SHORT TITLE

Histochemical Studies on Pathogenesis of the Elm Disease

Camilien Gagnon

HISTOCHEMICAL STUDIES ON THE PATHOGENESIS OF THE ELM DISEASE INCITED BY *CERATOCYSTIS ULMI* (BUISM.) C. MOREAU IN *ULMUS AMERICANA* L.

Camilien Joseph Xavier Gagnon

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Plant Pathology
McGill University
Montreal, Canada.

July 1965.

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. Réal L. Pelletier, Department of Plant Pathology, Macdonald College of McGill University, for his interest and guidance in this investigation. Grateful acknowledgment is made to Officials and Staff, Forest Research Laboratory, Canada Department of Forestry, Quebec City, for facilities provided at this Laboratory, where most of the work was done. Thanks to Mr. R. Gagnon, to whom I am indebted for the development of films and printing of black and white photographs.

TABLE OF CONTENTS

I. INTRODUCTION

The elm disease incited by Ceratocystis ulmi (Buism.) C. Moreau, generally named Dutch elm disease, especially in North America, was discovered in The Netherlands soon after the first world war. The designation, Dutch elm disease, should be avoided, according to Westenberg (1932), because it is misleading. The name elm disease is used in this work, following in this respect the nomenclature adopted in the Annual Report of the Quebec Society for the Protection of Plants (Pomerleau, 1961).

This desease has been the object of a considerable amount of rather inconclusive, but nevertheless theorically important, physiological studies on host-parasite relationship, especially on the mechanism of wilting.

Early studies on the pathogenesis of the elm disease were based on histological observations of infected elms. Various changes were observed in the xylem vessels of infected trees: formation of tyloses, production of brown gummy substances, and discolouration of cell walls. Later on the interest turned to the production of toxic substances by the causal organism. More recently investigations have been made on the production in vitro of extracellular enzymes by C. ulmi. The fact, however, that a pathogen produces in culture certain toxic substances and extracellular enzymes does not necessarily mean that it does so in its host.

Thus, it appeared that much useful information could be obtained by studying the biochemical changes occurring in the xylem during the development of the disease in white elms. Indeed, these

changes could be investigated to a certain extent by means of the classical biochemical methods, i.e. the preparation of tissue homogenates, extraction with suitable solvents, and analyses of the compounds. But that type of procedure has serious disadvantages, since changes in the actual compounds are likely to happen during the experiments whatever care is taken. Furthermore, this does not give adequate information concerning the sites of formation of the compounds in the infected trees. This may be very important in the case of an investigation on enzyme activity since both the enzyme and its substrate may be present in a preparation, thus giving a reaction, and it would be concluded that the reaction occurs in vivo, but it may be that the reaction does not occur in nature because the two substances are located in different sites, or the reaction is blocked otherwise.

Considering these facts, it appeared that histochemistry would be the best approach to the study of the biochemical development of the disease in elm trees. With histochemical techniques it is possible to locate compounds and enzymatic reactions in tissues, and even in cells. Indeed the specificity of certain tests is debatable, and no tests have been developed as yet to determine certain compounds and enzyme systems, but this rather new science in the field of plant pathology is sufficiently evolved to study most of the chemical compounds and enzymes suspected of significant importance in the pathogenesis of the elm disease, such as the formation of brown gummy substances, the alteration of cell wall constituents, the oxidation of phenolic compounds, and the oxidative enzyme systems of the causal fungus. These compounds and enzymes are the object of this work.

II. REVIEW OF LITERATURE

The following review of literature deals mainly with the mechanism of pathogenesis in the elm disease with a few references to other wilt diseases followed by a brief description of the histochemical studies in plant pathology.

A. Mechanism of Pathogenesis

1. Introduction

Dimond (1955) discussed the pathogenesis of wilt diseases in a review covering the literature up to August 1954 dealing with diseases induced by Fusarium and Verticillium spp., two groups of organisms which invade their hosts through the roots. He did not mention, however, the elm disease, the causal agent of which usually enters xylem tissues through wounds made on small branches by the insect carriers (Collins, 1941; Pomerleau, 1947), although the disease is occasionally transmitted also through the root system by grafts between adjacent trees (Verral and Graham, 1935; Zentmeyer et al. 1946; Himelick and Neely, 1962; Neely and Himelick, 1963).

However, when once the pathogens become established in the xylem vessels it seems that there is no great difference in the subsequent development of the diseases caused by Fusarium and Verticillium spp. on one hand, and C. ulmi on the other, because the root system of infected plants, in all cases appears to absorb water normally. In the case of diseases incited by Fusarium and Verticillium extensive damage to the root system occurs only when aerial parts show severe symptoms (Nelson, 1950; Ludwig, 1952), and in the case of the elm

disease spores of the fungus are carried down to the base of the stem fairly rapidly, but C. ulmi does not develop extensively in the root system (Banfield, 1937, 1938, 1941, 1941a; Ouellette, 1960).

The evolution of ideas on the mechanism of pathogenesis in the elm disease is closely related to that in other wilt diseases. The different theories which were put forward to explain the pathogenesis of the elm disease have been summarized in a recent paper (Gagnon, 1964).

2. Plugging of water conducting vessels

Most workers until the mid-forties attributed the rapid wilting and dying of infected elms to the plugging of the water conducting vessels by gums, tyloses, and degradation products of parenchyma cells (Wollenweber, 1927; Clinton and McCormick, 1936; Broekhuizen, 1929; Pope, 1943). More recently, Ouellette (1960) attributed the acute symptoms of this disease to the plugging of the xylem vessels by fungal growth, cytoplasm and other products from adjoining cells, and particles originating from alterated cell walls. He stated that this plugging occurs in small branchlets where the vessels are of short length and small diameter. The presence of brown gummy substances in the vessels of infected elms has been observed by all the investigators of this disease; it is only the amount of these substances and the time of their apparition in the vessels that led to question the importance of the plugging effect. Schwarz (1922) reported that the formation of tyloses was the first change observed after inoculation; that, later on, these tyloses disintegrated, the walls then being brown, gummy and swollen. Broekhuizen (1929) ob-

served a more or less granular gum varying from yellow to brown, which originated from longitudinal parenchyma and ray cells, and which was extruded through pits. He interpreted this colour variation as a possible gradual transformation of the gum into lignin. He was unable to produce a similar gum by injection of culture filtrates of the fungus, organic acids, and other compounds, but he observed numerous tyloses following these injections. Kerling (1955) stated that the first alteration after inoculation was the discolouration of the vessel walls, followed by changes in the appearance of the content of living cells which became darker, and by exudation through pits of gum droplets from longitudinal parenchyma and ray cells. These gum droplets, originally yellow, turned brown, and united in large masses. She observed that tyloses appeared one week after inoculation in non-discoloured parts of the vessels and originated from longitudinal parenchyma as well as ray cells.

No reports were found on the characterization of the materials extruded in the vessels and the biochemical changes occurring in the living cells of the xylem before the discolouration appears in these cells.

3. Toxin production by Ceratocystis ulmi

In the forties, the consensus of opinion tended towards a toxigenic theory of causation of wilt diseases. Much evidence in support of the toxin theory was derived from the injection into healthy plants of cell-free filtrates of culture of a pathogen. If there happened to be a close agreement between the symptoms produced by the culture filtrate or some of its components, and the symptoms resulting

from infection by the pathogen, then, it was presumed that the disease condition of the host was, at least to a certain extent, the result of a toxemia.

Broekhuizen (1929) had shown a toxic effect induced by injection of culture filtrates of the elm pathogen, but Zentmeyer (1942, 1942 a) was the first to report the production of toxin as such in culture medium in which C. ulmi had grown. Following injection of culture filtrates into elms he observed a wilting of the test plants, the discolouration of the cell walls, and the formation of gums in the vessels. This toxin complex was thermostable, according to Zentmeyer and co-workers (1946), but Feldman et al. (1950) reported that it was slightly inactivated by heat.

Dimond (1947) separated two fractions in the toxic filtrate: An alcohol insoluble polysaccharide responsible for the upward curling and marginal withering of the leaves, and an alcohol soluble fraction which produced a severe interveinal necrosis when injected in elms. Dimond and co-workers (1949) found that the toxin titre of still cultures increased from the sixth day onward, that the toxin was produced much more rapidly in shake cultures than in still ones, but that there was no difference in the symptoms produced by filtrates of still and shake cultures. Feldman and his associates (1950) stated that the polysaccharide of the culture filtrate was only of minor importance in the toxin complex. They reported also that the toxin was inactivated almost completely at pH 6.0 and above. This suggests that the toxin complex produced by the fungus in culture may have one or more enzymes as constituents.

Gagnon (1960) attempted to obtain such toxins from diseased elms by forcing distilled water through sections of stems and branches. The extracts, passed through a Seitz filter, were assayed together with extracts from healthy trees, and distilled water, by dipping uprooted elm seedlings in these fluids. Wilting appeared earlier in seedlings dipped in the extracts from diseased elms than all the others, and it was concluded that toxic materials were present in the sap of diseased elm trees. In tests made with extracts obtained by soaking chips of diseased elm wood showing discolouration, the wilting of the seedlings was even faster. It must be pointed out, however, that these toxic extracts were brown in colour, and probably contain toxins quite different from those produced by the fungus in vitro which are colourless.

4. Enzyme action

Wood (1960) reviewed the literature on the role of pectic and cellulolytic enzymes in plant diseases, and discussed briefly the action of these enzymes in the wilt diseases. He concluded that there is good evidence that pectic enzymes are important in the final stage of some wilt diseases, when the plant is moribund, but that there is no conclusive evidence that they are important in the second stage during which symptoms become visible. But, Dimond (1955), in his review on the pathogenesis in the wilt diseases cited a number of workers who produced evidence of the production of plugging materials resulting from the action of pectic enzymes. Ludwig (1952) was probably the first to point out the role of these enzymes in the formation of colourless or grey materials which plugged the vessels of tomato plants in early stage of infection by Fusarium.

In respect to the elm disease, Holmes (1954) indicated the presence of pectic enzymes in culture filtrates of C. ulmi. Beckman (1956) demonstrated the production of pectin depolymerase, cellulases, and a growth-promoting substance in the cultures of this fungus. The production of a polygalacturonase, and a cellulase capable of hydrolyzing carboxymethylcellulose was reported by Dimond and Husain (1958). The latter enzyme was ineffective against native cellulose, nevertheless they concluded that the role of these enzymes was to provide food for the fungus. Thus, the role of hydrolytic enzymes in the pathogenesis of the elm disease has been derived from in vitro studies, and similarity with other wilt diseases. No investigation has been reported on the role of oxidative enzymes of the tree or of the fungus in the mechanism of this disease.

B. Histochemical Studies

When this work was undertaken, little use had been made of histochemical techniques for the study of plant diseases. Most of the original methods in enzyme histochemistry were developed for animal tissues. However, histochemical techniques have been used in recent years by botanists for the study of the changes occurring in plant tissues during their differentiation, such as in root tips (Jensen, 1956; Avers, 1958; Van Fleet, 1959), and in developing and ripening fruits (Reeve, 1959, 1959a, 1959b), and in cell differentiation in fungi (Zalokar, 1959).

Mace (1963) reported on the histochemical localization of phenols in healthy and diseased banana roots, a disease caused by

Fusarium oxysporum f. cubense, and which is characterized by vascular browning, as is the elm disease. The role of phenol oxidases (Mace and Wilson, 1964) and peroxidases (Mace, 1964) of both the host and the pathogen was also investigated by histochemical techniques. No such histochemical studies have been reported on the elm disease, or other wilt diseases, as far as I know.

III. MATERIALS AND METHODS

A. Elm Materials

White elm (Ulmus americana L.) seedlings two to four years old and young white elm trees up to twelve feet high were used in this work. Some of the seedlings were planted in a greenhouse while the others and the young trees were nursery plants. A number of these plants were inoculated at the base of the stem with a spore suspension of C. ulmi, prepared by washing with sterile water the surface of one-week old cultures grown on potato dextrose agar (PDA) in Petri dishes. The inoculation of the seedlings was made with the aid of an hypodermic needle (Stubbs Gauge 16) and a syringe, and the young trees were inoculated by placing, with the aid of a dropper, one or two drops of the spore suspension in cuts made in the xylem by means of a flamed knife.

A few other seedlings, ten for each test, were injected with dilute citric acid-phosphate and veronal buffer solutions prepared by adding to distilled water just enough of the stock buffer solutions (0.2 M) to obtain the following pH values 4.0, 5.0, 6.0 and 7.0 with the citric acid-phosphate buffer, and 7.0 and 8.0 with the veronal buffer solutions. Solutions of aqueous ethanol 5, 10 and 20 per cent (v/v) were also injected in elm seedlings.

The technique of injection consisted in inserting, in the stem of seedlings, hypodermic needles connected by means of rubber caps to glass tubes 14 mm in diameter and 15 cm in length which were

filled before insertion of the needles in order to avoid the penetration of air in the xylem vessels. The open end of the tubes was covered with a plastic sheath fastened to the tube with a rubber band (Fig. 1).

B. Preparation of Sections

Tangential and cross sections of stems and twigs used in preliminary tests were cut with the aid of a hand microtome, but in subsequent experiments a sliding microtome (American Optical Co. Model 860) was used to cut the sections. Cross sections were cut 15 microns, and tangential sections 10 microns thick. Sections of fresh material were used in all the tests. In addition to these, for polyphenol tests, dehydrated, paraffin embedded, and frozen sections cut in a cryostat (Fig. 2) were used; and for peroxidase tests, celloidin infiltrated and frozen sections cut in the cryostat were used.

C. Culture of the Fungus

Different isolates of C. ulmi were used in the histochemical tests made to detect the presence of various oxidative enzymes in their thallus. These isolates were obtained from infected elm trees during the summers of 1961 to 1963, inclusively. These samples were collected in the following localities: Quebec City, Montreal, Senneville, and Grand'Mere, P.Q., and Campbellton, N.B. These isolates were grown on PDA slants, and kept at 4°C in a refrigerator. Because no physiological and morphological differences were observed between these isolates, they were used indifferently in the course of this work.

The fungus was transferred from stock cultures to PDA in Petri plates, and allowed to grow at least one week before it was used

Fig. 1. The injection of a solution in an elm seedling by means of hypodermic needles connected to glass tubes with rubber caps.

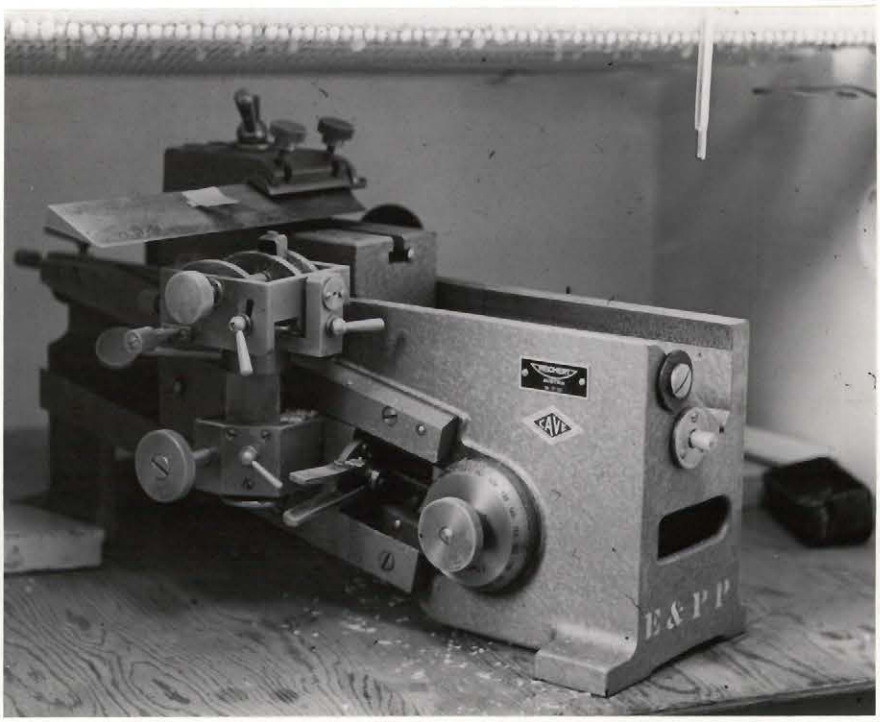


Fig. 2. Sliding microtome on the bottom shelf of a vertical freezer of the domestic type which had been converted into a cryostat.

for culture on cellophane foil prepared as following. Square pieces of cellophane foil, 2 x 2 cm, were sterilized by autoclaving in water for 15 minutes at 15 lb. pressure, or by washing in 70 per cent ethanol. The cellophane squares were laid upon the surface of PDA in Petri plates, and the plates were set aside for a day to allow for the evaporation of excess liquid. Fungus spores were deposited, with the aid of a flamed needle, on the center of the cellophane squares, and the cultures were incubated at room temperature (22° to 25°C).

D. Testing Technique with Foil Cultures

Four days after seeding, the pieces of cellophane, half covered by the fungus, were lifted from the agar medium, cut into four parts at right angles with scissors, and transferred to the tests reagents in the cavities of porcelain test plates placed on a heating plate set at 37°C, or standing at room temperature (22° to 25°C). The fungus colonies were detached from the cellophane by shaking during incubation and subsequent washings. The fungus colonies were then spread on microscope slides by floating them in a drop of water. The excess of water was drained off, and the colonies were mounted in Aquamount (Ed. Gurr) or CMC-10 (Turtox). At least ten colonies were used for each of the ten experiments carried out, and each test was repeated three times.

E. Histochemical Tests

The histochemical tests for lignin, pectin, polyphenols, phenoloxidase, peroxidase and beta-glucosidase were performed on sections of healthy and of diseased elms. The number of sections examined varied from 50 to 200 for each experiments. Each test was

made two or three times during a growing season and repeated two consecutive years. Foil cultures of the pathogen were tested for phenoloxidase, peroxidase, beta-glucosidase and also dehydrogenases and diaphorases.

1. Lignin test

Different tests were used to find the location of lignin in the xylem tissues of healthy and of diseased elms. The phloroglucinol test (Johansen, 1940; Jensen, 1962) was applied to fresh sections. Sections were placed in a drop of a saturated aqueous solution of phloroglucinol in 20 per cent hydrochloric acid on a microscope slide. The sections were then covered with a cover slip and examined under a microscope. The following modification of this procedure (Sass, 1951) was tried also. Sections were floated in a drop of one to two per cent solution of phloroglucinol in 95 per cent ethanol, and covered with a cover glass. Then, a small drop of concentrated, or 1:1 diluted, hydrochloric acid is placed at one edge of the cover glass, and the reaction is followed under the microscope.

The chlorine-sulfite test (Siegel, 1953; Jensen, 1962) also was tried on about 50 fresh sections. The sections were immersed for five minutes in a saturated, acidified solution of calcium hypochlorite for chlorination; then they were transferred into a one per cent sodium sulfite solution for the development of the red colour, characteristic of lignin.

Since the colour which develop in the above tests is unstable and does not permit later examination of the sections, in other tests, lignin was stained with Schiff's reagent, as recommended by

Jensen (1962). Fresh sections were placed for four hours in Schiff's reagent, rinsed in water, placed in a two per cent solution of sodium metabisulfite for two minutes, rinsed in running water for ten minutes, and mounted.

2. Pectin tests

Two different methods were used to locate the pectic compounds in healthy and in diseased elms. The first method, which was introduced by Krajcinovic et al. (cited by Pearse, 1960), and was claimed to be specific for pectins, depends on the formation of an addition product between pectic acid and benzidine. This compound has a free NH_2 group which can be diazotized and subsequently can form an azo dye by treatment with beta-naphthol. Fresh sections were treated as follows: Treatment for two minutes in one per cent solution of hydrochloric acid; washing in three changes of absolute ethanol; immersion for 45 minutes in a 0.1 M benzidine solution in alcohol; washing in four changes of absolute ethanol; diazotization for one minute by immersion in a solution made of three parts of 0.1 N hydrochloric acid and one part of 0.1 N sodium nitrite; washing in water; treatment for one to thirty minutes in 0.1 M beta-naphthol.

The second method, called the hydroxylamine-ferric chloride reaction, was developed by Reeve (1959a 1959c). It is said to be specific for esterified pectins. It is based on the formation of a pectin-hydroxylamine complex when esterified pectin is treated with hydroxylamine in alkaline medium. This complex when acidified and treated with ferric chloride forms red iron complexes. The procedure followed was similar to that described by Jensen (1962). The sections

were placed for five minutes in a fresh alkaline hydroxylamine solution prepared just before its use by mixing equal volumes of 14 per cent sodium hydroxide and 14 per cent hydroxylamine hydrochloride, both in 60 per cent ethanol. Then the reaction mixture was acidified by adding an equal volume of a solution composed of one part of concentrated hydrochloric acid and two parts of 95 per cent ethanol. Five minutes after acidification the sections were transferred for five to ten minutes into a 10 per cent ferric chloride solution in 60 per cent ethanol, acidified with hydrochloric acid (0.1 N).

3. Polyphenol test

The nitroso reaction, called the Hoepfner-Vorsatz test, was used to locate the polyphenols in the xylem tissues of healthy and diseased elms. The method described by Reeve (1951) consists in incubating the plant material in a mixture of equal volumes of 10 per cent sodium nitrite solution, 20 per cent urea solution (acts as a stabilizer), and 10 per cent acetic acid solution. After two to three minutes of incubation at room temperature, enough 2 N sodium or potassium hydroxide solution is added for the development of the characteristic coloration which varies from yellow to red and brown depending on the phenolic compounds.

This mixture of sodium nitrite, urea, and acetic acid gradually loses its property after standing for a few weeks and the reaction becomes slower and slower. After experimenting with this technique the following procedure was adopted with satisfactory results. The

sections were placed immediately after cutting in the cavities of a porcelain test plate and incubated in a mixture of an equal number of drops of sodium nitrite and acetic acid. Because the role of urea in the reagent mixture proposed by Reeve is that of a stabilizer, this compound was not necessary with the present technique, since the reagents were mixed just before each test. The reaction was allowed to proceed for about two minutes, and then the alkali solution was added. The incubated sections were mounted in glycerine jelly or Aquamount. Control sections which were not treated with the reagent mixture were examined to determine the extent of the pathological discolouration of the tissues of infected trees.

4. Phenoloxidase tests

Tests to determine the topological distribution of phenoloxidases were performed on healthy and on diseased elms, and on the fungus. Because in preliminary experiments the tests devised for the elm tissues were negative when used on the fungus, another procedure was followed for the latter.

(i) <u>In elm tissues</u> - To locate and evaluate the activity of phenoloxidases in the xylem tissues of healthy and of diseased elm trees, fresh sections were incubated in solutions of various phenolic compounds, which upon oxidation catalysed by phenoloxidases give coloured compounds. Aqueous 0.005 M solutions of p-cresol, pyrocathecol, pyrogallol, orcinol, resorcinol, and hydroquinone and a saturated aqueous solution of tyrosine, an amino acid with a phenolic group, were used.

Solutions were stored at 2°C in a refrigerator. They were allowed to warm up to the incubation temperatures, 25 and 37°C, before the freshly cut sections were placed in them.

