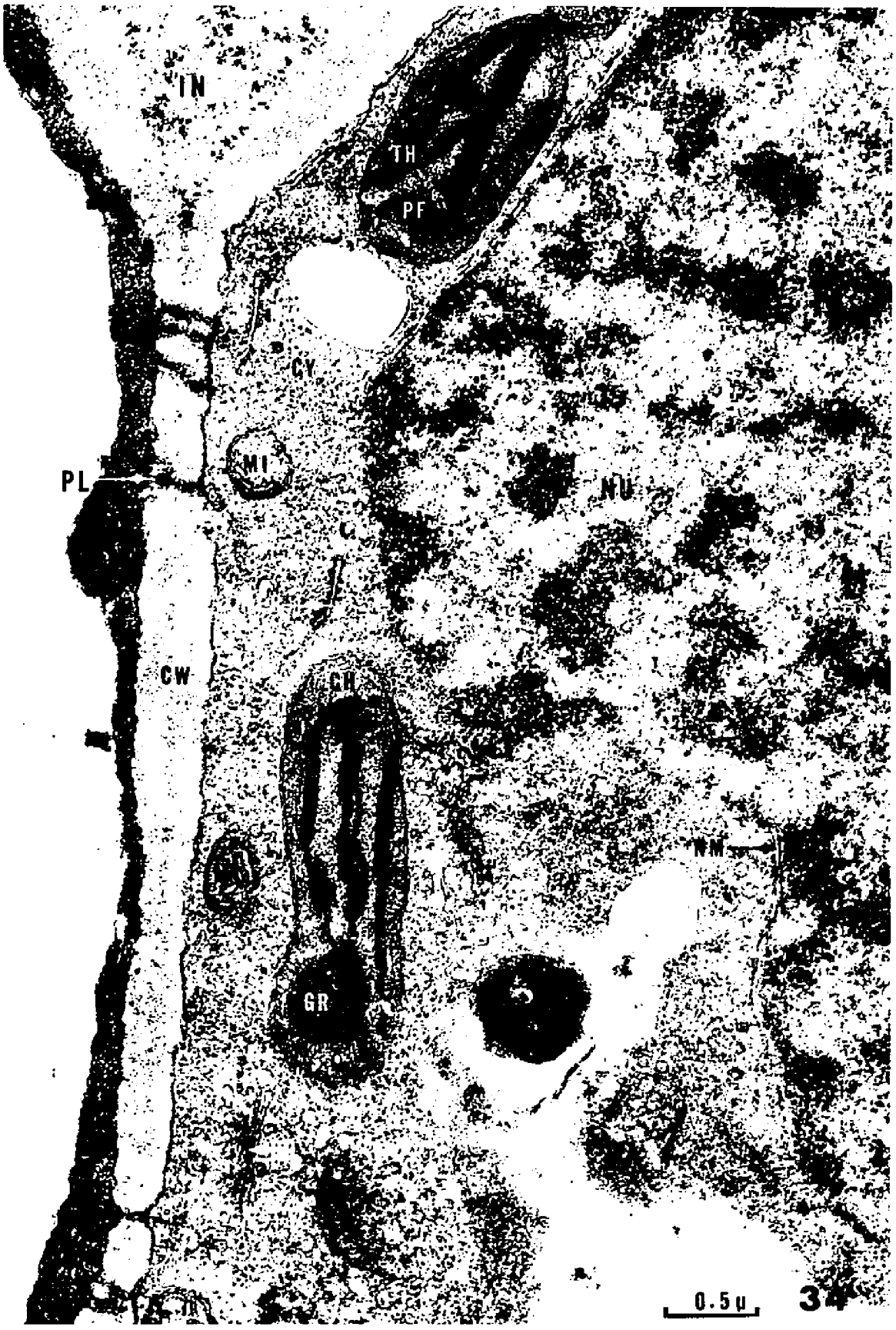

CH
GR
MI
CH
GR
CH
CY
PL
IN
MI
MI

Fig. 34. A portion of cortical tissue treated with 5µl/l of ethylene for five hours prior to incubation in diaminobenzidine. Several plasmodesmata (PL) cross the cell wall (CW) at different places. There is peroxidase staining in the intercellular spaces (IN) of the cell wall. Phytoferritin granules (PF) are present in the matrix of the chloroplast (CH). A single membrane-bound granular component (GR) and the thylakoids (TH) demonstrate peroxidase reaction product. Rough endoplasmic reticulum (RER) and mitochondria (MI) are present in the cytoplasm (CY). The nuclear membrane (NM) is visible surrounding the nucleus (NU). Golgi (GO) are present in the cytoplasm (CY). The Golgi (GO) and rough endoplasmic reticulum (RER) show evidense of darkening but the post-staining may be partially for some degree of darkening. Osmium tetroxide fixation with uranyl acetate post-staining. Approximately X31,000.


IN
TH
PF
CV
MI
PL
NU
CW
CH
WM
GR
0.5μ
34