Since cathecol gave the best reaction, this phenol was used to determine the best conditions for the test. Catechol solutions were adjusted to the following pH values: 5.5, 6.0, 6.5, 7.0, and 7.5, with appropriate 0.05 M phosphate buffers. Potassium cyanide (KCN) and sodium azide (NaN_3) at 0.05 M concentrations were used separately as inhibitors. Controls were incubated in distilled water. Possible autooxidation of the phenolic solutions was checked upon by keeping portions of the reaction mixture under the incubation conditions, but with no sections added.

(ii) In the fungus - Fungus colonies were incubated in solutions of the various phenols as described in the tests for elm materials. Because negative results were obtained, another method was used to determine if the fungus could oxidize phenols added to the culture medium. The fungus was grown on PDA media to which pyrocatechol was added, in some cases before and in others after sterilization in the autoclave. The following concentrations of catechol were used: 0.025 M, 0.005 M, 0.001 M, 0.002 M, and 0.0001 M, with control cultures on PDA with no catechol added. Two fungus isolates were utilized in these tests; one (S isolate) growing characteristically on the surface of the culture medium, and the other (A isolate) producing abundant white aerial filaments.

5. Peroxidase tests

The benzidine test of van Duijn (in Pearse, 1960) was applied to detect the presence of peroxidase in elm sections as well as in the fungus. The method consists in incubating the plant material in the following mixture: One ml of a saturated aqueous solution of ammonium chloride, one ml of a five per cent aqueous solution of ethylenediaminetetraacetic acid (EDTA), nine ml of a saturated aqueous solution of benzidine, and one drop of a three per cent solution of hydrogen peroxide. The hydrogen peroxide is added just before placing the sections in the incubation mixture. Controls consist in sections incubated in the above mixture with no hydrogen peroxide added and in sections treated in the complete mixture to which one ml of 0.01 M potassium cyanide is added. The blue coloration, which turns brown and then colourless with time, indicates the presence of peroxidase.

(i) <u>In elm tissues</u> - Fresh and frozen (cryostat) materials were used in these tests, and also materials which had been infiltrated with celloidin solution in ether-alcohol (1:1) for periods of 18, 24, 36, and 48 hours.

The sections were transferred immediately after cutting to the reaction medium of benzidine-hydrogen peroxide, or the controls, as described above. But, it was soon realized that it was more convenient to place directly in the holes of a porcelain test plate one drop of the saturated ammonium chloride solution, one drop of the EDTA solution, nine drops of the benzidine solution, and one drop of hydrogen peroxide diluted to one per cent or 0.1 per cent. Another modification

of the technique consisted in adding the peroxide only after the sections had been for one minute in the otherwise complete incubation media.

The blue colouration began to appear in the incubation medium as soon as the sections were immersed. The reaction was allowed to proceed for different periods of time, washed in distilled water, mounted on microscope slides, and examined immediately.

The leuco-patent blue method of Dunn (in Pearse, 1960) also was tried for the demonstration of peroxidase activity. This method is based on the oxidation to a coloured product of a dye which is colourless in the reduced form. Before the test, the dye is reduced by boiling with powdered zinc and glacial acetic acid until the colour of the original solution disappears. The incubation medium was prepared by mixing the following reagents in the holes of a test plate: Ten drops of leuco-patent blue solution, two drops of glacial acetic acid, and one drop of diluted hydrogen peroxide. For control sections, the hydrogen peroxide was omitted in the incubation medium.

(ii) <u>In the fungus</u> - Since the benzidine method and its variants described above gave erratic results when applied to fungus colonies, the concentration of hydrogen peroxide in the incubation medium was varied in order to arrive at a good, repeatable coloured reaction. With the same quantities of the other reagents, one to ten drops, one ml, one and a half ml, and two ml of hydrogen peroxide were added to the incubation reagents as described in the original method. Finally a satisfactory procedure was found. It is described with the results. The leuco-patent blue method was used also.

6. Beta-glucosidase test

The method proposed by Pearse (1960) for the histochemical demonstration of beta-glucosidase is derived from the method used by Rutenberg et al (1958) for the demonstration of beta-galactosidase in animal tissues. Fresh sections of wood and fungus colonies were incubated for eight to 24 hours in a solution of 6-bromo-2-naphthyl-beta-D-glucopyranoside prepared in the following manner. Fifty mg of the glucoside was dissolved in 10 ml of methanol, diluted with 100 ml of distilled water at 70°C, and then the solution was allowed to cool at room temperature. It was further diluted with 40 ml of pH 5.0 phosphate-citrate buffer. After incubation in the glucoside substrate, the section and the fungus colonies were washed for three minutes in each of three changes of distilled water, transferred to a freshly prepared solution of Fast Blue B at 4°C, containing one mg of stain per ml of solution, and adjusted to pH 7.4 to 7.8. A final washing was made in cold water before mounting on microscope slides.

Tests were made also by dipping elm cuttings in the solution of glucoside, sectioning, and post-coupling with Fast Blue B.

7. Succinic dehydrogenase tests

In the tests for succinic dehydrogenase, the tetrazolium salt used was 2,2'-di-p-nitrophenyl-5-5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride, also called Nitro-BT or Nitro blue tetrazolium. This chemical acts as an hydrogen acceptor for succinic dehydrogenase, and is a blue insoluble formazan in the reduced state. The Nitro-BT solution was prepared by dissolving first the tetrazolium salt in a few drops of ethanol, and adding distilled water

slowly and with constant stirring until the desired volume was obtained. The malonic acid, a specific inhibitor for succinic dehydrogenase, used in the reagent mixtures was neutralized with concentrated sodium hydroxide.

The use of quinacrine was suggested by the electron pathway proposed by Hartree (reproduced by Pearse, 1960) in which it acts as a mid-chain block between the reduced nucleotide and the flavoprotein, consequently it should stop the reduction of the tetrazolium salt by coenzyme-dependent dehydrogenases. Phenazine methosulfate was reported by Pearse (1960) to speed up the initial velocity of the succinic dehydrogenase and diaphorase reactions, and for this reason it was included in some of the incubation media.

The following solutions were used at the volumes indicated in various combinations to test the activity of succinic dehydrogenase. The concentration given is that of the stock solution, not that of the compound in the complete reaction mixture.

0.25 ml sodium succinate (0.2 M)

0.25 ml Nitro-BT (0.5 mg per ml)

0.25 ml quinacrine hydrochloride (10^{-2} M)

0.25 ml phenazine methosulfate (5.0 mg per ml)

0.25 ml malonic acid (0.1 M)

0.2 M phosphate buffer, pH 6.5 or 7.6 to make total volume of 1.5 ml.

Stock solutions of these chemicals were kept at 4°C in a refrigerator. The action of these solutions in the test was studied by using them in the following 28 combinations.

1. Sodium succinate
   Nitro-BT
   Buffer

2. Sodium succinate
   Nitro-BT
   Malonic acid
   Buffer

3. Nitro-BT
   Buffer

4. Nitro-BT
   Malonic acid
   Buffer

5. Sodium succinate
   Nitro-BT
   Quinacrine
   Buffer

6. Sodium succinate
   Nitro-BT
   Quinacrine
   Malonic acid
   Buffer

7. Sodium succinate
   Quinacrine
   Buffer

8. Sodium succinate
   Quinacrine
   Malonic acid
   Buffer

9. Nitro-BT
   Quinacrine
   Buffer

10. Nitro-BT
    Quinacrine
    Malonic acid
    Buffer

11. Quinacrine
    Buffer

12. Quinacrine
    Malonic acid
    Buffer

13. Sodium succinate
    Nitro-BT
    Phenazine methosulfate
    Buffer

14. Sodium succinate
    Nitro-BT
    Phenazine methosulfate
    Malonic acid
    Buffer

15. Sodium succinate
Phenazine methosulfate
Buffer

16. Sodium succinate
Phenazine methosulfate
Malonic acid
Buffer

17. Nitro-BT
Phenazine methosulfate
Buffer

18. Nitro-BT
Phenazine methosulfate
Malonic acid
Buffer

19. Phenazine methosulfate
Buffer

20. Phenazine methosulfate
Malonic acid
Buffer

21. Sodium succinate
Nitro-BT
Quinacrine
Phenazine methosulfate
Buffer

22. Sodium succinate
Nitro-BT
Quinacrine
Phenazine methosulfate
Malonic acid
Buffer

23. Sodium succinate
Quinacrine
Phenazine methosulfate
Buffer

24. Sodium succinate
Quinacrine
Phenazine methosulfate
Malonic acid
Buffer

25. Nitro-BT
Quinacrine
Phenazine methosulfate
Buffer

26. Nitro-BT
Quinacrine
Phenazine methosulfate
Malonic acid
Buffer

27. Quinacrine
    Phenazine methosulfate
    Buffer

28. Quinacrine
    Phenazine methosulfate
    Malonic acid
    Buffer

A series of tests were conducted with each the two buffers mentioned, and also with fungal colonies that were previously immersed for one hour, in water at 80°C.

8. DPN and TPN diaphorases

These two flavoprotein enzymes, as mentioned by Pearse (1960) catalyzes the transfer of hydrogen and electrons from the reduced coenzymes, i.e. DPNH and TPNH, to the tetrazolium salt and no intermediate acceptor is required. The reduced coenzymes were used as substrates. The following volumes of stock solutions, at the concentrations indicated, of the various reagents were used in ten different combinations in the tests for DPN and TPN diaphorases:

0.3 ml Nitro-BT (5.0 mg per ml)

0.2 ml DPNH or TPNH (5.0 mg per ml)

0.3 ml quinacrine (10^{-2} M)

0.3 ml malonic acid (10^{-1} M)

0.3 ml phenazine methosulfate (5.0 mg per ml)

0.2 M phosphate buffer pH 6.5 or 7.6 to make the total volume of incubation media 1.5 ml.

The following combinations of reagents were mixed just before the tests.

1. Nitro-BT
   DPNH or TPNH
   Buffer

2. Nitro-BT
   DPNH or TPNH
   Malonic acid
   Buffer

3. Nitro-BT
   DPNH or TPNH
   Quinacrine
   Buffer

4. Nitro-BT
   DPNH or TPNH
   Quinacrine
   Malonic acid
   Buffer

5. Nitro-BT
   DPNH or TPNH
   Phenazine methosulfate
   Buffer

6. Nitro-BT
   DPNH or TPNH
   Phenazine methosulfate
   Malonic acid
   Buffer

7. Nitro-BT
   DPNH or TPNH
   Quinacrine
   Phenazine methosulfate
   Buffer

8. Nitro-BT
   DPNH or TPNH
   Quinacrine
   Phenazine methosulfate
   Malonic acid
   Buffer

9. DPNH or TPNH
   Phenazine methosulfate
   Buffer

10. DPNH or TPNH
    Phenazine methosulfate
    Malonic acid
    Buffer

9. DPN-linked dehydrogenases

Two DPN-dependent dehydrogenases were tested. These were malate dehydrogenase and lactate dehydrogenase. The substrate for the reaction consisted of solutions of L-malic or DL-lactic acids, final concentration 0.1 M, adjusted to pH 7.0 with 0.2 M Tris buffer, i.e. tris (hydroxymethyl) aminomethane. The incubation media were prepared by mixing the indicated volumes of the following stock solutions at the concentration mentioned.

0.1 ml substrate solution (1.0 M)

0.1 ml DPN solution (5 mg per ml)

0.1 ml sodium cyanide (0.1 M)

0.25 ml phosphate buffer, pH 7.5 (0.06 M)

0.25 ml Nitro-BT solution (1 mg per ml)

0.1 ml malonic acid (1.0 M)

0.1 ml distilled water.

In some tests, sodium cyanide was replaced by other respiratory inhibitors, namely: sodium azide, amytal, and quinacrine. The addition of malonate was to inhibit the reaction occurring in the absence of exogeneous substrate.

10. TPN-linked dehydrogenases

Malic decarboxylase and glucose-6-phosphate dehydrogenase were the two TPN-dependent dehydrogenases tested; the substrates for these enzymes were 1.0 M solutions of DL-malic acid and gluco-pyranose-6-phosphate disodium salt respectively. The incubation media were as follow:

0.1 ml substrate solution 1.0 M

0.1 ml TPN solution containing 5 mg per ml

0.25 ml tris buffer 0.2 M, pH 7.5

0.25 ml MTT solution containing 1 mg per ml

0.1 ml malonic acid 1.0 M

0.05 ml cobaltous chloride 0.5 M

As for the DPN-dependent dehydrogenase test, 0.1 ml of either sodium azide, amytal, or quinacrine were added to the incubation media as terminal or mid-chain blocks in the respiratory chain.

IV. RESULTS

A. Extent of Discolouration in Injected and Inoculated Elm Seedlings.

Healthy elm seedlings have uniform light coloured sapwood. Control plants injected with distilled water showed sapwood discolouration at the points of puncture. The largest discoloured zones did not exceed three mm in diameter from two days to two weeks after injection. The elm seedlings injected with veronal buffers or acetic-acid-phosphate buffers reacted as follows: At pH's 6.0, 7.0, and 8.0, there was no evidence of xylem discolouration beyond that due to puncturing; at pH 5.0 brown streaks extended an average of 12.6 cm above the points of injection and about five cm below (Fig. 3); at pH 4.0 brown streaks were formed in about 50 per cent of the plants, and they were usually less than five cm in total length, i.e. above and below the points of injection. In elms injected with solutions of ethyl alcohol brown streaks appeared in the sapwood and extended about 10 cm above and 2.5 cm below the points of injection. Discolouration developed much faster in elm injected with 20 per cent alcohol than in those injected with five and ten per cent.

The absorption of liquids was significantly reduced and even stopped after four hours in the seedlings which later on showed brown streaks, while the seedlings which received buffers at pH 6.0, 7.0, and 8.0 absorbed the liquid at about the same rate as distilled water during the whole injection period which lasted five days.

In artificially inoculated elm seedlings the wood discolouration was visible to the naked eye one week after inoculation,

Fig. 3. Brown discolouration in the xylem of a seedling injected with pH 5.0 veronal buffer. X 0.5

and gradually extended up to the leaves. Two weeks after inoculation almost all the sapwood above the inoculation points had become discoloured, and the plants were wilted.

The discolouration produced in injected elm seedlings was similar to that produced in inoculated trees. When examined under the microscope, the discolouration seemed to have been formed first in the ray cells and in the xylem parenchyma cells surrounding the vessels, and finally in the water conducting elements and even in the fibers. However, there was almost no brown material plugging the vessels of the injected plants while there was appreciable plugging of vessels by brown materials in infected specimens.

B. Pathological Alterations of the Cell Walls

The structure of the cell walls was affected as a result of infection. The first modification notices was a yellowing at the bordered pits, and a swelling of the pit membrane (Fig. 4). This was followed by a swelling of the walls of ray cells, vasicentric parenchyma cells, and vessels. The walls of fibers appeared to be somewhat thinner than those in healthy wood tissues.

The middle lamella, particularly in the advanced stages of the disease was pathologically altered. Tissues that had been infected for two weeks were very difficult to section with the microtome, especially in the transverse direction, because the cells separated more easily than those of healthy tissues. Even longitudinal sections had a tendency to split apparently because the middle lamella was more or less destroyed.

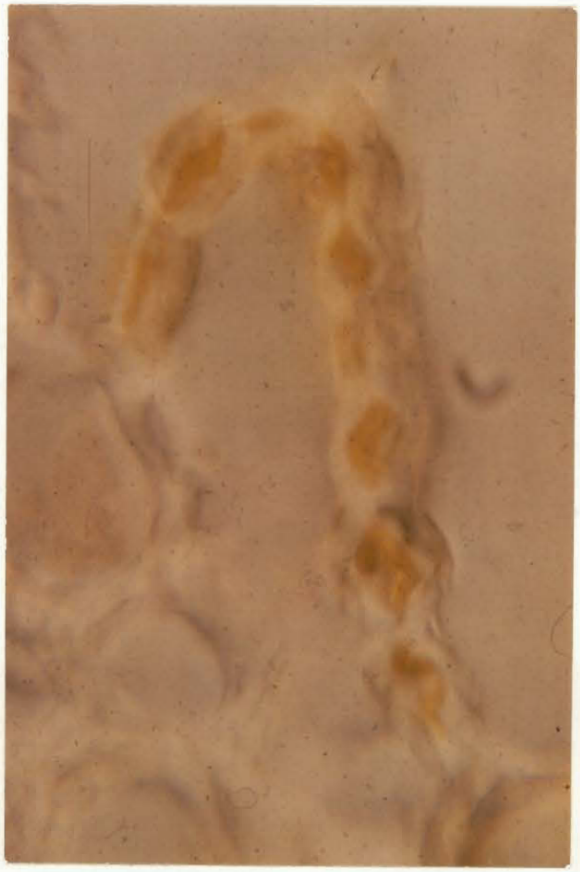


Fig. 4. Cross section through the stem of a diseased elm seedling showing yellowing end swelling of pit membranes. X1500

Many other pathological alterations were detected by means of histochemical tests. They are described below.

C. Tests for Lignin

1. Comparison of different procedures

The technique described by Sass (1957) was used first but gave unsatisfactory results. The reaction was not uniform over the whole section. This was apparently due to an uneven distribution of hydrochloric acid. According to this technique one drop of this reagent is placed at one edge of the cover glass, and allowed to diffuse between the slide and the cover glass. Consequently the colouration started on one side of the section and progressed slowly across the section. When the reaction began to show on the side of the section opposite to the starting point, it had already begun to fade out at its initial point. However, this technique permitted to observe the development of the reaction and locate the lignin in the cell walls, but it was hardly possible to compare the amount of lignin in two different parts.

When the hydrochloric acid solution was mixed previously with the phloroglucinol reagent a more uniform reaction was obtained; and it gave a brilliant red violet colour supposedly specific for lignin. Therefore, this modification of the test was used to determine the topological distribution of lignin in tissue sections of healthy and of diseased elms.

The chlorine-sulfite and the Schiff's reaction tests were not satisfactory. The chlorine-sulfite test produced little reaction, if any, in the cell walls. Occasionally, a pale rose reaction appeared

in the walls of spring vessels, but this colouration faded out rapidly. With Schiff's reagent, the test was negative also, even when the incubation period was extended to 24 hours. The period of incubation recommended by Jensen (1962) is 15 minutes to 4 hours.

2. On healthy elms

The reaction in the phloroglucinol-hydrochloric acid test was negative in the walls of cells formed in the spring when sections were tested at the end of June, and negative also, throughout the growing season in the walls of newly formed cells. But a positive reaction was obtained in the middle lamellae of ray cells, parenchyma cells, and fibers formed early in the growing season, when the tests were performed in mid-summer (Figs. 5 and 6, Pl. 1). At the same time a strong positive reaction developed in layers in the walls of spring vessels (Figs. 5, 7 and 8, Pl. 1). At the end of the growing season a positive reaction was evident in the middle lamella of all cells; even those near the cambium reacted positively. A positive reaction was obtained also in the wood of previous years, as the middle lamella and layers of the secondary walls stained red.

3. On diseased elms

In general the location of lignin in diseased elm trees was similar to that in healthy elms. However, the reaction was much stronger in the walls of diseased spring vessels when tested at the end of June than in walls of corresponding vessels in healthy trees (Fig. 9, Pl. 2). Tests made in mid-summer showed that the reaction was strong in the walls of summer vessels and in the middle lamella

of spring and early summer elements (Figs. 10 and 11, Pl. 2). At the end of the summer, when growth had about ceased, the reaction was much stronger in diseased elms than in healthy trees as can be seen by comparing Figs. 7 and 8 to Figs. 9 and 10. Reaction in laminations of cell walls was also more evident. A reaction was also observed in some of the particles of the materials plugging the vessels (Figs. 11 and 12, Pl. 2).

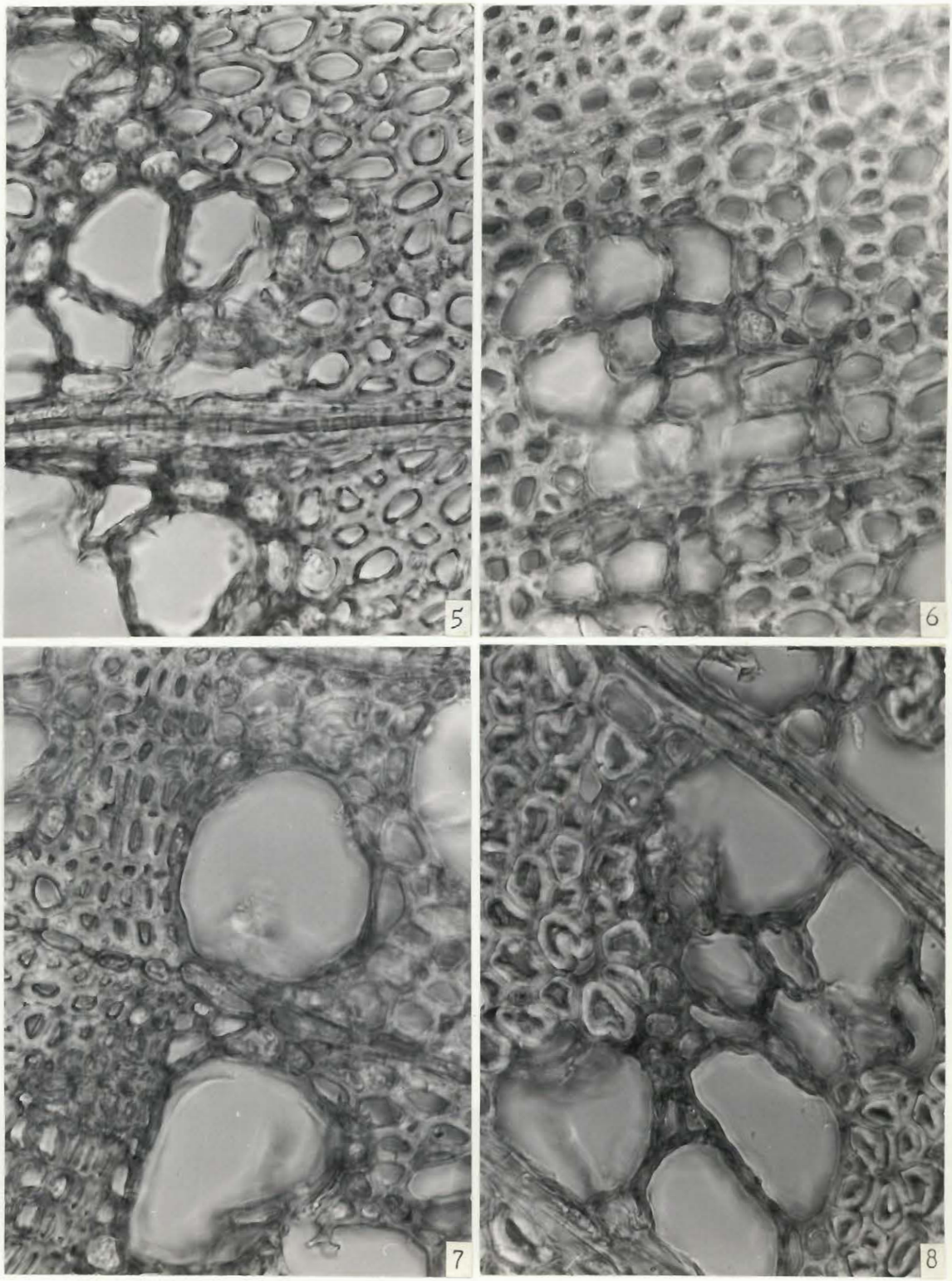
Figs. 5 - 8. Cross section through stem of healthy elm seedlings showing the reaction in phloroglucinol test for lignin performed in mid-summer. X650

Fig. 5. Reaction of layers of vessel walls and of middle lamella between ray cells, fibers and longitudinal parenchyma cells.

Fig. 6. Reaction of layers of walls of summer vessels and middle lamella between cells.

Figs. 7 and 8. Reaction of layers of walls of spring vessels.

Plate 1



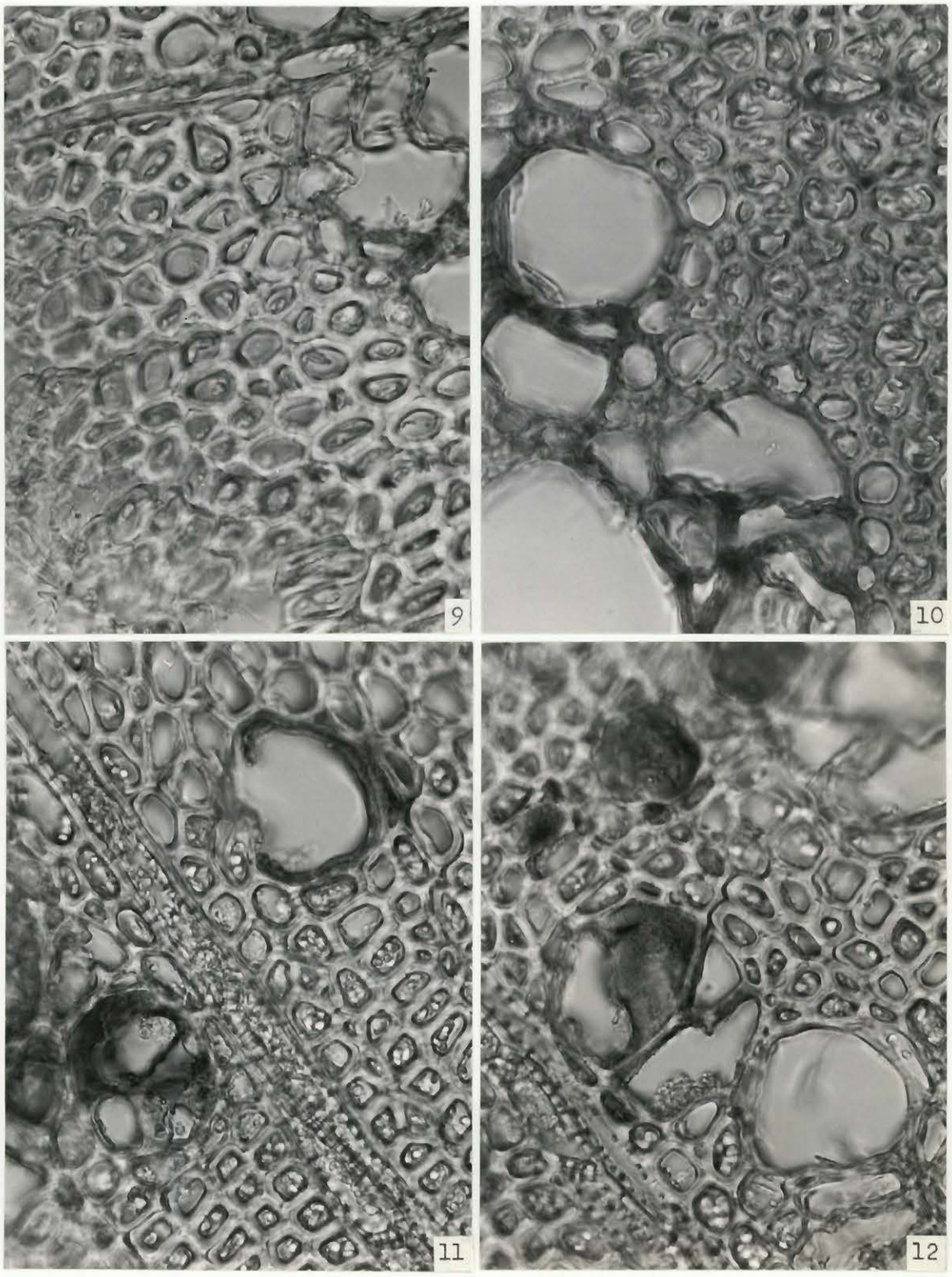
Figs. 9 - 12. Cross section through the stem of diseased elm seedlings showing reaction in phloroglucinol test for lignin. X650

Fig. 9. Test performed at the end of June. Note the reaction of lamella between cells and of walls of vessels.

Fig. 10. Test performed in mid-summer. Note the strong reaction of layers of cell walls near spring vessels, and of middle lamella of summer cells.

Figs. 11 - 12. Test performed in mid-summer. Note the strong reaction of particles in materials plugging the vessels.

Plate 2



D. Tests for Pectins

1. Comparison of different procedures

The hydroxylamine-ferric chloride test gave a pale reddish colouration located mainly in the middle lamellae in healthy tissues. Doubling the time of incubation in either one or both the hydroxylamine and the ferric chloride solution did not improve the intensity of the reaction.

Esterification of the pectins by one-hour treatment in boiling methanol containing 0.5 N hydrochloric acid, or overnight in a similar solution at room temperature changed completely the picture of the reaction to subsequent treatment with hydroxylamine and ferric chloride reagents. Cell walls were completely stained pink, but the middle lamellae had a somewhat more reddish colour. There appeared to be very little differentiation in pectin content of different layers of cell wall in healthy tissues. However, in injected tissues, there was a more or less definite striation due to differences in the intensity of the colour reaction in the vessel walls, thus differentiating various layers. In some cases the material plugging the vessels gave a pink reaction, but in most cases it remained yellowish brown in colour, which can have masked the pink colour reaction.

De-esterification of the pectins in control sections by a five-minute treatment with 1% per cent sodium hydroxide in 60 per cent ethanol resulted in almost complete absence of colour reaction in the hydroxylamine-ferric chloride test. On the whole, this procedure, although it yielded useful indications on the nature and distribution of pectins in healthy and diseased woody tissues of elms, was not quite satisfactory because of the faintness of the colour that developed.

The Krajcinovic method, which depends on the formation of an addition product between pectic acids and benzidine, and the formation of an azo dye after diazotization of the free NH_2 group from benzidine when treated with beta-naphthol in alkaline solution, gave a fairly strong reaction. The technique described in Pearse's book (1960 p. 840) calls for a two-minute treatment in one per cent hydrochloric acid to liberate pectic acid for reaction with benzidine. Trials of 1, 2, 3, 5, 10 and 20-minute treatments in HCl revealed that with our material, the five-minute treatment gave the best final results in most cases. Increasing further the duration of the treatment to 10 and 20 minutes did not increase appreciably the intensity of the colour reaction, nor modify its location.

2. On healthy elms

In the healthy wood of the current year's growth, the middle lamella of differentiating cells at the end of June stained light red. There was very little reaction in the walls of fibers and medullary rays, except for a hardly detectable pinkish colour in the middle lamellae. A strong red reaction was observed in the vessel walls, especially those formed early in the spring. Similarly, the walls of the parenchyma cells surrounding the vessels gave a strong reaction (Figs. 13 and 14, Pl. 3). In all cases, the middle lamellae were intensely coloured, and cell walls with alternating layers of pale and dark colour were clearly visible (Figs. 15 and 16, Pl. 3).

Towards the end of the growing season there was a strong reaction in the walls of spring and summer vessels, and xylem parenchyma cells. A somewhat less intense, but still distinct reaction occurred in the walls of fibers formed in late summer. This reaction appeared to be located mainly, but not exclusively, in the middle lamella. The reaction in the wood of previous growth rings was similar to that in the xylem elements of the current year at the end of the growing season.

3. On diseased elms

Tissues of diseased trees reacted, in pectin tests, somewhat differently than tissues of healthy trees. The colour seemed to be more intense, and to develop faster in diseased than in healthy tissues. Maximum reaction was obtained with a two-minute treatment in HCl whereas a five-minute treatment was needed for healthy wood. The colour reaction was particularly intense in the walls of spring vessels and parenchyma cells around them soon after infection (Fig. 17, Pl. 4). As the disease progressed, the colour reaction became visible in some ray and vasicentric parenchyma cells (Fig. 18, Pl. 4), and in the vessels (Figs. 19 and 20, Pl. 4) besides the reaction in middle lamella and layers of vessel walls normally found in healthy wood. Furthermore, pectic materials appeared to be swollen in several infected vessels as mentioned previously (Figs. 19 and 20, Pl. 4). In some cases, this swelling had reduced the vessel lumina considerably. Lamellation of walls was much more evident in diseased than in healthy tissues as can be seen by comparing Figures 14 and 18. At the end of the growing season, the reaction was strong in the walls

of cells formed in late summer. There was a strong reaction in the vessel walls of the apparently healthy summer wood that was formed over the pathologically discoloured spring wood in infected young trees. A positive pectin test reaction was observed in the materials contained in those vessels.

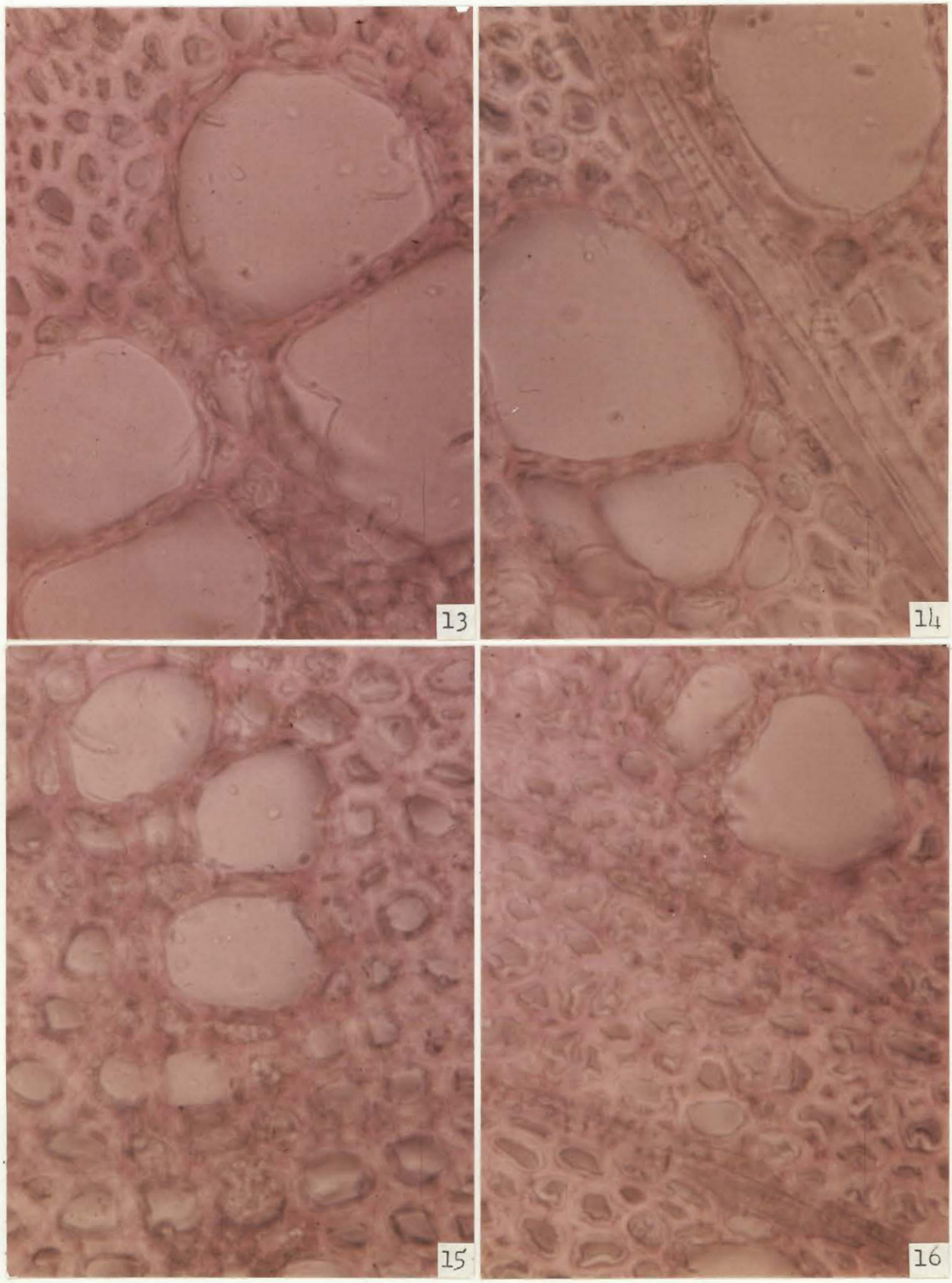
Figs. 13 - 16. Cross sections through stem of healthy elm seedlings showing reaction in test for pectins. X650

Fig. 13. Note reaction of cell walls of spring wood test at the end of June.

Fig. 14. Note reaction of vessel walls and middle lamellae of spring wood tested in mid-summer.

Figs. 15 - 16. Note reaction of layers of cell walls of summer wood tested in mid-summer.

Plate 3



Figs. 17 - 20. Cross section through stem of diseased elm seedlings showing reaction in tests for pectins. X650

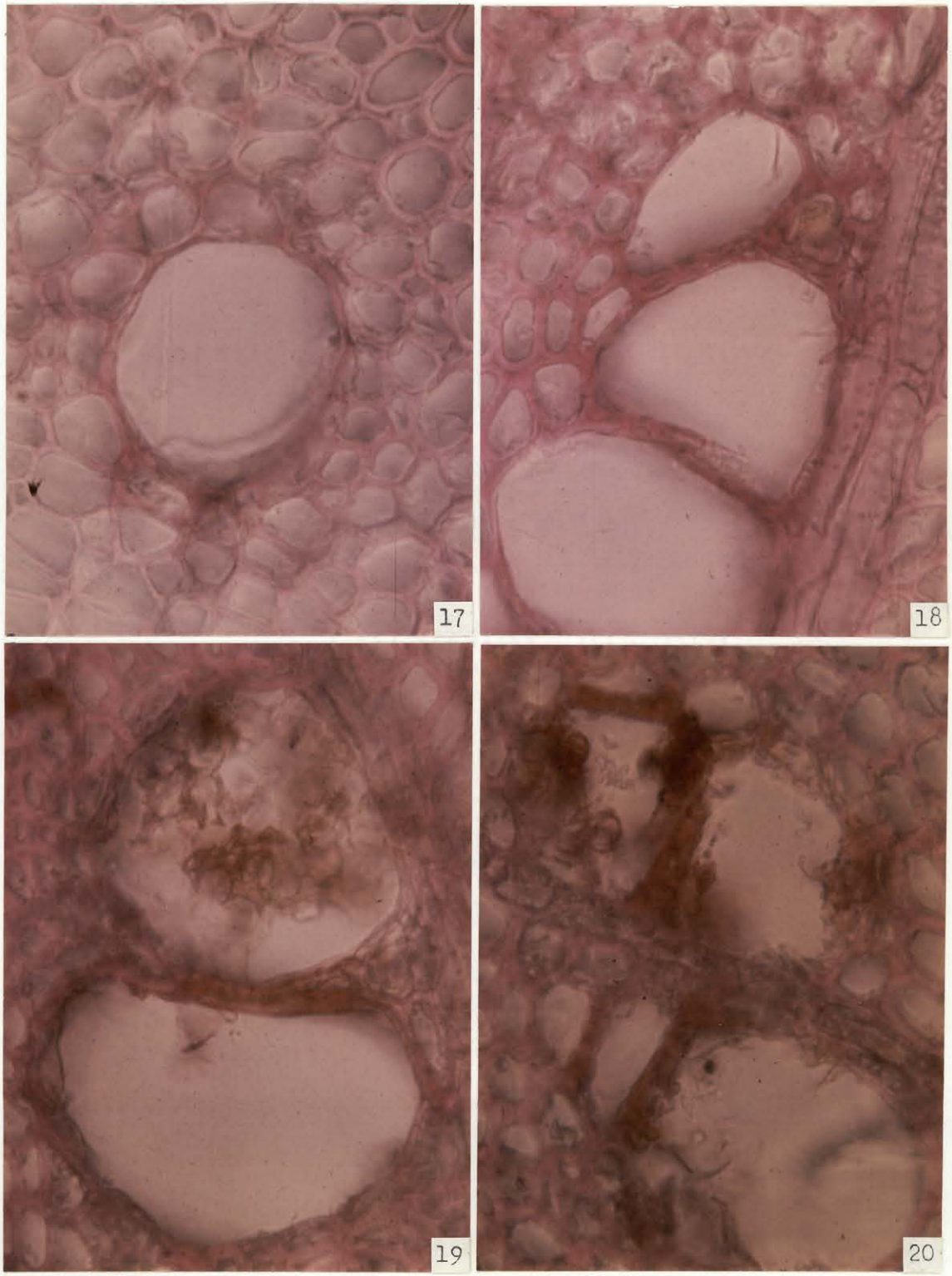
Fig. 17. Note the reaction of middle lamella of cells near the vessel and of the vessel wall.

Fig. 18. Note the strong reaction of cell walls of recently infected tissues.

Fig. 19. A more advanced stage of infection than above. Note reaction of vessel plugging material.

Fig. 20. Reaction of disintegrating cell walls that are swollen.

Plate 4



E. Tests for Polyphenols

1. Comparison of different procedures

The nitroso reaction gave yellow to red and brown colourations in sections of wood. The pattern of distribution of polyphenols in sections cut at room temperature was identical to that of sections cut in a cryostat at -20°C. Therefore, most of the work was performed at room temperature. Care was taken to immerse the sections in the reagents immediately after cutting. Thus, the browning due to oxidation in the air was avoided because it took at least two minutes for this browning to become noticeable.

There was no difference in the general histological distribution of polyphenols between sections cut from fresh tissue samples, those cut from samples dehydrated in a series of alcohol, and those cut from similarly dehydrated and subsequently embedded in paraffin. However, in the last two kinds of samples, the cells that gave a positive reaction contained roughly spherical globules (Fig. 21, Pl. 5), whereas the cellular content was more evenly distributed in fresh material.

2. On healthy elms

The concentration of polyphenol, as it could be visually estimated from the nitroso reaction, was very low in the xylem of healthy elms. A pale pinkish reaction was found in newly differentiated xylem tissues and in cambial cells, indicating the presence of polyphenols in very low concentration. Usually older cells were negative although the cell walls had a very slight yellowish colouration. Occasionally, a red colour reaction was observed in some ray

cells, and still more rarely in parenchyma cells surrounding the vessels (Figs. 21 and 22, Pl. 5). Stained cells occurred with increasing frequency in medullary rays with increasing closeness to the cells surrounding the pith, i.e. the cells of the medullary sheath, where the red colour reaction was the most intense (Figs. 23 and 24, Pl. 5). The proportion of the cells that gave a positive reaction increased in the inward direction from the younger to the older rings of wood, particularly in wood from two to four years of age.

3. On diseased elms

In infected seedlings, and in twigs of diseased older trees, the reaction in the medullary sheath was similar to that in the medullary sheath of healthy elms, but in the other parts it was quite different. A yellow colouration was produced at the bordered pits in the vessels near the point of inoculation, as early as two days after inoculation. Later on, this positive reaction was obtained in advance of the progressing discolouration visible to the naked eye. Control sections in distilled water had a less visible colouration at the vessel pits.

As the disease developed further a red colouration was produced in ray cells (Fig. 25, Pl. 6) and in longitudinal parenchyma cells near the inoculation point, indicating a greater concentration of polyphenols in these cells than in corresponding cells in healthy trees, and in uninfected parts of diseased trees. Five days after inoculation, polyphenols could be detected in apparently large amounts in almost all the ray cells and the xylem parenchyma cells surrounding

the vessels, for about five inches above the points of inoculation (Figs. 26 and 27, Pl. 6). At that time, discolouration was not yet clearly visible to the naked eye, although corresponding untested sections examined microscopically revealed that the protoplasm in these cells had started to develop a granular appearance and was somewhat discoloured. At that time also, some vessels and fibers gave a positive reaction (Fig. 28, Pl. 6). Later on, as the disease progressed, the vessels became more abundantly filled with compounds that gave various shades of yellow, red, and brown colours in the test for polyphenols. The fibers nearest the infected vessels gave a dense red colour reaction. A similar reaction spread to fibers more and more distant from the vessels as the disease developed, until finally almost all cells in dying trees appeared to be filled with polyphenols (Fig. 31, Pl. 7).

Pits seemed to serve as passage ways for the movement of polyphenolic compounds from one cell to another, particularly after pits had been disintegrated by the fungus. In many cases it was possible to trace the polyphenol positive materials in the vessels back to the nearby xylem parenchyma or ray cells (Figs. 29-30, Pl. 7). Tyloses that protruded from those cells and gave a positive reaction occurred once in a while. In advanced stages of the disease, few tyloses were distinguishable, because they were more or less disintegrated. A positive reaction was commonly observed in layers of cell walls as shown in Figs 31 and 32.

In the brownish discoloured streaks formed after injection of alcohol solutions and of pH 5.0 buffers, the test for polyphenols

was positive and gave reactions similar to those described for infected xylem parenchyma and ray cells. The protoplasm of these cells was granular and turned to various shade of red and brown colours. However, there was much less accumulation of polyphenols in the fibers, the vessels, and the tracheids of these plants than in those of the infected plants. The cambial region, on the other hand, seemed to be more affected in the injected plants than in the infected ones.

Fig. 21. Cross section through healthy elm stem showing positive reaction in the polyphenol test of ray cells. Note the near spherical globules. (Paraffin embedded tissues) X750

Fig. 22. Cross section through healthy elm stem showing a more intense positive reaction than above in the polyphenol test of ray cells near the pith, and of longitudinal parenchyma cells near vessels. (Paraffin embedded tissues) X750

Fig. 23. Cross section through healthy elm stem showing positive reaction in the polyphenol test of cells near the pith. Note that some of the fibers are filled with polyphenol-positive materials (Paraffin embedded tissues) X750

Fig. 24. Cross section through healthy elm stem showing positive reaction in the polyphenol test of the cells of the medullary sheath (Paraffin embedded tissues). X750

Plate 5

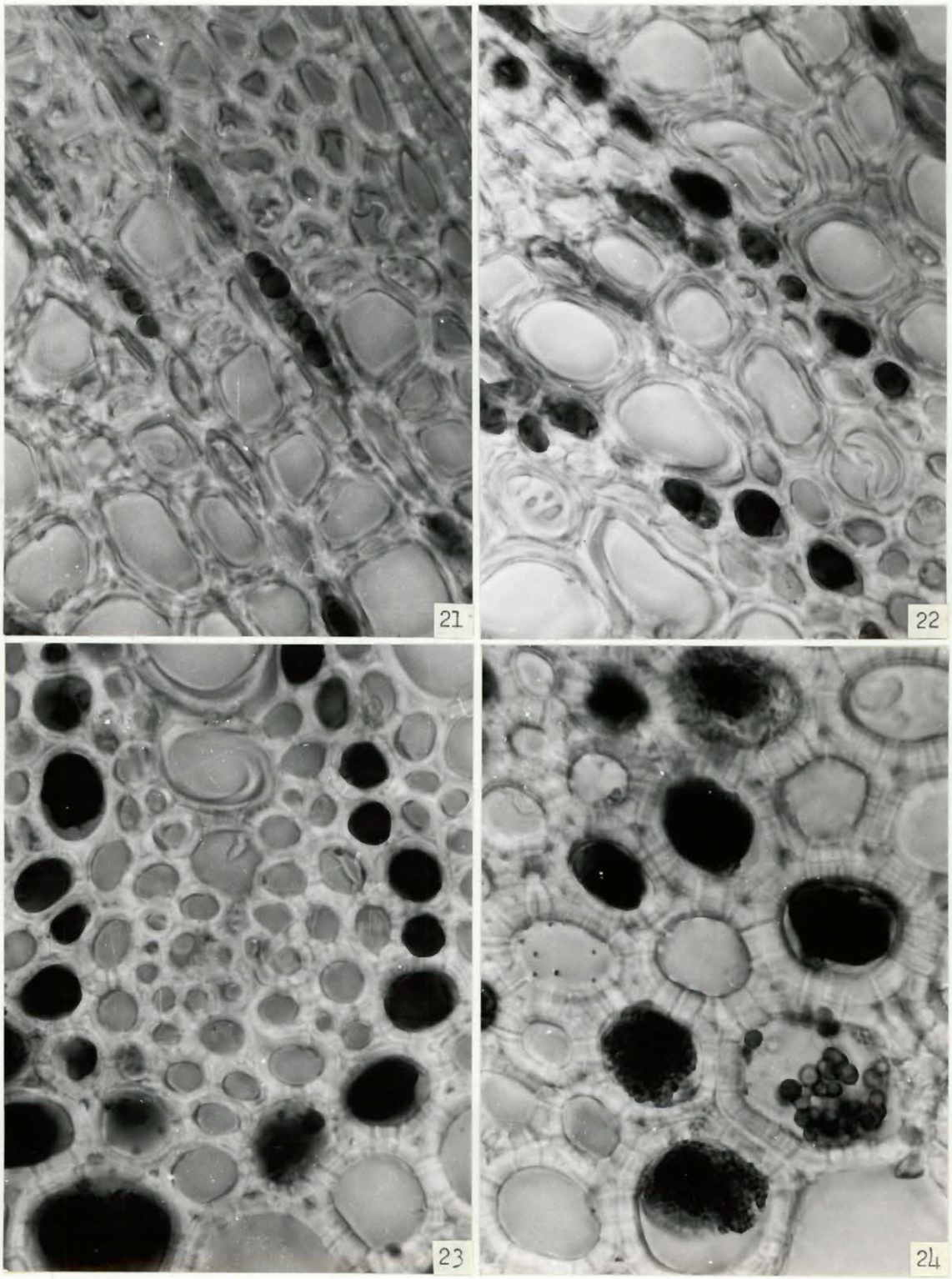


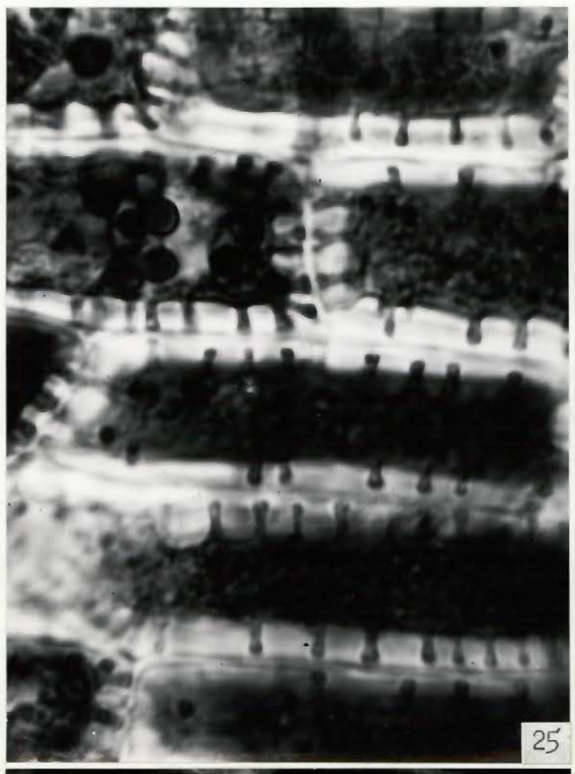
Fig. 25. Longitudinal section through a diseased elm stem showing the positive reaction in the test for polyphenols of the ray cells. X1350

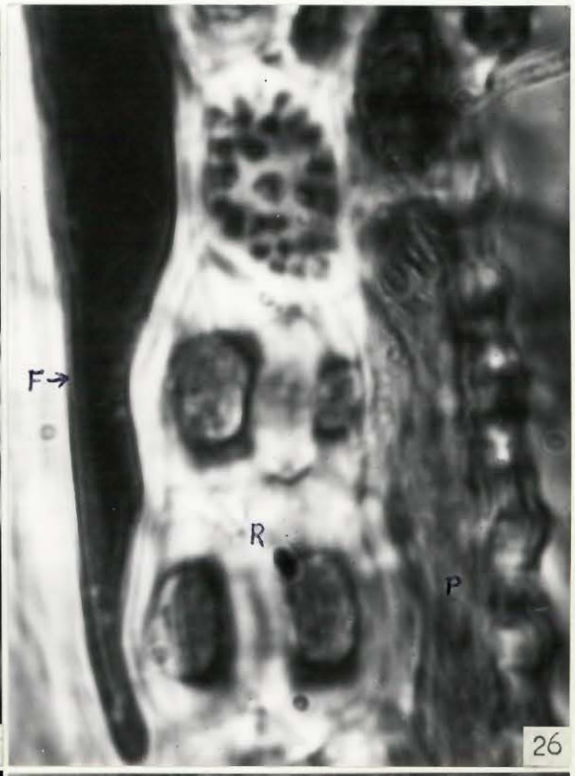
Fig. 26. Tangential section through a diseased elm stem showing the reaction in the test for polyphenols of longitudinal parenchyma cells (P), ray cells (R) and fibers (F) X1350

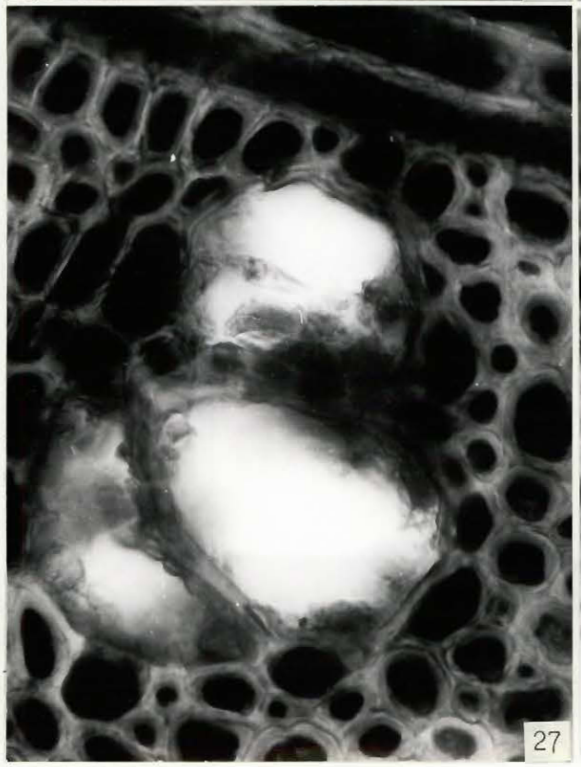
Fig. 27. Cross section through a diseased elm stem showing the reaction in the test for polyphenols of ray cells, longitudinal parenchyma cells, fibers, and vessels. X650

Fig. 28. Cross section through a branch of a diseased elm showing the reaction of fibers to the test for polyphenols. X650

Plate 6







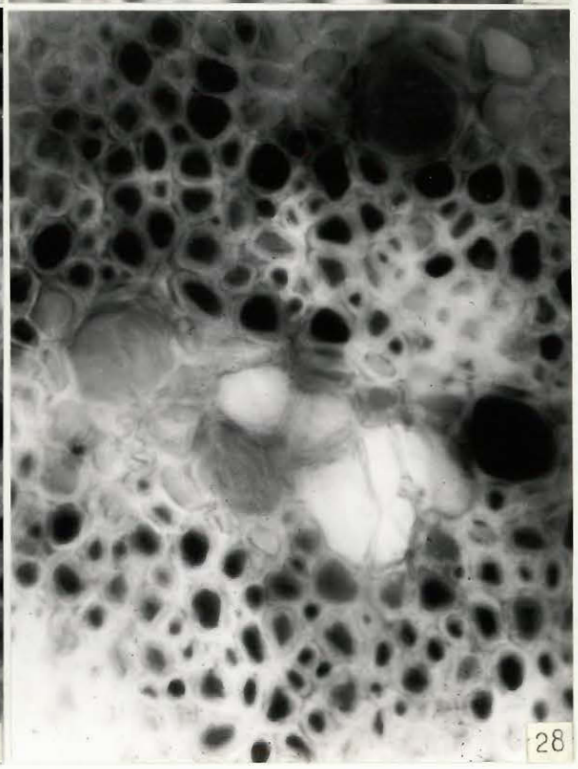


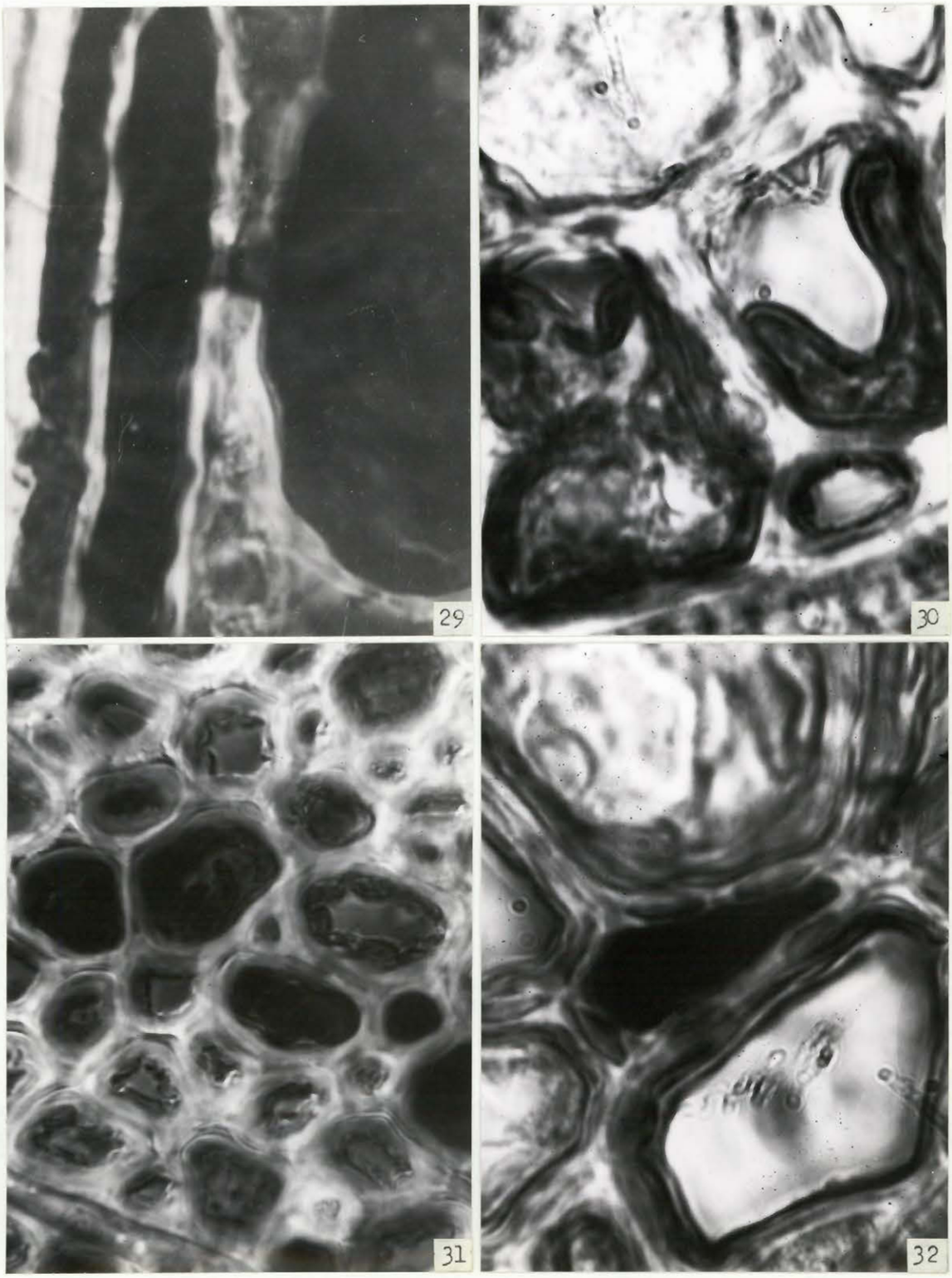
Fig. 29. Cross section through the stem of an infected elm seedling showing the continuity of polyphenol containing materials in two ray cells and a vessel. X1350

Fig. 30. Cross section through the stem of an infected elm seedling showing continuity of polyphenol containing materials in two longitudinal parenchyma cells and a vessel. Note the positive reaction close to the wall in another vessel. X900

Fig. 31. Cross section through the stem of an infected elm seedling showing positive reaction in test for polyphenols. Note the reaction in fibers and in some cell walls. X750

Fig. 32. Cross section through the stem of an infected elm seedling showing polyphenol reaction in fibers and in layers of the cell walls, particularly those of vessels. X900

Plate 7



F. Tests for Phenol Oxidases

1. Comparison of different procedures

Observations with the naked eye on elm sections revealed that the colour reactions did not develop equally well with all the substrates used. Sections in resorcinol and p-cresol gave very weak straw colour reactions that appeared after more than one hour of incubation. Also tyrosine, orcinol, and hydroquinone reacted slowly, giving light brown colourations of the sections after one hour of incubation. With pyrogallol a brilliant yellow colour reaction that turned to yellowish brown and then to brown took place rapidly. But some oxidation of the pyrogallol solution occurred spontaneously in the absence of wood sections, particularly at pH 6.5 and above. This oxidation was even faster with the addition of neutral solutions of the enzyme inhibitors potassium cyanide and sodium azide, although somewhat less with the latter than with the former. These results clearly showed that at least part of the oxidation of pyrogallol was non-enzymic.

Pyrocatechol gave the fastest tissue reaction of all the phenolic compounds tried. The colour was greenish at first, then brown, and finally it turned almost black. The period of incubation was usually limited to 90 minutes, but the colour developed much earlier. Incubation extending to eight hours did not show any spontaneous oxidation in solutions exposed to air with no wood sections as was the case for pyrogallol solutions. But exposure to the air in the holes of a porcelaine test plate, at room temperature for 24 hours showed oxidation. This oxidation could have been due to air

contaminants, because no oxidation occurred in solution kept in a bottle standing on the same laboratory table for the same length of time.

Pyrocatechol appeared to be the most suitable substrate. It was chosen as substrate in further experiments carried out to find the most favourable conditions for the histochemical tests. The results of other experiments on the effect of pH and of inhibitors on the development of colours in catechol solutions and incubation solutions are recorded in Table I. Ten sections were used in each treatment. The experiment was carried out three times.

Table I. Indices[a] of the colour density of solutions at pH from 5.5 to 7.5 after 90 minutes of incubation with (+) and without (-) sections of wood.

		pH				
Solutions	Sections	5.5	6.0	6.5	7.0	7.5
Catechol	+	5	5	7	9	9
Catechol	-	0	0	0	0	0
Catechol + KCN	+	2	2	3	5	5
Catechol + KCN	-	0	0	0	0	0
Catechol + NaN_3	+	1	1	2	3	5
Catechol + NaN_3	-	1	1	2	2	5
Water	+	0	0	0	0	0

a) 0 indicates no colour; 1 to 9 indicate densities from light to dark brown.

The greatest colour intensities were obtained when elm sections were incubated in pyrocatechol without inhibitors. No colour appeared, at any of the pH levels tested, in the catechol solutions standing without elm sections, nor in water in which wood sections were incubated. On the other hand, a colour developed in pyrocatechol to which either potassium cyanide, or sodium azide was added, when elm sections were incubated in them. But, contrary to the catechol-potassium cyanide solutions that remainded colourless for the duration of the experiment when no elm sections were incubated in them, a pink colour appeared in the catechol-sodium azide solutions even in the absence of elm materials. This table shows also the effect of pH on the density of the colour in the pyrocatechol solutions. When a brown colour was formed, it was intensified as the pH of the solution was increased. Maximum colour intensity was obtained with neutral and alkaline solutions.

The time required for the appearance of colour in catechol solutions of various pH levels and in the elm sections incubated in them is given in Table II. These results were obtained with ten sections in each treatment, and in four experiments. In general, the colour developed as fast in the sections as in the solutions. The time required for the appearance of the colour decreased as the pH was increased from 5.5 to 7.5. The optimum pH was 6.5, because it was at that pH that the greatest difference between treatments was detected. Potassium cyanide and sodium azide partially inhibited the colour development. However, the latter appeared to be a more powerful inhibitor than the former. But a pink colour that formed

spontaneously in the solutions containing sodium azide and pyrocatechol probably masked the early indications of enzymatic oxidation of pyrocatechol. Therefore, a solution of pyrocatechol at pH 6.5 was used in subsequent tests, because it permitted a fairly short incubation period for colour development relative to that for controls with inhibitors.

Table II. Time (minutes) required for the appearance of colour in incubation solutions (S), at pH from 5.5 to 7.5, in presence of wood, and in wood sections (W) incubated in them.

Solutions	Wood (W) or Solution (S)	pH 5.5	6.0	6.5	7.0	7.5
Catechol	W	20	15	3	1½	1
Catechol	S	20	15	3	1½	1
Catechol + KCN	W	60	45	25	20	15
Catechol + KCN	S	60	45	25	20	15
Catechol + NaN_3	W	(a)	(a)	(a)	40	15
Catechol + NaN_3	S	90	60	50	40	25
Catechol + NaN_3 (without wood)	S	90	90	60	40	30

(a) No colour after 90 minutes

2. On healthy elms

Phenol oxidase, as indicated by the oxidation of pyrocatechol, was particularly active in newly formed cells of the xylem of healthy trees, as shown in Fig. 33, Pl. 8). Cells near

the cambium, ray cells, and vasicentric xylem parenchyma cells of spring wood turned black when sections were incubated in pyrocatechol. A similar distribution of the activity was observed in the spring wood when tested at the end of June. But when tested in mid-summer the distribution was somewhat different: A strong reaction took place in parenchyma cells surrounding the spring vessels, and at the pits of the vessels (Fig. 34, Pl. 8); a weaker reaction in some of the fibers (Figs. 34-36, Pl. 8); and a variable one in the ray cells of the spring wood (Fig. 36, Pl. 8).

The reaction was located mainly in the protoplasm of all these cells, although cell walls, especially vessel walls, exhibited a reaction localized in their inner layer and their pits (Figs. 33-36, Pl. 8).

3. On diseased elms

The distribution of phenol oxidase activity in diseased elms was similar to that in healthy elms as far as newly formed cells were concerned. Almost all cells near the cambium turned black on incubation with pyrocatechol whether tested at the end of the spring or in mid-summer (Fig. 37, Pl. 9). However, there were some differences in the intensity and in the location of the activity in the older elements of the xylem in the test performed in mid-summer. In general, the reaction was somewhat stronger in the ray and parenchyma cells of the spring wood of diseased elms than in those of healthy trees (Fig. 38, Pl. 9). But the greatest difference was the occurrence of activity in the materials plugging the vessels of diseased trees (Figs. 39 and 40, Pl. 9).

A similar distribution of phenol oxidase activity was obtained when pyrogallol, instead of pyrocatechol, was used as a substrate.

Figs. 33 - 36. Cross sections through the stem of healthy elm seedlings showing the reactions in tests for phenol oxidase. X650

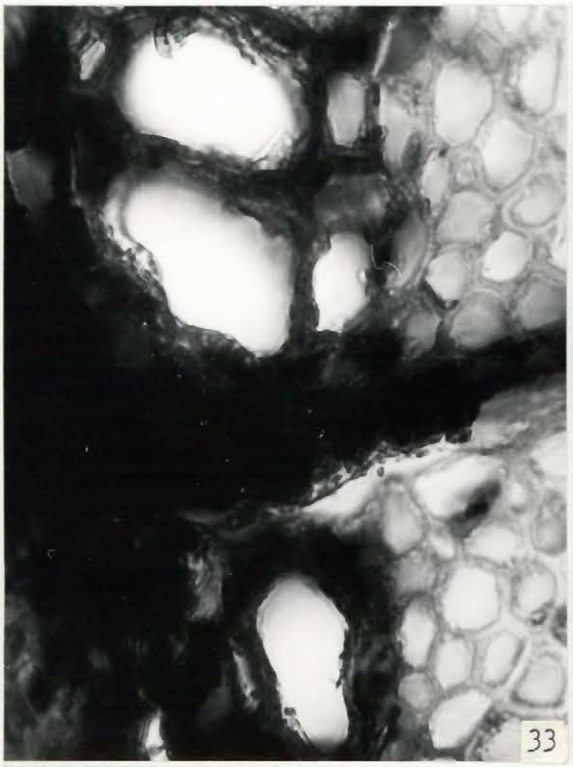
Fig. 33. Note the dark colour reaction of newly formed cells, in parenchyma cells around the vessels, and in ray cells. Test made at the end of June.

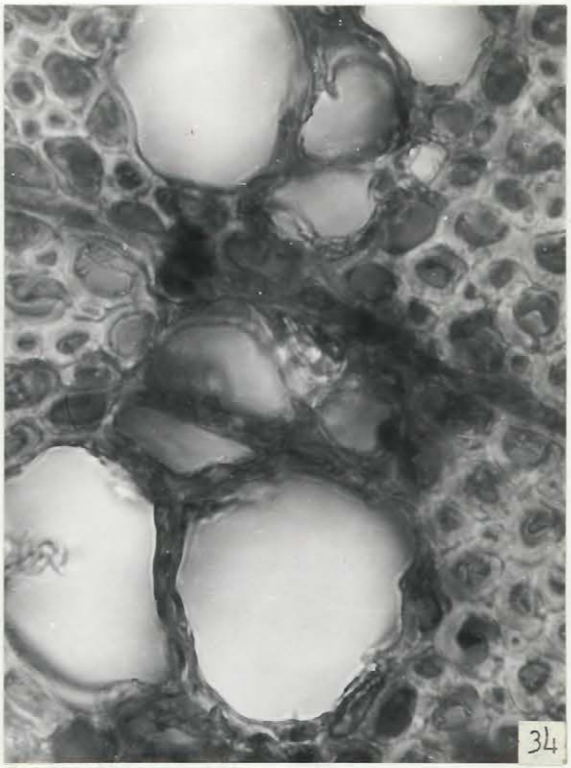
Fig. 34. Reaction of vasicentric parenchyma and ray cells of springwood tested in mid-summer. Note reaction also of pits of spring vessels, and a weaker reaction in some fibers.

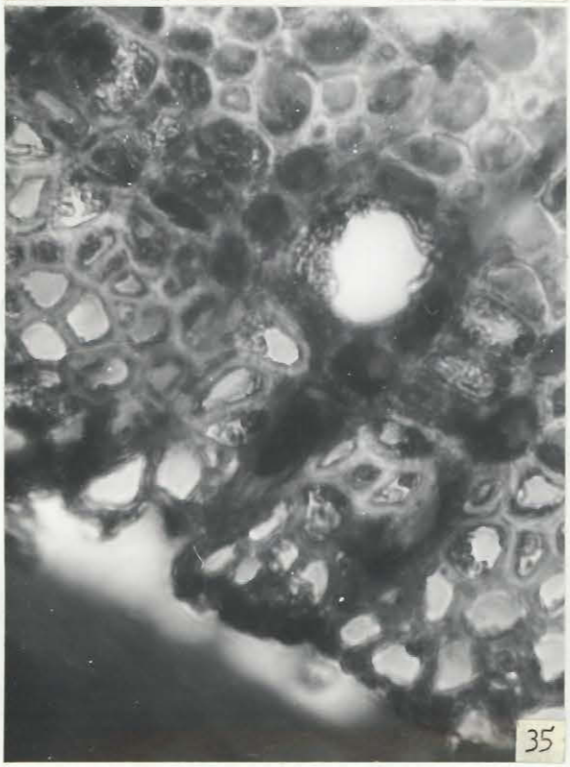
Fig. 35. Reaction of vasicentric parenchyma cells and fibers of summer wood tested in mid-summer.

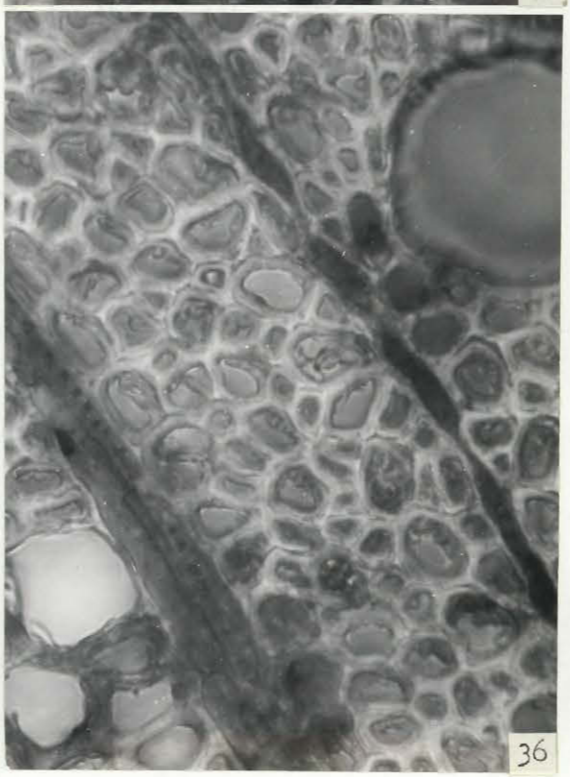
Fig. 36. Variable reaction of ray cells of spring wood tested in mid-summer. Note reaction also of inner layer of a vessel.

Plate 8









Figs. 37 - 40. Cross sections through the stem of diseased elm seedlings showing reactions in tests for phenol oxidase. X650

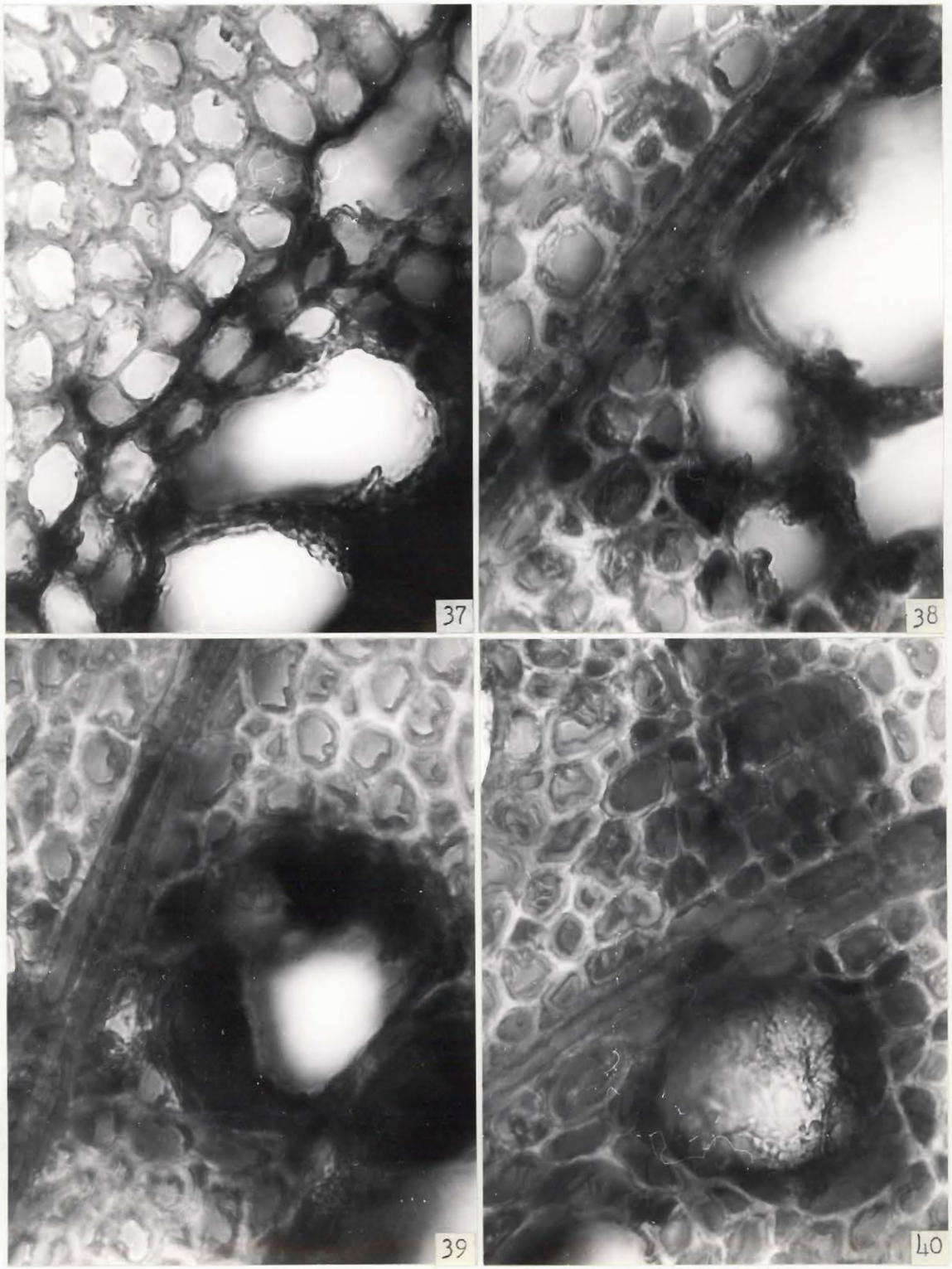
Fig. 37. Reaction of newly formed cells in tests performed in mid-summer. Note the reaction of cell walls and protoplasm of living cells.

Fig. 38. Reaction of ray and vasicentric xylem parenchyma cells of spring wood tested in mid-summer.

Fig. 39. Reaction of vasicentric parenchyma cells and of material within the smaller vessel.

Fig. 40. Reaction of material within an infected vessel.

Plate 9



4. On fungus colonies

As indicated before, incubation of colonies of C. ulmi in pyrocatechol and other phenolic compounds gave negative results, i.e. no colour developed. However, by culturing the fungus on PDA media containing five different concentrations of pyrocatechol ranging from 0.0001 M to 0.025 M, striking effects on the growth and colour of the fungus were obtained.

At the highest concentration (0.025 M) of pyrocatechol the growth of both isolates A and S was completely inhibited, whether the pyrocatechol had been incorporated into the substrate before or after sterilization in the autoclave.

The growth obtained at the other concentrations of pyrocatechol incorporated before autoclaving the culture media is summarized by the growth curved illustrated in Figs. 41 and 42. Two diameters of each colony in five plates were measured in mm, at right angle of each other, daily from the third to the twelfth day, and the average diameters were plotted. The experiment was repeated three times. It can be seen in comparing Fig. 41 to Fig. 42 that the two isolates behaved differently. The colony of isolate A, which produces aerial filaments, increased in diameter at a somewhat slower rate than that of isolate S, which is surface growing. Perhaps more significant is the fact that colony extension by isolate A was retarded significantly by all concentrations of pyrocatechol, except the lowest (0.0001 M), whereas that of isolate S was significantly inhibited only at the 0.005 M concentration and above. The growth on the medium containing 0.001 M pyrocatechol followed closely

that on the control with no pyrocatechol. Moreover, the fungus appeared to be slightly stimulated by 0.0001 M and 0.0002 M concentrations.

When the pyrocatechol was added aseptically in the culture media after sterilization in the autoclave, the results were somewhat different, as evidenced by the growth curves shown in Figs. 43 and 44. No stimulatory effect was obtained with the isolate S as it happened when pyrocatechol was added to the culture media before sterilization. In general it appeared that the inhibition was stronger in all cases. This is shown in Table III where the colony diameter in each treatment is expressed in percentage of that of the corresponding controls on the 12th day.

Table III. Diameter (per cent of control) of 12-day old colonies of C. ulmi grown on PDA containing different concentrations of pyrocatechol added before and after autoclaving the culture media

Pyrocatechol concentration	Addition before autoclaving		Addition after autoclaving	
	Isolate A	Isolate S	Isolate A	Isolate S
Control	100	100	100	100
0.0001 M	96	103	83	91
0.0002 M	82	109	70	90
0.001 M	68	97	68	65
0.005 M	49	42	41	25
0.025 M	0	0	0	0

Fig. 41. Growth of isolate A on PDA and PDA added with four different concentrations of pyrocatechol before autoclaving.

Fig. 42. Growth of isolate S on PDA and PDA added with four different concentrations of pyrocatechol before autoclaving.

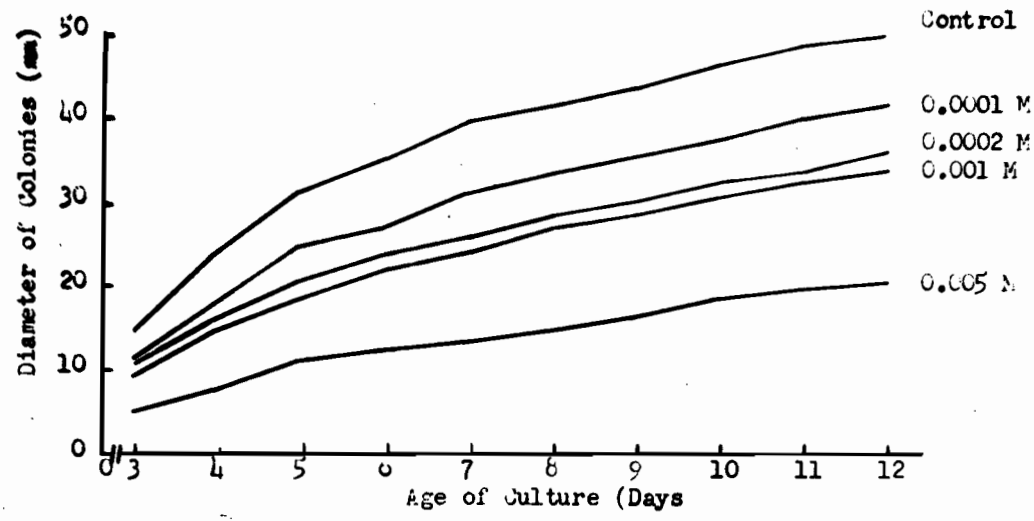


Fig. 43. Growth of isolate A on PDA and PDA added with four different concentrations of pyrocatechol after autoclaving.

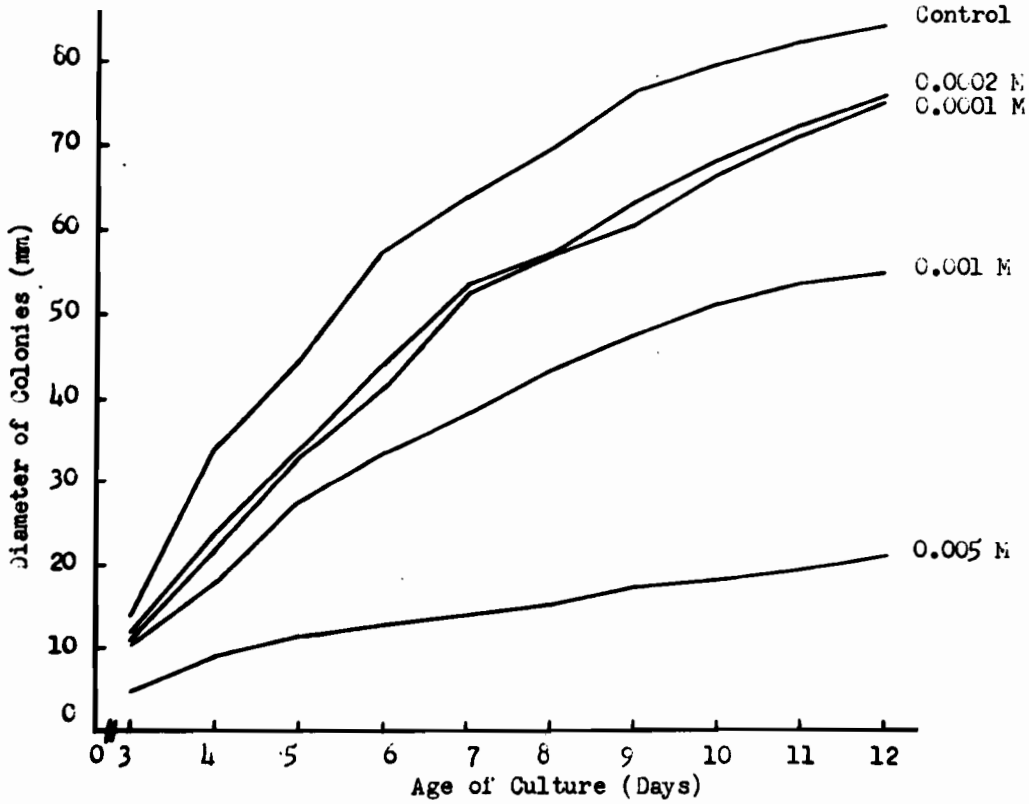


Fig. 44. Growth of isolate S on PDA and PDA added with four different concentrations of pyrocatechol after autoclaving.

Another interesting reaction of isolate S grown on PDA added with pyrocatechol was the formation, in the mycelium, of a dark brown colouration mostly visible under the colonies after ten to 15 days of growth. Figure 45 shows two cultures of isolate S. The one without pyrocatechol on the left remained white, the other, on the right, was grown on a PDA containing 0.0002 M pyrocatechol, and was dark brown. Brown pigment was found in the medium and in the fungus. The pigmentation, in varied intensity, was observed in all the cultures of isolate S when grown on media containing pyrocatechol, whether this compound was added to the media before or after autoclaving. The isolate A did not produce this brown pigment under these conditions.

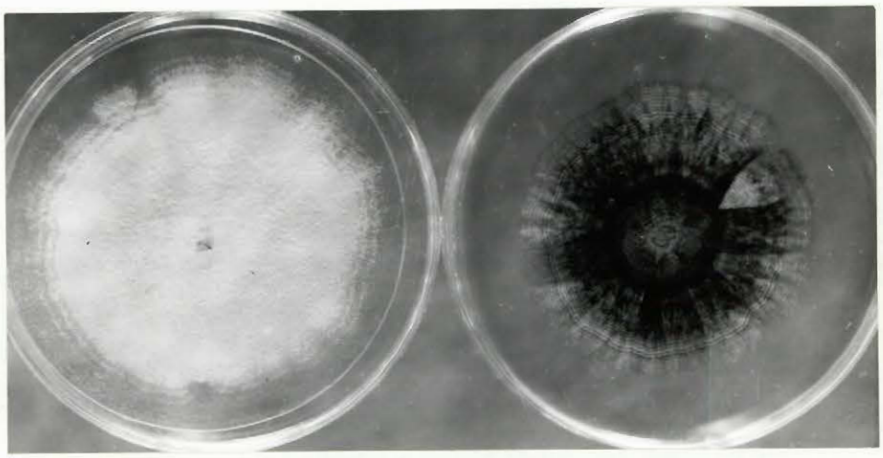


Fig. 45. Culture of Ceratocystis ulmi, isolate S, grown on PDA with no pyrocatechol (left), and on PDA containing 0.0002 M pyrocatechol (right).

G. Tests for Peroxidase

1. Comparison of different procedures.

The benzidine reagent solution started to become deep blue immediately after the sections were immersed in the incubation medium. Crystals began to form in the solution within one hour of incubation of the wood sections. The reaction in the solution appeared to be faster when sections from healthy elms were incubated than when sections from diseased trees were tested. However, the reaction, in the solution, with sections from healthy wood could be slowed down to about the same speed as that with sections from diseased plants when pieces of branches or stem for healthy elm trees were previously immersed overnight in a four per cent solution of celloidin in ether-alcohol (1:1). This decreased the diffusion of the enzyme from the sections into the solution. With shorter celloidin impregnation treatment (18 hours) less colour appeared in the tissues, but the reaction in the solution was faster than with the treatment for 24 hours. With 36 hours of impregnation in celloidin solution, the colour reaction was greatly reduced, both in the tissues and in the incubation solutions, whereas with a 48 hours treatment, the colour reaction was completely prevented.

Two different colour reactions were observed in the sections: a blue reaction and a violaceous brown reaction. Neither of these reactions appeared in sections incubated in the reagent solution without hydrogen peroxide, or when potassium cyanide was added to the complete incubation media.

2. Location of the reactions in elm tissues

The brown reaction was found mainly in the cell walls. The intensity of the colour was the strongest in the cambial region of the xylem, and decreased gradually inwards, although occasionally group of cells in the two- or three-year-old growth rings gave as strong a reaction as the cells near the cambium. There was little difference in colour intensity of the cell walls of ray cells, fibers, xylem parenchyma cells or vessels of the same age. There was no appreciable difference either in the intensity of the reaction between sections of healthy and of diseased elms, as seen in comparing Fig. 46 to Fig. 47 (Pl. 10).

The blue reaction on the other hand was almost exclusively located in the protoplasts. Contrary to the brown reaction, the blue colour was much more intense and widespread in infected xylem tissues than in non-infected ones. This difference was observed not only between sections from diseased trees and those from healthy elms, but also between sections from infected and those from non-infected parts of diseased trees. However, the blue reaction occurred also in healthy wood that had been impregnated with celloidin (Fig. 46, Pl. 10). The best results were obtained with sections from the first two millimeters of segments of branches or stems immersed in a celloidin solution for 24 hours. With these, as mentioned before, the rate and intensity of the colour reaction taking place in the incubating solutions of sections from healthy stems were equal to those obtained in the solutions in which sections

from diseased elm trees were incubated. Concerning the reaction in the tissues, it appeared that it was somewhat less pronounced in celloidin-impregnated sections from healthy wood than in comparable sections from infected tissues.

The cells that showed the strongest reaction to the benzidine test in healthy tissues were the cambium, ray, and parenchyma cells surrounding the vessels (Fig. 48, Pl. 10).

In infected tissues, the reaction occurred in cambium cells, ray cells, xylem parenchyma cells, fibers, and in occluded vessels (Fig. 49, Pl. 10, Figs. 50 and 51, Pl. 11). The latter two were negative in healthy stems except for very young fibers. Also the colour in the cambium and parenchyma cells was more intense in the infected than in noninfected tissues. The results of these tests are summarized in Table IV.

Table IV. Indices of colour density in the xylem cells of healthy and diseased elms after peroxidase test.

Type of cell	Healthy elm		Diseased elm
	Fresh tissues	Celloidin impregnated tissues (24 hours)	
Cambium	2[a]	4	5
Ray	1	3	4
Parenchyma	1	3	4
Fiber	0	0	3
Vessels	0	0	4

(a) 0 indicates no colour; 1 to 5 indicate densities from light to darker blue.

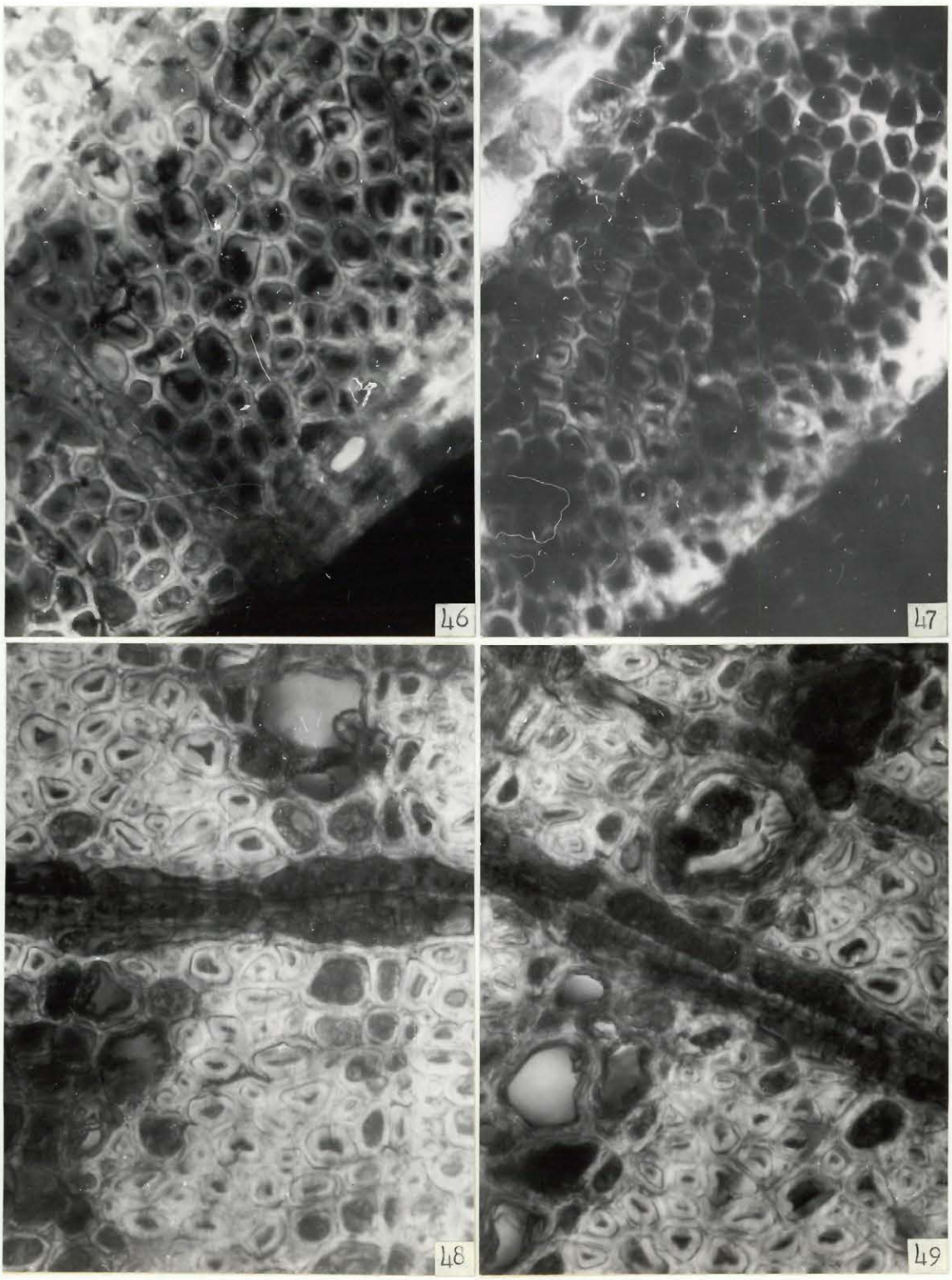
Fig. 46. Cross section through the stem of a healthy elm seedling showing the reaction of newly formed cells in test for peroxidase. Note the blue colour inside cells and the brown reaction of the cell walls. Tissues impregnated with celloidin for 24 hours. X650

Fig. 47. Cross section through the stem of a diseased elm seedling showing the reaction of newly formed cells in benzidine test for peroxidase. X650

Fig. 48. Cross section through the stem of a healthy elm seedling showing blue reaction in benzidine test for peroxidase of ray cells, vasicentric parenchyma cells and a few fibers. Tissues impregnated in celloidin for 24 hours. X650

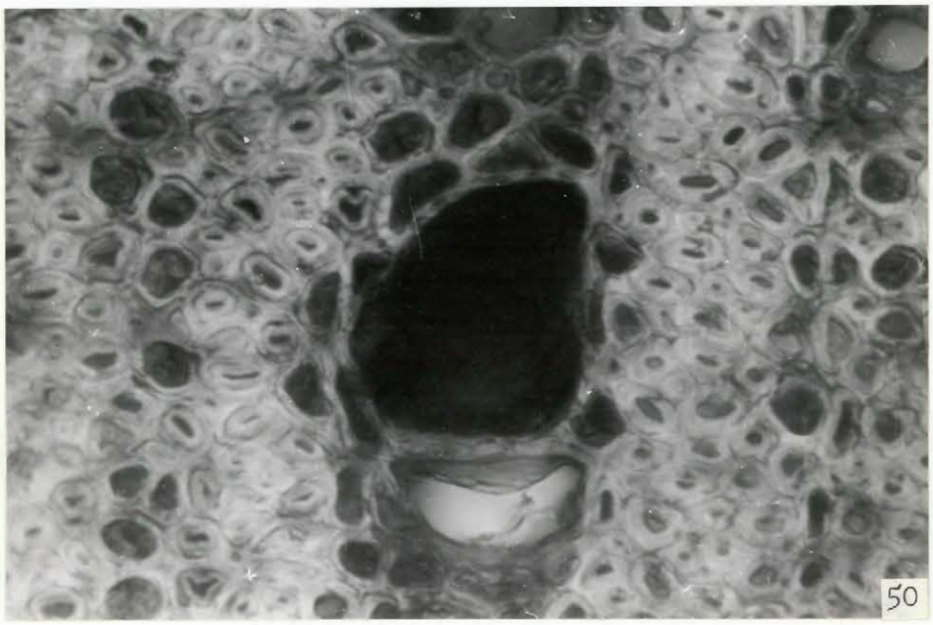
Fig. 49. Cross section through the stem of a diseased elm seedling showing blue reaction in benzidine test for peroxidase of ray cells, vasicentric parenchyma cells, fibers, and vessels. X650

Plate 10

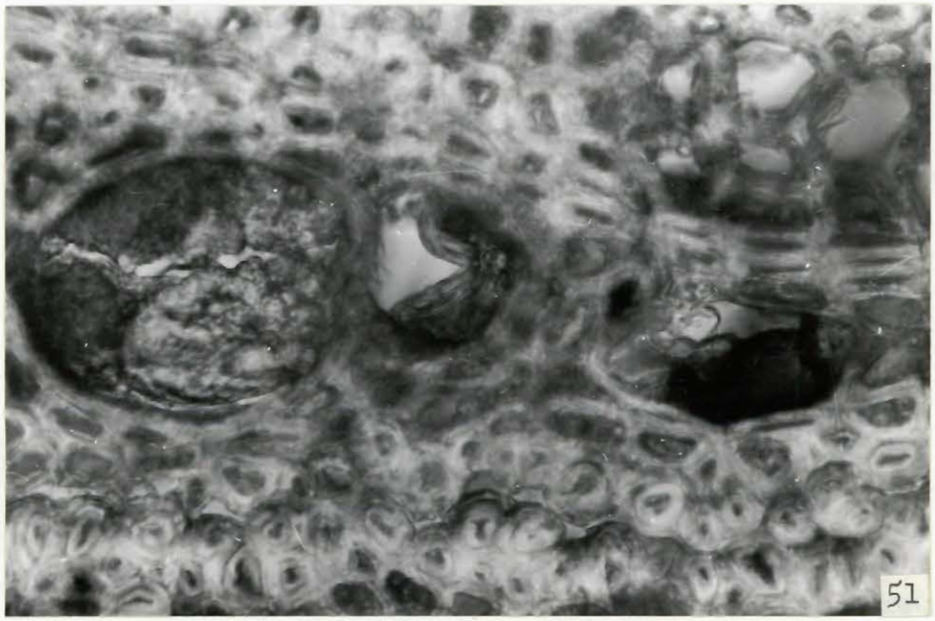


Figs. 50 - 51. Cross sections through stem of diseased elm seedlings showing reaction in benzidine test for peroxidase. Note the variation in blue reaction of the material plugging large vessels. X650

Plate 11



50



51

3. Colour reaction in fungus colonies

The benzidine test as described by van Duijn (Pearse, 1960) did not give satisfactory results. The reaction, if any, was slow to appear, and it developed in irregular spots in the fungus colonies. However, by increasing the amount of hydrogen peroxide in the incubation medium, the typical blue colour reaction of partly oxidized benzidine was obtained fairly regularly and more uniformly in the colonies. This colouration gradually turned to a violaceous brown colour, and then to a yellowish brown colour after about four hours. This last colour had all but disappeared after 24 hours.

Tests with increasing concentrations of hydrogen peroxide, from one drop to two ml in a total of 11 ml of complete incubation medium permitted to determine the amount of hydrogen peroxide required for the best reaction. With less than 0.5 ml of hydrogen peroxide in the incubation medium, the reaction was poor; because colouration appeared only occasionally, and was limited to spots in the fungus colonies. With 0.5 ml of hydrogen peroxide, the reaction was sometimes very good, but most often the blue colouration developed only at the margin of the colonies. With 1.0 ml, the reaction was the best, as far as it could be determined with the naked eye. The blue colouration began to show after five minutes of incubation, and reached its maximum development after about 15 minutes. With 1.5 and 2.0 ml of hydrogen peroxide in the incubation medium, gas bubbles evolved when the fungus colonies were immersed in the media, and the blue colouration appeared only after 25 minutes of incubation. The reaction reached its maximum intensity after one hour.

Frozen colonies were tested as well, on the assumption that freezing and thawing would increase the permeability of the fungal cells to the reagents. Although the colonies appeared blue in the incubation medium when seen with the naked eye, almost no blue colouration could be detected within the fungus structures under the microscope. The incubation medium itself had become blue, but not the fungus. Apparently the peroxidase had diffused out of the fungus into the medium.

The reaction was inhibited by potassium cyanide and sodium azide as shown in Fig. 52. This photograph, which was taken on an infrared film with a red filter (Kodak Wratten 25 A), shows the reaction of four colonies of the fungus to the benzidine test. The colour development in colonies 2 and 4 was completely inhibited by six drops of 0.01 M sodium azide.

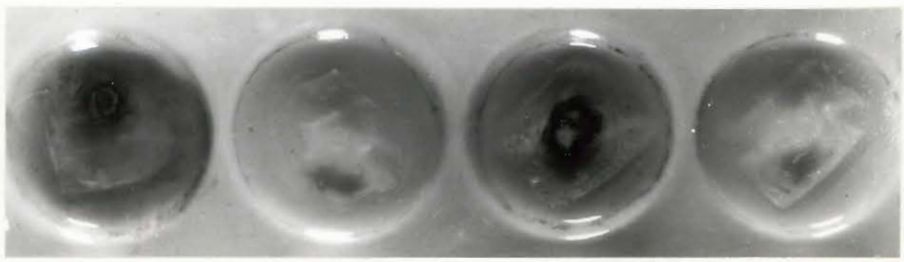


Fig. 52. Reaction of four colonies of C. ulmi in the benzidine test for peroxidase. Note the complete inhibition of the reaction by sodium azide in second and fourth colonies (from left to right).

The evolution of gas bubbles from the incubation medium during the tests for peroxidase was apparently due to the decomposition of hydrogen peroxide by the enzyme catalase. This enzyme could possibly interfere with the test for peroxidase by decomposing the substrate. By using different amounts of sodium azide, which is an inhibitor of both catalase and peroxidase, it was found that sodium azide, at very low concentrations, inhibited catalase partially, i.e. less gas bubbles were evolved. Peroxidase activity, on the other hand, was not reduced at these low concentrations of inhibitor; on the contrary, the colour reaction developed faster and more uniformly in the fungus colonies. The procedure finally adopted involved the addition of one or two drops of 0.01 M sodium azide to an incubation medium that contained eleven drops of the other reagents.

4. Location of peroxidase in the fungus

Peroxidase was found in spores and in hyphae. In tests without sodium azide, the blue reaction product appeared as small, more or less regular granules or globules. Some hyphae showed a densely coloured granulations, others had only a few blue particles (Figs. 53 and 54, Pl. 12). Younger hyphae had apparently more peroxidase activity than older ones, and the granulation usually was smaller in the older than in the younger mycelium. However, this distribution pattern can be a result of the catalase activity which appeared to be higher in the centre of the colonies than at the margin. In some cases, the highest peroxidase activity, as determined by the number and size of the coloured particles and the intensity of the colouration, was observed a few microns from the hyphal tips. A

strong peroxidase activity was observed also in the conidiophores, particularly near the points of attachment of the spores.

When one or two drops of sodium azide were added to the test reagents, the colour reaction was not restricted to small particles, but it appeared as large irregular patches in the protoplasm (Figs. 55-57, Pl. 12). Again with this technique, there was a great variation in the reaction of different hyphae and spores to the benzidine reagent. Spores and hyphal cells varied from colourless to almost completely stained (Figs. 55-58, Pl. 12).

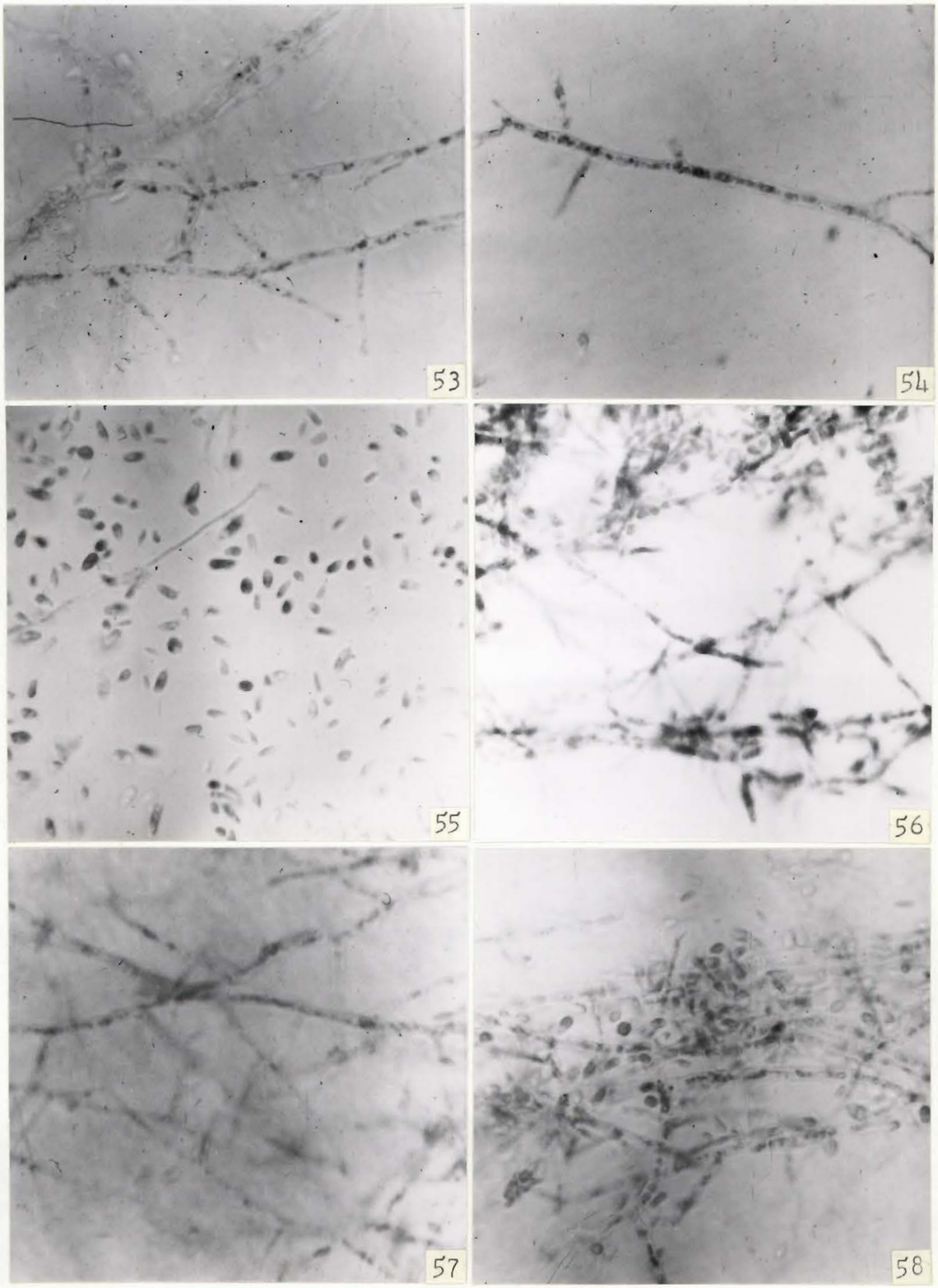
The leuco-patent blue test did not give satisfactory results. Varying the hydrogen peroxide concentration of the incubation medium did not improve the results, contrary to the reaction with the benzidine reagent. A grayish blue colouration developed in the leuco-patent blue incubation medium when left overnight exposed to the air at room temperature. But this reaction was not due to the peroxidase activity of the fungus, because it appeared also in the medium in the absence of the fungus. Therefore, it is doubtful that this reaction was enzymic.

Figs. 53 - 58. Reaction of Ceratocystis ulmi in benzidine test for peroxidase. X650

Figs. 53 and 54. Note the variation in the intensity of the blue colouration that appeared on definite structures resembling microendospores in young hyphae. No sodium azide in the incubation medium.

Figs. 55 - 58. Note the variation in the intensity of reaction in spores and hyphae. No definite structures were stained when sodium azide was added to the indubation medium.

Plate 12



H. Tests for Beta-Glucosidase

The test suggested by Pearse (1960) for the demonstration of beta-glucosidase was applied to sections from diseased and from healthy elms, and to colonies of C. ulmi grown on pieces of cellophane foil laid upon PDA in Petri plates. No colour reaction could be obtained in either cases. The conditions of incubation were varied in attempts to obtain a positive colour reaction. Materials were incubated in the glucoside solution for periods ranging from eight to 24 hours without success. Changing the pH of the incubating solution from 5.0 to 7.0, and incubating at 37°C instead of at room temperature did not change the negative results. Sections obtained from elm cuttings stood in a solution of the glucoside for up to 24 hours, did not developed a colour reaction after subsequent incubation in Fast Blue B. It was noted, however, that very little solution was absorbed by these cuttings relative to the amount of water taken up by similar cuttings stood in distilled water. So the failure of this procedure to demonstrate beta-glucosidase activity might be attributed to an insufficient amount of the glucoside translocated by the cuttings to the site of the beta-glucosidase. However, this would not explain the negative results obtained with the first procedure where a lack of substrate is less likely to happen.

I. Tests for Succinic Dehydrogenase

1. Comparison of different procedures

The tests for the demonstration of succinic dehydrogenase activity were applied to colonies of C. ulmi grown on cellophane foil.

During the incubation of the fungus colonies, reagent mixtures14, 16, 17, 18, 19, 20, 24, 26, 27 and 28 (see pages 24-26) turned brown and formed a fine precipitate. This colouration and precipitation was apparently due to a reaction of phenazine methosulfate, since all the above mixtures contained this compound; however, other reagent mixture, 13, 15, 21, 22, 23 and 25, also containing phenazine methosulfate did not show this reaction. Further evidence that this reaction was due to phenazine methosulfate was obtained later, when the same colouration and precipitation took place in test tubes containing mixtures of phenazine methosulfate and malonic acid kept at room temperature. But the colouration and precipitation cannot be attributed to a reaction of malonic acid with phenazine methosulfate, since they were present in mixtures 17, 19 and 27 which did not contain malonic acid. However, the reaction developed much slower in the last three mixtures than in the others. This reaction might be due to a small change in the pH of the medium. All the other incubation mixtures containing phenazine methosulfate, 13, 15, 21, 22, 23 and 25 turned to greenish blue and blue.

The addition of quinacrine solution, which is yellow, to incubation mixtures containing phenazine methosulfate tended to accentuate the green colouration. In other media, quinacrine had apparently no effect on the colour reaction.

The reduction of Nitro BT, a colourless ditetrazolium salt yields a blue diformazan product. The reaction occurred in colonies incubated in all mixtures containing Nitro BT (1, 3, 5, 9,

13, 17, 21, 25) except those containing also malonic acid (2, 4, 6, 10, 14, 18, 22, 26) which inhibited partly the reduction of Nitro BT to the blue formazan. Although the colour appeared after a lapse of time in the presence of malonic acid, it never reached the intensity of that in incubation media in the absence of this inhibitor.

The colour reaction was observed in colonies incubated in media 3, 9, 17 and 25, although no sodium succinate was added as substrate for the dehydrogenase system.

2. Intracellular location of the reaction product

The blue reaction product appeared as small granules or particles inside the spores and hyphae as shown in Figs. 59-62 (Pl. 13). Microspores inside spores and hyphae, and free in the medium exhibited the reaction (Figs. 59 and 60, Pl. 13). The cytoplasm never stained as a whole. The colouration was located mainly at the tip of hyphae (Figs. 61 and 62, Pl. 13) where the reaction particles were sometimes so numerous and close together as to give at first the impression that the whole cytoplasm was stained, but in all cases a closer examination revealed that the reaction was located on particles. As shown in Figs. 60-62 (Pl. 13), the reaction in older parts of hyphae was limited to scattered microspores and other cytoplasmic particles located mainly in cells near hyphal branching, and near septations.

All spores exhibited a certain degree of reaction, but microspores were the most reactive. It was not possible to relate the intensity of the colour reaction in large spores with their age,

although there was a great deal of variation in the intensity of the reaction, because the age of the spores could not be determined with the technique used.

Figs. 59 - 62. Tests for succinic dehydrogenase in Ceratocystis ulmi. X650

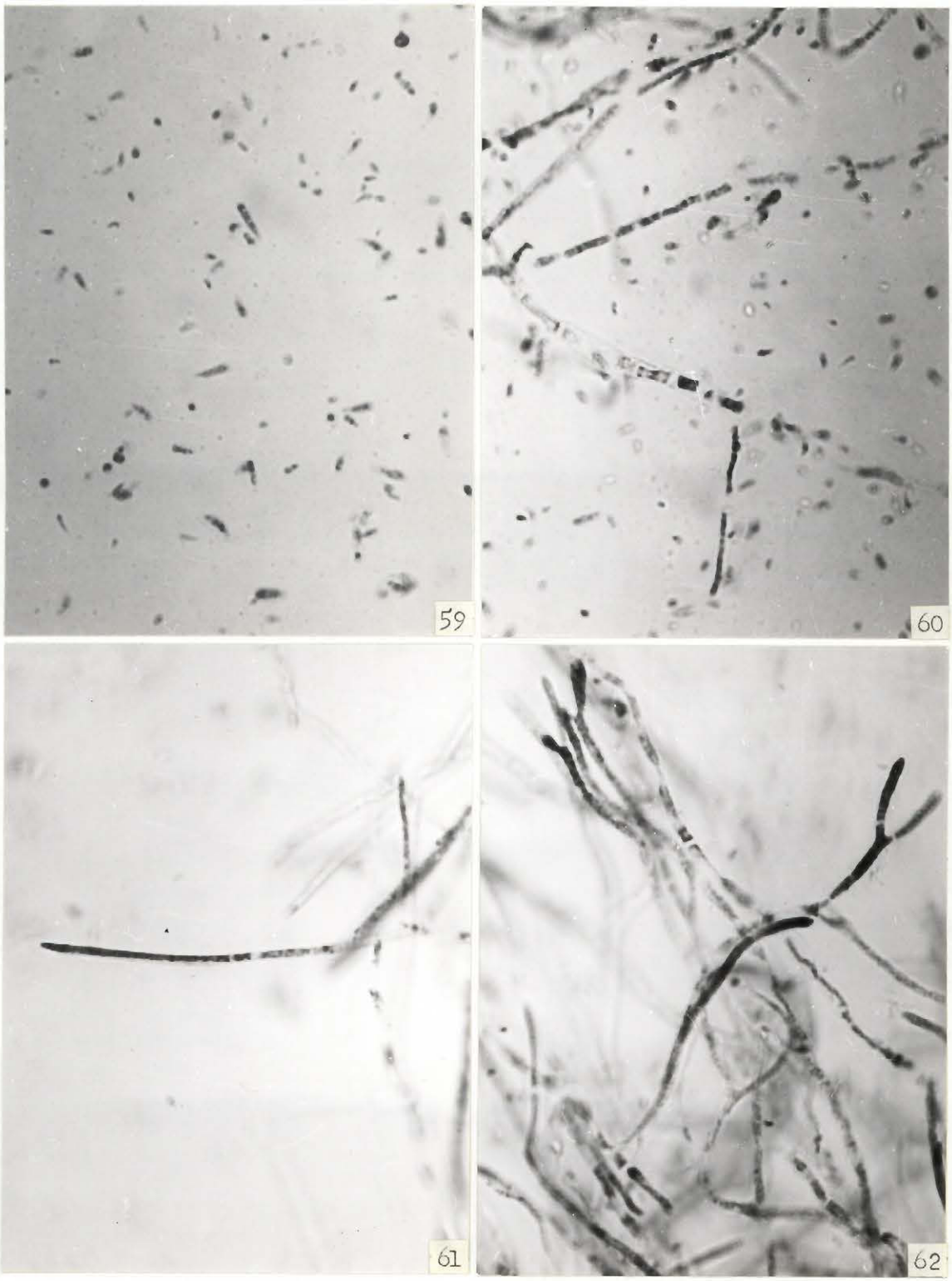
Fig. 59. Reaction of particles inside spores of C. ulmi in test for succinic dehydrogenase.

Fig. 60. Reaction of particles inside hyphae in test for succinic dehydrogenase, located in branching hyphae, and in cells near branches.

Fig. 61. Test for succinic dehydrogenase. Note the concentration of reaction products on particles located in the apical cell of an hypha, and near septa in older parts of mycelium.

Fig. 62. High concentration of reaction products located in hyphal tips and near septa.

Plate 13



J. Tests for DPN and TPN Diaphorases

1. Comparison of different procedures

Malonic acid had to be used in the incubation media to prevent the reduction of Nitro BT that occurred with no substrate as reported in the previous section. In the tests for succinic dehydrogenase, malonic acid inhibited partly the reaction, but did not inhibit the reaction due to the diaphorase. The reaction was less intense after incubation in media 2 (see page 27) which contained either DPNH or TPNH and malonic acid than in media 1, which contained the same nucleotides. This difference in intensity of the reaction in these two tests apparently was not due to an inhibitory effect on the diaphorase by malonic acid in medium 2, but was probably due to partial inhibition of the activity of succinic dehydrogenase or other similar enzymes normally active in the fungus, even without addition of substrate. Results in the previous section on succinic dehydrogenase activity have demonstrated this possibility.

The addition of quinacrine (media 3 and 4, see page 27) contrarily to what was observed in the tests for succinic dehydrogenase seemed to accelerate and intensify the reaction to a certain extent. The addition of phenazine methosulfate to the incubation media (5, 6, 7, 8, 9 and 10) did not give better results, on the contrary it masked the colour reaction produced by the reduction of Nitro BT. In fact, the addition of phenazine methosulfate resulted in an immediate deep blue reaction in the solution, even when Nitro BT was not added to the incubation solutions, (media 9 and 10). A precipitate was formed

in these media after standing for a while at room temperature. This indicated a spontaneous reduction of phenazine methosulfate in the presence of DPNH or TPNH.

2. Location of DPN diaphorase

The reaction product in the test for DPN diaphorase was located on intracellular particles of the size of microspores and smaller. The mycelium and all spores gave a variable but nevertheless positive reaction. Young mycelium had more coloured particles than the mycelium in the middle of the colonies. In general, the hyphal tips did not show more activity than the hyphal parts one mm from the tips, although a few of them appeared exceptionally to be somewhat more reactive.

3. Location of TPN diaphorase

The size of the coloured particles giving a positive reaction in the test for TPN diaphorase was smaller and the intensity of the reaction, in general, was less in the tests for TPN diaphorase than in those for DPN diaphorase. The distribution of the coloured particles in hyphae and spores was very similar to that described above for DPN diaphorase. The reaction was observed in all spores and hyphal threads, but mainly in young hyphae.

K. Test for DPN-Linked Dehydrogenases

1. Colour Reaction

The colour reaction in these tests was identical to that described before since it involved also the reduction of Nitro BT. The substitution of sodium cyanide by sodium azide as terminal respiratory chain block did not influence the speed nor the intensity

of the reaction. However, the use of mid-chain blocks such as amytal or quinacrine instead of sodium cyanide seemed to speed up and intensify the reaction slightly.

2. Location of the reaction

(i) Malic dehydrogenase - When malic acid and DPN were used together in the incubation medium, the blue colour reaction was located on granules in spores and hyphae as in the tests for succinic dehydrogenase and for DPN diaphorase. There were differences, however, in that the reactive particles were less numerous in these tests than in the test for DPN diaphorase, and they were more evenly distributed between younger and older mycelia (Figs. 63 and 64, Pl. 14). Reactive particles were not as concentrated in hyphal tips in this test as in the test for succinic dehydrogenase. The reaction occurred to various degrees in all spores: Some of the large spores were almost completely filled with stained particles while others had only a few such granules (Figs. 65 and 66, Pl. 14).

(ii) Lactic dehydrogenase - The tests using lactic acid as substrate resulted in little colour reaction. The activity was located on particles in spores and hyphae. The intensity of the reaction was much weaker than in all the preceding tests. Most of the reactive particles were in young mycelium (Figs. 67 and 68, Pl. 15).

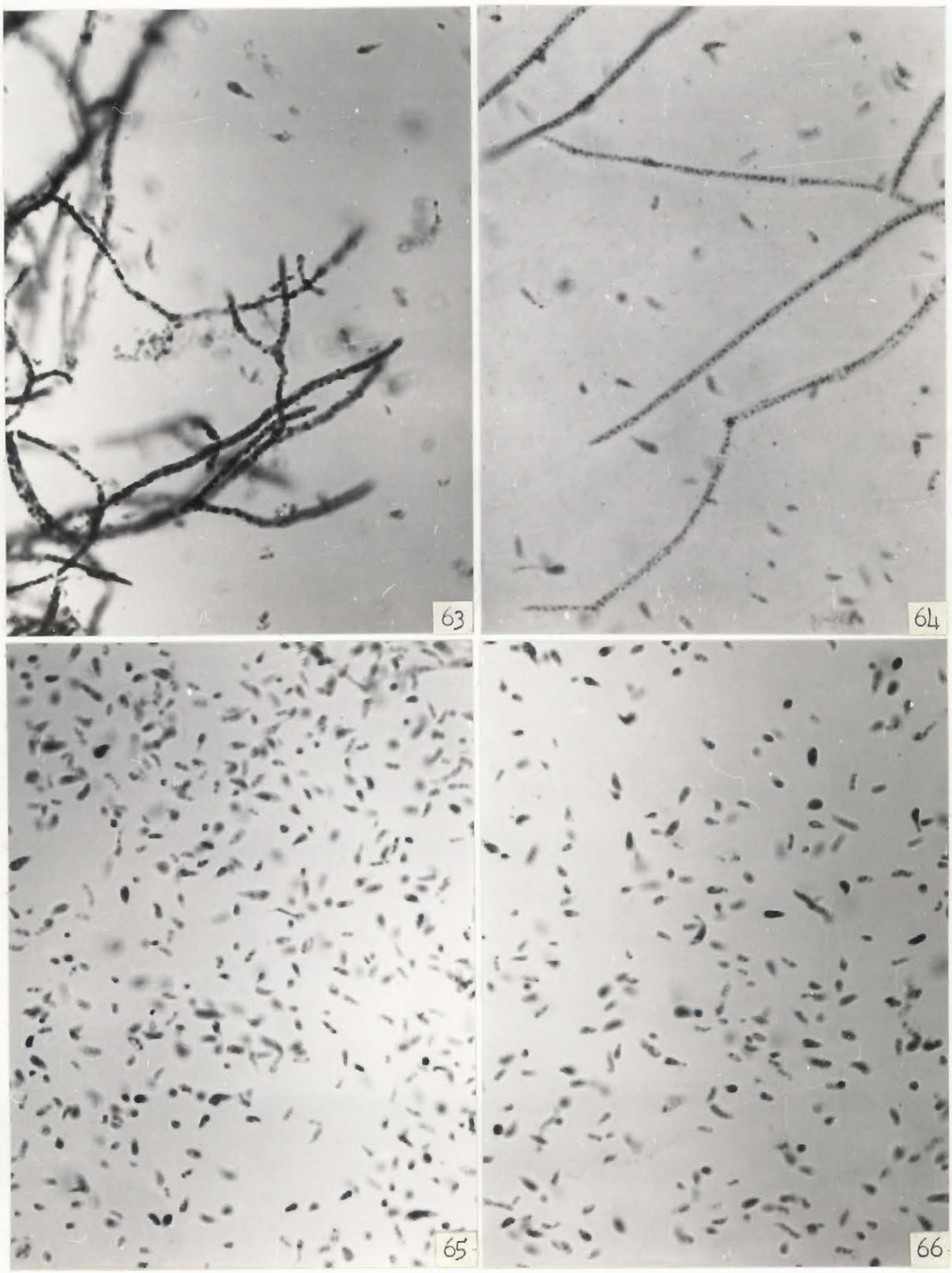
Figs. 63 - 66. Tests for malic dehydrogenase in Ceratocystis ulmi. X650

Fig. 63. Location of the reaction products in mycelium using quinacrine as a respiratory mid-chain block.

Fig. 64. Location of the reaction products in mycelium with no respiratory inhibitor. Note the location on globular particles resembling microendospores.

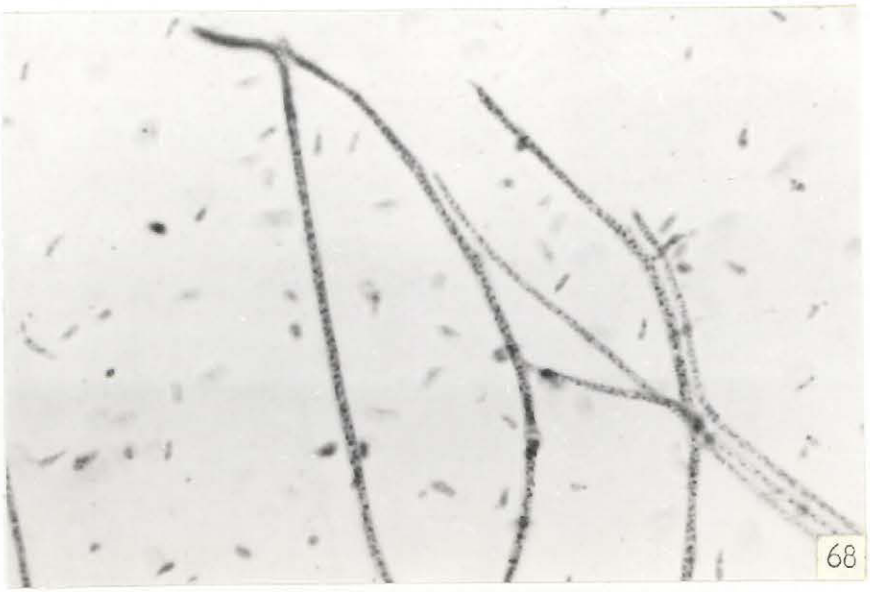
Figs. 65 and 66. Location of the reaction products in spores. Note the size of smallest stained spores compared to the size of some stained particles in hyphae of Figs. 63 and 64.

Plate 14



Figs. 67 and 68. Tests for lactic dehydrogenase in Ceratocystis ulmi. Note the blue reaction products located on particles in the mycelium and spores. Some spores showed little reaction. X650

Plate 15



L. Tests for TPN-Linked Dehydrogenases

1. Comparison of different procedures

In these tests MTT was used as an acceptor of the hydrogen from TPN-linked dehydrogenases. A fine deposit of black cobalt formazan was formed. It gave a very diffuse gray colour to the cytoplasm. The characteristic black colour was very slow to appear and did not always develop. For this reason, the technique used for the demonstration of the DPN-linked dehydrogenases was adapted to TPN-linked dehydrogenases by replacing DPN by TPN in the incubation media which contained the appropriate substrates, that is, malic acid, or glucose-6-phosphate. As a result, the characteristic blue colour of reduced Nitro BT was observed constantly on particles in spores and in mycelium. However, there was also a diffuse red colour reaction in the cytoplasm. This colour had not been observed in previous tests using Nitro BT.

2. Location of the reaction

(i) Malic decarboxylase - A positive reaction was found in about 25 per cent of the spores when TPN and malic acid were present in the incubation medium, compared with 100 per cent when DPN was the nucleotide provided for the reaction. But the spores showing the reaction were almost completely stained, being filled with reactive particles (Figs. 69 and 70, Pl. 16). In the mycelium, the reaction also was located on particles that were apparently larger than in previous tests, and these were rather evenly distributed (Figs. 71 and 72, Pl. 16).

Figs. 69 - 72. Tests for malic decarboxylase in Ceratocystis ulmi. X650

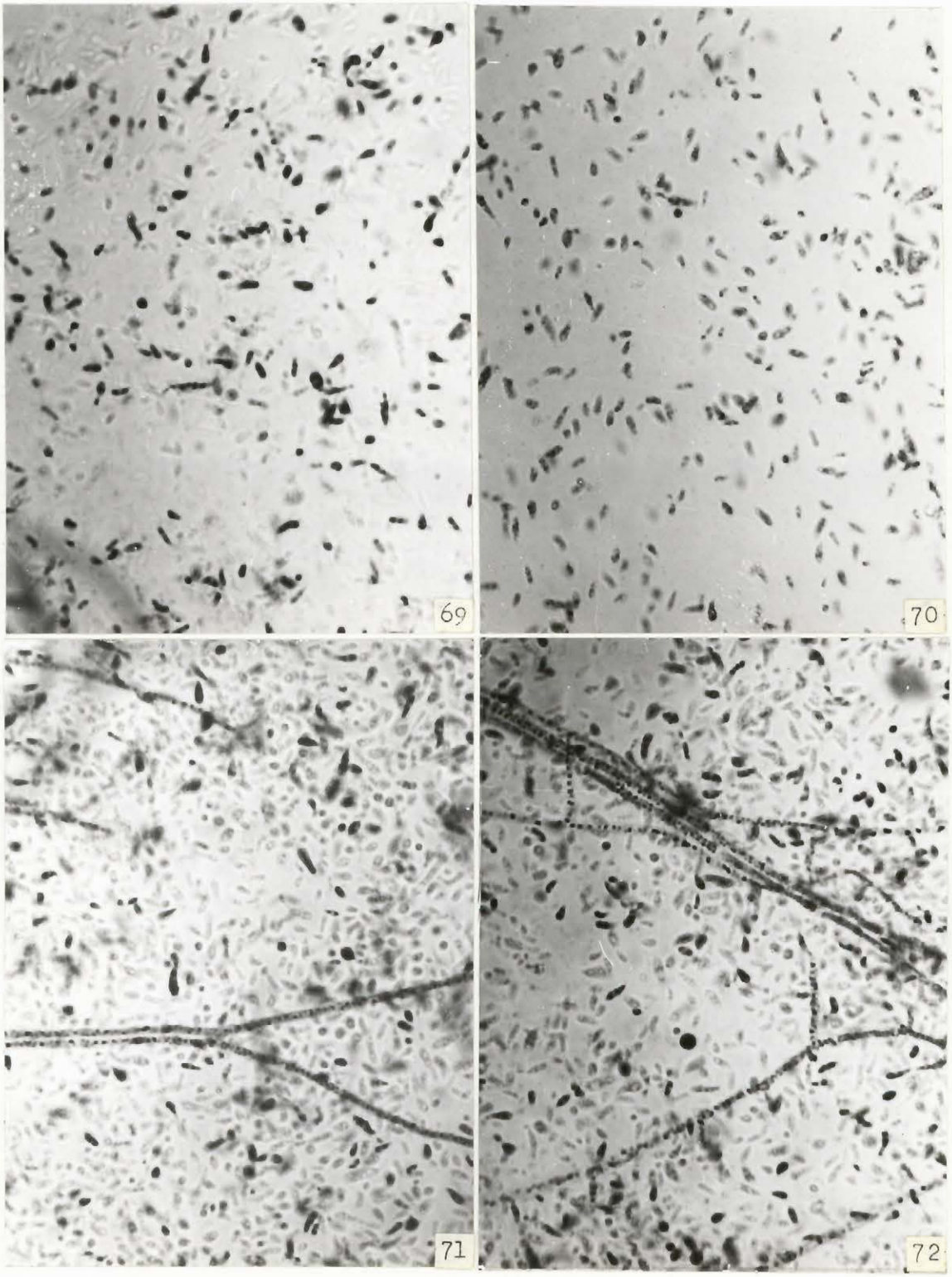
Fig. 69. Note the dense reaction products located in about 25 per cent of the spores.

Fig. 70. Note the reaction products located on particles in spores resembling microendospores.

Fig. 71. Blue reaction products located on particles in hyphae, and a diffused red colouration in hyphal tips.

Fig. 72. Blue reaction products located on particles in hyphae and resembling microendospores.

Plate 16



(ii) Glucose-6-phosphate dehydrogenase - The blue colour reaction that took place when glucose-6-phosphate was used as substrate was located mainly in young hyphae, but not especially in hyphal tips. The reaction appeared mostly on particles of the size of microspores. In addition, it showed as small dots in the protoplasm. A diffuse red colour mostly located in older hyphae or parts of hyphae was detected. About 50 per cent of the large spores contained reactive particles.

The intensity of the reaction was one of the highest among all the coenzyme dependent dehydrogenases tested, indicating that the glucose-6-phosphate dehydrogenase was relatively one of the most active.

M. Growth of C. ulmi on Cellophane Foil

For a few transfers the fungus grew normally on pieces of cellophane foil laid upon the surface of PDA. The colonies reached a diameter of 10 mm in three to four days. At that time, the growing colonies were used for histochemical tests, and they could be detached easily from the cellophane during the incubation in the appropriate reagents, indicating that the fungus had not grown through the membranes. Thus it was possible to mount the whole fungus colonies on microscope slides with little intermixing of young hyphae with old mycelium. Of course, the spores could not be kept in their original position, and they were distributed throughout the mounting media.

After a few transfers on cellophane foil the fungus developed much more aerial filaments than it did in direct contact

with PDA. This characteristic was maintained when these aerial structures were allowed to grow in direct contact with PDA. It is not known through how many transfers on PDA this characteristic can persist.

The original isolates did not produce coremia, when grown in direct contact with PDA, until after three to four weeks of growth. But the same isolates, when grown separated from PDA by a cellophane membrane started to produce coremia abundantly after only one week of incubation. This was probably due to a reduced availability of certain nutrients, because the onset of sporulation is often related to a reduction in the food supply (Hawker, 1957).

V. DISCUSSION AND CONCLUSION

The interpretation of the results of histochemical tests present a number of difficulties. For several tests, positive results are difficult to interpret because the specificity of these tests is disputed. However, the greatest difficulty is with negative results. As pointed out by Vanden Born (1963) it would not be justified to accept negative results as a proof of the absence of an enzyme system, or a compound, since the conditions of histochemical tests are usually established to differentiate between high activity, or concentration, and low activity, or concentration, or none. Thus, a negative result may mean: a) The low activity of an enzyme or the low concentration of a compound, b) the absence of the enzyme or the compound studied, or c) even the failure of the technique used.

Most of the time it is impossible to distinguish between these three conditions. The last one, especially, should not be neglected in the interpretation of negative results in histochemical tests with plant tissues, because most histochemical methods, enzyme ones in particular, were developed for animal histochemistry, where among other things, the conditions of cell permeability are different. Hence almost all the techniques have to be modified to suit plant material. Even with techniques developed for plant tissues, the procedures often must be varied because frequently these techniques have been developed for meristematic tissues, and the conditions in these tissues and the conditions, for example in xylem tissues of woody plants can be quite different, particularly with respect to permeability of the cells to the substrates.

A. Sequence of Pathohistological Alterations

In the present investigation the pathological alteration that appeared first was the yellowing of the vessel walls at the pits. Subsequently this yellowing extended within the vessel walls to produce eventually a continuous discolouration. This extended discolouration of the vessel walls was reported by Kerling (1955) as the first noticeable alteration after inoculation; but she did not mentioned that it had started at the pits.

The next change to become visible took place in the living parenchyma cells surrounding the vessels. The protoplasm of these cells became increasingly granular. This was followed by a yellowing and then a browning of the entire contents of these cells. Similar transformations were observed by Broekhuizen (1929). As the disease progressed the pits of vessel walls disintegrated, as previously mentioned by Ouellette (1960), and the contents of the longitudinal parenchyma and ray cells that were similarly affected, exuded as "gum" droplets in the vessels. These "gum" droplets coalesced in the vessels, and, together with other materials, often completely plugged the vessels. Broekhuizen (1929), Kerling (1955), and Ouellette (1960) reported similar observations. As the disease developed further, the brown "gummy" material appeared in fibers, suggesting that these cells still had cytoplasm when the tree was infected. This will be further discussed below.

The development of tyloses, considered by Schwarz (1922) as the first detectable change after inoculation, and reported by Kerling (1955) as occurring one week after inoculation in non-discoloured

parts of the vessels was observed also by Ouellette (1960). In this investigation, true tyloses were seen occasionally originating from ray cells and longitudinal vasicentric parenchyma cells. However, tylose-like structures were very common in infected vessels. Cytoplasmic material extruding in vessels from adjoining parenchyma cells very often presented a tylose-like appearance. Because there was no walls around these protrusions, they are not considered here as true tyloses. This is in accordance with the concept of tyloses accepted by plant anatomists (Esau, 1953; Foster 1964), but at variance with the nomenclature often used by wood anatomists (Cited by Esau, 1953). The latter concept has given rise to a great deal of confusion and should be avoided. Empty spores of the fungus can assume also the appearance of small tyloses (Ouellette, 1960). In addition, we have found that debris of vessel walls can resemble empty tyloses.

Other pathological modifications of the cell walls are swelling, and lamellation, especially of the vessel walls. These changes can be of a great significance in the pathogenesis of the elm disease, because such alterations, especially in the vessel walls, possibly interfere with the movement of sap. Vessel walls are though to have a role to play in the ascent of sap. Their structure could affect the stability of the water columns in the vessels. This stability is a requirement of the cohesion theory of the ascent of sap (Kramer, 1959). These alterations of the vessel walls could possibly affect the translocation of water through cell walls. Translocation through

this path is considerable according to Strugger (cited by Kramer, 1959). However, this theory of the ascent of sap through walls has few tenents nowadays, for it has been shown that plugging of the vessel lumina resulted in a much reduced movement of water (Kramer, 1959).

B. Pathological Alterations in Lignin

Xylem discolouration in infected elms attributed by Broekhuizen (1929) to the transformation of "gum" into a lignin-like compound similar to natural "wound-gum". Ouellette (1960) obtained a weakly positive reaction in the phloroglucinol-HCl test for lignin in the dark brown material plugging the vessels, but the light brown almost yellow substance was negative. Since the light brown material was observed mostly during the early stages of disease, and the dark brown material at later stages, it seems that the former was transformed into the later, and therefore into lignin-like "gum". Because the theory behind the phloroglucinol reaction with lignin is imperfectly known, and lignin is formed from phenolic compounds, Ouellette (1960) concluded that the positive reaction in his preliminary tests for lignin in vessels might be indirect evidence for the theory (Dimond, 1955) that phenolic compounds are involved in the formation of brown discolouration. Though the chemical reaction of phloroglucinol with lignin is not completely understood, it is reasonably specific according to Jensen (1962).

In this study, a positive reaction was also obtained with some particles of the material plugging vessels. But these particles

were probably debris of the vessel walls which were disintegrating at the stage of disease when this reaction was observed. It is concluded that the "gum" was not transformed into lignin-like substance, but that it contained lignin originating from disintegrating vessel walls.

The main sites of positive reaction to the phloroglucinol test for lignin were the wall layers of the spring vessels, of summer wood vessels, and in the middle lamellae of other types of cells. The more intense reaction in vessel walls of diseased trees than in those of healthy trees can be interpreted in a number of ways. Two of these will be discussed. First, it can indicate that there was more lignin deposited in the vessel walls of diseased trees than in those of healthy trees. This explanation is compatible with the observed thickening of the vessel walls in infected tissues, and also by the observed pathological increase in phenolic compounds in the xylem of diseased trees, since phenolic compounds are basic units in the synthesis of a lignin. A second interpretation of the pathological increase in lignin reaction is that the lignin in the vessel walls of the diseased trees was rendered more sensitive to the histochemical test, either because of a change in the permeability of the walls to the reagents, or because of the degradation of other wall constituents, such as pectins, which impregnate the lignin in normal tissues. Evidence obtained in the present study on pectin degradation will be discussed below. These postulated mechanisms can be involved either singly or jointly. The latter one seems more likely than the first

one because the first mechanism supposes an increased metabolic activity in the diseased trees relative to that in healthy ones, whereas the second only implies the production of pectolytic enzymes by the pathogen, C. ulmi, and that was demonstrated by Beckman (1956) and Dimond and Husain (1958).

The negative results obtained with the chlorine-sulfite test and the Schiff's reaction for lignin is difficult to interpret, since the chlorine-sulfite test, in particular, is supposedly more sensitive than the phloroglucinol-HCl test according to Siegel (1953). This might be related to differences in lignification with different plant species.

C. Pathological Alterations in Pectins

The first anatomical alteration that can be attributed to a modification of the pectin in the cell walls of diseased elms was the swelling of the vessel walls. The Krajcinovic test for pectin revealed that the swollen parts reacted more intensely than other parts, thus the chemical test confirmed the anatomical observation.

The histochemical tests for pectin demonstrated that the plugging material in the vessels of infected trees had pectin as one of its main constituents. There is a similarity here with the situation in Fusarium-infected tomato where plugging is though to be due to pectic substances (Ludwig, 1952; Gothoskar et. al., 1953; Dimond, 1955; Pierson et al., 1955; Waggoner and Dimond, 1955). But this hypothesis was questioned by Saaltink and Dimond (1964) who reported that most of the plugging was caused by fungus plugs, and that the

granular plugs were not pectic substances, although they stained with ruthenium red. However, in the present studies the pectic nature of the plugging material was determined by a test supposedly more specific for pectin (Pearse, 1960) than staining with ruthenium red that is considered only partially specific by Jensen (1962). Consequently it is concluded that pectins form a major constituent of the vessel plugging materials in diseased trees, although another test, devised by Reeve (1959c), and reported to be highly specific for esterified pectin (Jensen, 1962), gave a rather poor reaction with elm tissues, even after methylation of the pectins. However, the condition of the pectins in the xylem tissues of elm can differ considerably from that for which it was originally devised.

The positive results obtained can be considered as direct evidence that pectins derived from cell walls, especially those of vessels, contribute, together with other materials, to the plugging of the water conducting vessels. As a corollary, it can be supposed that the pectic enzymes produced by C. ulmi (Beckman, 1956; Dimond and Husain, 1958), besides their direct role of providing food to the fungus, as mentioned by Dimond and Husain (1958) as their main function, have an indirect role in the pathogenesis of the elm disease by decreasing or stopping the movement of water through alteration of the vessel walls and plugging of the vessels.

D. Polyphenols in the Elm Disease

The technique used for the demonstration of polyphenols was a modification of the technique described by Reeve (1951), who

considered it as specific for polyphenols. Therefore, from the results obtained in this study, it is concluded that polyphenols are present in low amount in newly differentiated cells near the cambium, and in large amount in very few longitudinal parenchyma cells and ray cells, but are generally abundant in the cells of the medullary sheath. This is in accordance with the general view that the cells of the medullary sheath are filled with tannins, which are phenol derivatives (Esau, 1953).

It is concluded also that polyphenols are produced abundantly in the xylem tissues of elms after infection by C. ulmi, or injection of buffer solutions adjusted to pH 5.0 and dilute solutions of ethanol. Thus it seems that the formation of phenolic compounds in the elm disease is not a direct effect of the fungus, but seemingly results from a change in the normal physiology of the tissues following a disturbance of the host. Furthermore, the negative results obtained in tests for beta-glucosidase seem to indicate that these phenolic compounds are not derived from glucosides by the action of a beta-glucosidase according to the theory suggested by Dimond (1955). However, these negative results can be due to the inadequacy of the technique used.

The presence of polyphenols and of discolouration in the fibers nearest to the infected vessels in early stages of the disease, and the fibers progressively more distant from the vessels in later stages, indicates also that the fibers of the current year growth ring contained protoplasm, as do wood fibers in other species (Fahn and Arnon, 1963, Fahn and Leshem, 1963). Substances responsible for

the positive polyphenol reaction in the fibers could conceivably have originated in longitudinal parenchyma and ray cells. But this seems unlikely because of the lack of continuity of the material giving positive reaction in the fibers and that in parenchyma cells. However, continuity of the substance plugging the vessels and material of apparently the same nature in adjoining parenchyma or ray cells was often observed.

It is concluded that polyphenols are important constituents of the plugs in the water conducting vessels. These phenols, as mentioned before, are apparently formed in longitudinal parenchyma and in ray cells and are extruded through the pits into the vessels.

The positive reaction observed between layers of cell walls is difficult to interpret. It can be due to a reaction with either free phenolic groups of lignin, with phenols liberated from lignin, or with polyphenols that had diffused from parenchyma cells.

The role of these phenolic compounds in the pathogenesis of the elm disease can be manifold. First, their oxidation and subsequent condensation with the proteins of the living cells in which they are formed can denaturate and even coagulate the protoplasm and thus cause the death of these cells. On the basis of accumulating evidence that living cells are involved in translocation of sap, (Greenidge, 1955, 1957; Postlethwait and Rogers, 1958) the death of these cells would contribute to the shortage of water in the leaves and the resulting wilting. Another mechanism that can be responsible for wilting is the extrusion of coagulated protoplasm in the vessels

to form "gum" plugs. A third possibility is that phenolic compounds are translocated to the leaves by the sap stream before it is stopped. In leaves, they could act as toxins causing the irregular brown spots often observed especially on young trees before the appearance of flacidity.

It seems that polyphenols could have also a role in resistance to the disease. This is evidenced by the inhibition of the growth of the fungus in culture containing phenolic compounds, and the restricted growth of the pathogen in living elms compared to its profuse development in killed trees. This could be used perhaps in the selection of resistant American elms if this theory is verified.

E. Phenoloxidase Role in the Elm Disease

Phenoloxidase tests using pyrocatechol indicated that polyphenoloxidase was present in the xylem of healthy and diseased elms.

The greatest polyphenoloxidase activity was located in the same kinds of cells that reacted positively in the test for polyphenols, and that showed discolouration in diseased elms. Thus it seems that the phenoloxidase of the tree was responsible for the oxidation of the polyphenols formed during the development of the disease.

The apparent increase of the phenoloxidase activity in the diseased trees compared to that in healthy elms can be ascribed to the enzyme of the fungus, even though tests on incubation of fungus colonies in pyrocatechol solutions gave negative results, because the pathogen either possesses or can develop an enzyme capable of oxidizing phenolic compounds in diseased trees, since an isolate developed a dark pigment, in cells and in media, when grown on media containing pyrocatechol. However, this apparently increased activity in diseased

trees might be due also to the pathological discolouration which, added to the reaction of the test, produced a more intense colour.

It seems more likely that the oxidation of the polyphenols in diseased elms was brought about mainly by the phenoloxidase of the host, and that the phenoloxidase of the pathogen had little to do in this reaction, mainly because the fungus seems to have a low phenoloxidase activity. Furthermore browning could be induced in the absence of C. ulmi by injection of alcohol solutions or of buffers at pH 5.0. In this respect, the vascular browning produced in the elm disease probably differs from that in Fusarium-infected banana roots, since Mace (1964) showed that phenoloxidases from the host and from the pathogen may participate in the oxidation reaction of polyphenols.

F. Peroxidase Role in the Elm Disease

The results of the peroxidase tests using benzidine showed that peroxidase was present in the host as well as in the pathogen. However, the reaction was more readily produced in elm tissues than in fungus colonies. In the fungus, the benzidine was oxidized first to benzidine blue, and gradually further oxidized to benzidine brown. The last step, according to Pearse (1960) does not require the action of peroxidase. In the elm tissues, besides the blue reaction in the protoplasts, there was a violaceous brown reaction in the cell walls. The violaceous brown product formed in the cell walls seemed different from the brown product resulting from complete oxidation of benzidine to benzidine brown in cytoplasm, but this difference was possibly due to different absorption effects in the cell wall and in the cytoplasm.

Van Fleet (1959) reported that in the youngest cells of the protoderm, benzidine was oxidized to benzidine blue, whereas in the derived and older cells of the epidermis, benzidine was oxidized to benzidine brown. These reactions are similar to those of elm tissues in which the blue product was formed in the protoplasm and the brown product in the cell walls. The fact that the blue reaction was almost non-existent in fresh, noninfected tissues, whereas it was found abundantly in various cells of infected tissues and in noninfected tissues impregnated with celloidin, can be explained as follows. The peroxidase responsible for the blue reaction and located in the protoplasm diffused out of the cells when fresh sections were incubated in reagent solution, but with sections from material previously impregnated with celloidin, there was much less diffusion of the peroxidase into the incubation medium. Presumably, the protoplasm was better retained in the cells impregnated with celloidin. Consequently the colour reaction appeared in the cells and in the test solution.

The presence of peroxidase activity in fibers, and in vessels of infected tissues, does not invalidate this reasoning, since it was shown that protoplasm occurs in fibers and tracheids of woody plants (Fahn and Arnon, 1963; Fahn and Leshem, 1963). In fact, this is another indication that xylem fibers of elm contain protoplasm, at least those formed recently, as discussed in the section on polyphenols.

The results reported above are not in accordance with Van Fleet's statement (1959) that "benzidine blue end product was found in the locus of future cell divisions or in cells in active division but not in older resting cells" because the blue end product was found in fully divided cells of the xylem of elm. However, results of this investigation, in agreement with those of Van Fleet (1959), indicated a gradual decline in peroxidase activity from cambium to older xylem tissues.

Peroxidases have been recognized for a long time as oxidative enzymes of phenolic compounds under appropriate conditions, but Kenten (1955) and Van Fleet (1959) reported the inhibition of peroxidase activity by phenols. Moreover, Van Fleet (1959) associated the decline in peroxidase activity with the built up of phenols in older cells. In the present investigation, the greatest peroxidase activity was found in cells which showed high concentration of polyphenols in infected tissues. Consequently, it seems that peroxidase activity was not inhibited noticeably by the polyphenols present in the infected elms. The location of both polyphenols and peroxidase activity in the same cells suggests that peroxidase acted as an oxidative enzyme for the polyphenols, resulting in the browning of the tissues, pending on the formation of hydrogen peroxide by the action of other enzymes. Mace (1964) has suggested also that peroxidase can play a part in the oxidation of phenolic compounds in the Fusarium-infected banana roots.

Since peroxidase was produced also by C. ulmi, it is possible that the peroxidase of the pathogen contributed to the browning of the infected tissues. But considering the high concentration of hydrogen peroxide needed to reveal the presence of peroxidase in the fungus compared to that for the host enzyme, it seems that the peroxidase of the fungus played a minor role in the oxidation of the polyphenols in the elms infected by this fungus.

G. Dehydrogenase Role in C. ulmi

The demonstration of dehydrogenase activity by histochemistry, using tetrazolium salts, has been discussed by several authors (see Lison, 1960; Pearse, 1960; and Burstone, 1962). The specificity of the reaction for succinic dehydrogenase seems to be generally accepted; but there is considerable disagreement as to the specificity of the tests for DPN- and TPN-linked dehydrogenases. These enzymes are specific for their substrate, therefore a greater reaction in the plus substrate compared to the minus substrate reaction mixture indicates the presence of the dehydrogenase specific for this substrate. But the transfer of hydrogen from the substrate to tetrazolium salts, in the case of DPN- or TPN-linked dehydrogenase, is not direct, but is mediated by diaphorases. Therefore, the reaction indicates directly the location of the diaphorase, and indirectly the site of the dehydrogenase, assuming that the dehydrogenase and the diaphorase are at the same sites. Also, one must assume that DPN or TPN are firmly bound to the tissue, especially after reduction, and that the dehydrogenase is not solubilized and reacts in sites where it is normally present.

In animal histochemistry (Lison, 1960; Pearse, 1960), no reduction of the tetrazolium salt is produced unless a substrate is added to the incubation medium, except at high pH and in the presence of DPN or TPN, then the tetrazolium salt can be reduced by a mechanism called "nothing dehydrogenase". With colonies of C. ulmi, however, Nitro BT was reduced in the absence of exogenous substrate supply. This reaction was stopped by various inhibitors among which neutralized malonic acid, a specific inhibitor of succinic dehydrogenase. Therefore, it seems that the reduction of Nitro BT in the absence of exogenous substrate is due to the activity of succinic dehydrogenase, and that succinic acid was metabolized in the fungus in sufficient amount to produce this reaction. Consequently this reaction had to be inhibited in tests for dehydrogenase-diaphorase activity in colonies of this fungus. Otherwise the reaction in the minus-substrate controls, essentially blanks, was too high.

DPNH and TPNH were used as substrate in tetrazolium-reduction tests to locate their respective diaphorase. The reaction involves the direct transfer, by the diaphorase, of the hydrogen from the reduced pyridine nucleotide (DPNH or TPNH) to Nitro BT. The addition of exogenous dehydrogenases or substrates for endogenous dehydrogenase systems are not required. Thus the reaction indicated indubitably the activity of the diaphorase studied, unless diaphorases are artifacts, as suggested by a group of biochemists (Watari et al., 1963; Cremona et al., 1963).

The results of the tests for DPN-, and TPN-linked dehydrogenases indicated that all the enzymes tested, i.e. malic dehydrogenase and lactic dehydrogenase, two DPN-dependent enzymes, and malic decarboxylase and glucose-6-phosphate dehydrogenase, both TPN-dependent, were active in the spores and in the mycelium of C. ulmi, but at different degrees. These tests indicated also that the fungus can obtain its energy from the oxidation of glucose via the Kreb's cycle and the pentose shunt pathway, since, on one hand, two enzymes of the citric acid cycle; succinic dehydrogenase and malic dehydrogenase; and on the other hand, one enzyme of the pentose shunt, glucose-6-phosphate dehydrogenase, were active in C. ulmi. However, the relative importance of these two routes cannot be determined from the results presented here.

H. Mechanism of Pathogenesis: A Synthesis

The results reported here allow an appreciation of the different theories put forward to explain the mechanism of pathogenesis in the elm disease, and provide links for unification of the main elements of previous findings into a synthesis of events occurring in the pathological processus of the elm disease. This synthesis can serve as a working hypothesis in future research, even though a number of points deserve further investigation.

The pathogen of the elm disease, C. ulmi, is well adapted for rapid spread in elms. The microspores produced by this fungus (Ouellette and Gagnon, 1960; Ouellette, 1962) can be quickly drawn up with the transpiration stream and start numerous infections at different

sites along the vessels, especially so as a result of artificial inoculations at the base of the stem. In the case of natural inoculations by insects, the fungus is introduced in small branches, gets down to the roots in as yet an unknown way and spread throughout the aerial parts. The downward movement is fast, according to Banfield (1941a), who reported that C. ulmi was recovered at stump height 15 minutes after injection of large volumes of spore suspension 40 feet above.

The first pathological alterations are detected in vessel pits and in parenchyma cells around the vessels. Spores, especially the microspores, can be trapped in pits, where, upon germination, they attack the pit membrane and send hyphae in the adjoining parenchyma cells. This can be produced at several locations along the vessels and result in a multisite infection. Therefore, it seems that the first important pathological event is the invasion of the xylem parenchyma cells surrounding the vessels. The invasion of these cells is followed by a disturbance in their normal physiology. Either through the action of a beta-glucosidase on existing phenolic glucosides, through a blockage in lignin synthesis, or through a shift in carbohydrate metabolism, polyphenols accumulate in these cells, causing a denaturation of their protoplasm, eventually becoming visible as a brownish discolouration, and finally their death. Thus, if living vasicentric parenchyma cells are either directly or indirectly essential for the ascent of sap (Kramer, 1959), the invasion and killing of these parenchyma cells by the fungus would be certainly of prime importance in the pathogenesis of the elm disease.

Wilson (1965) reached a similar conclusion on the ground that the fungus is more abundant in these living cells than in vessels and tracheids. However, this was a right conclusion on a false assumption since we found, after Ouellette (1962a), that the fungus could be as abundant, if not more, in the vessels as in parenchyma cells, especially in fresh sections of small branches.

As the disease progresses, vessel walls are further altered, probably by the action of pectic and cellulolytic enzymes of the pathogen, especially at the pits. The disintegration of the pits and the swelling of the vessel walls can perhaps cause the disruption of the water columns and consequently the wilting of the leaves. But the most evident effect of the disintegration of the vessel at the pits is the extrusion of the contents of the parenchyma cells in the vessels where it forms plugs of "gum", with pectic material from cell walls, debris of walls containing lignin, and fungal growth. This plugging of the vessels, although apparently secondary in the wilting mechanism, adds to the primary pathological effect on living parenchyma cells in ultimately bringing the death of the infected trees.

The development of necrosis in leaf blades, especially at the margin, before the appearance of wilt was observed frequently in young elms. This could be regarded as a toxemia due to the translocation of the phenolic compounds formed in the xylem parenchyma cells and liberated in the vessel in the early stage of infection. This hypothesis is in agreement with the conclusion reached in previous experiments on the production of toxins in diseased elms

(Gagnon, 1960). However, it seems that the role of such toxins is of secondary importance in the pathogenesis of the elm disease.

VI. SUMMARY

Histochemical studies were carried out on white elms, Ulmus americana L., infected by the elm disease pathogen, Ceratocystis ulmi (Buism.) C. Moreau, and on culture of this fungus. Pathohistological alterations in the xylem of diseasedtrees were observed also in the course of these studies.

The first histological alteration observed in the xylem of infected trees was the yellowing of vessel pits accompanied by a granulation of the protoplasm of living parenchyma cells. This was followed by swelling of the vessel walls, disintegration of the pits, and extrusion into the vessels of the content of parenchyma cells.

In lignin tests, a more intense colour reaction was obtained in xylem cell walls of trees in advanced stage of disease than in corresponding locations in healthy elms. Part of the plugging material in the vessels reacted positively to tests for lignin, and appeared to be debris of vessel walls.

Pectins were distributed in layers in the cell walls of healthy and of diseased elms. The test reaction for pectins was faster and gave a more intense colour in diseased than in healthy trees. Pectins were found also in the material plugging the vessels.

Very little reaction was obtained in testing for polyphenols in healthy elms, except in cells near the pith. Polyphenols were present in the xylem parenchyma cells in early stage of disease development. In advanced stages, polyphenols were found in almost

all cells and were among the most important constituents of vessel plugs. These compounds are readily oxidized to coloured compounds, which would be presumably responsible for the discolouration of the xylem of diseased trees.

Phenoloxidase and peroxidase were found to be very active in the xylem tissues of healthy and diseased elms, and are believed to be responsible for the oxidation of phenolic compounds in diseased elms. The activity of these types of enzymes in the pathogen was low compared to that in the suscept, and therefore, it can be presumed that these enzymes of the fungus have little to do in the discolouration of infected tissues.

Tests for dehydrogenase activity have shown that DPN and TPN diaphorases, succinic dehydrogenase, malic dehydrogenase, lactic dehydrogenase, malic decarboxylase, and glucose-6-phosphate dehydrogenase are all active in the spores and mycelium of the fungus, but at various degrees in different parts of the mycelium as indicated by the intensity of the colour reactions. Consequently, it seems that both the citric acid cycle and the pentose shunt are active metabolic pathways.

A mechanism of pathogenesis in the elm disease is suggested. The fungus is distributed rapidly within the xylem by means of microspores. These invade and damage the living vasicentric parenchyma cells thus bringing about the first wilting symptoms. Alteration of vessel walls, and plugging of the vessels by protoplasm from adjoining parenchyma cells, cell wall materials, and fungal growth would

contribute to further interference with sap movement. Translocation of toxic substances would then contribute to necrosis of tissues in leaves, thus limiting the role of toxins in the pathogenesis of the elm disease to that of secondary factors.

VII. CLAIM OF ORIGINAL WORK

Except for microchemical tests made by Ouellette (1960) on lignin and tannins in infected elm tissues, no histochemical studies have heretofore been made on American elm, either healthy or infected with C. ulmi. The causal organism itself was submitted to cytochemical tests for fats and nucleic acids (Ouellette and Gagnon, 1960), and its cell wall was analysed chemically (Rosinski and Campana, 1964), but no tests on enzymic activity have been done previously by means of histo- or cytochemistry. Thus most of the studies reported herein are original contributions to the knowledge of the pathogenesis of the elm disease, of the physiology of the fungus and, in general way to the knowledge of the mechanism of wilting.

Furthermore, the observations on the pathological alterations in host tissues were performed in a way that clarified the sequence of these events. Most of these alterations had been reported by previous authors who, however, had not established that sequence with any degree of certainty.

VIII. REFERENCES

Avers, C.J. 1958. Histochemical localization of enzyme activity in the root epidermis of Phleum pratense. Amer. J. Botany, 45: 609-613.

Banfield, W.M. 1937. Distribution of spores of wilt inducing fungi throughout the vascular system of the elm by sap stream. Phytopathology, 27: 121-122 (Abstr.).

Banfield, W.M. 1938. The relation of sap flow, vessel length, spore distribution to development of Dutch elm disease in American elm. Phytopathology, 28: 3 (Abstr.).

Banfield, W.M. 1941. Relation of vessel length at infection points to extent of vascular invasion in American elm by Ceratostomella ulmi. Phytopathology, 31: 2 (Abstr.).

Banfield, W.M. 1941a. Distribution by sap stream of spores of three fungi that induce vascular wilt disease of elm. J. Agr. Res. 62: 637-681.

Beckman, C.H. 1956. Production of pectinase, cellulases, and growth-promoting substance by Ceratostomella ulmi. Phytopathology, 46: 605-609.

Broekhuizen, S. 1929. Wondreacties van hout; het ontstoan van thyllen en wondgom, in het bezonder in verband met de Iepenziekte. English translation by E.W.J. Reyers, In Plant Pathology Library, Cornell University, Ithaca, N.Y. (Typescript).

Burstone, M.S. 1962. Enzyme histochemistry and its application in the study of neoplasms. Academic Press, New York, London.

Clinton, G.P. and McCormick, F.A. 1936. Dutch elm disease Graphium ulmi. Conn. Agr. Exp. Sta. Bull. 389: 701-750.

Collins, C.W. 1941. Studies of elm insects associated with Dutch elm disease fungus. J. Econ. Ent. 34: 369-372.

Cremona, T., Kearney, E.B., Villavicencio, M., and Singer, T.P. 1963. Studies on the respiratory chain-linked DPNH dehydrogenase. V. Transformation of DPNH dehydrogenase to DPNH-cytochrome c reductase and diaphorase under the influence of heat, proteolytic enzymes, and urea. Biochem. Z. 338: 407-442.

Dimond, A.E. 1947. Symptoms of Dutch elm disease reproduced by toxins of Graphium ulmi in culture. Phytopathology, 37: 7 (Abstr.).

Dimond, A.E. 1955. Pathogenesis in the wilt diseases. Ann. Rev. Plant Physiol. 6: 329-350.

Dimond, A.E., and Husain, A. 1958. Role of extracellular enzymes in the pathogenesis of Dutch elm disease. Science, 127: 1059.

Dimond, A.E., Plumb, G.H., Stoddard, E.M., and Horsfall, J.G. 1949. An evaluation of chemotherapy and vector control by insecticides for combating Dutch elm disease. Conn. Agr. Exp. Sta. Bull. 531: 1-69.

Esau, K. 1953. Plant anatomy. John Wiley & Sons, Inc., New York, Chapman & Hall, Limited, London.

Fahn, A., and Arnon, N. 1963. The living fibres of Tamarix aphylla and the changes occurring in them in transition from sapwood to heartwood. New Phytologist, 62: 99-104.

Fahn, A., and Leshem, B. 1963. Wood fibres with living protoplasts. New Phytologist, 62: 91-98.

Feldman, A.W., Caroselli, N.E., and Howard, F.L. 1950. Physiology of toxin production by Ceratostomella ulmi. Phytopathology, 40: 341-354.

Foster, R. C. 1964. Fine structure of tyloses. Nature, 204: 494-495.

Gagnon, C. 1960. Studies on the physiology of Ceratocystis ulmi (Buism.) C. Moreau in culture and in association with its host. M. Sc. Thesis, Cornell University, Ithaca, N.Y.

Gagnon, C. 1964. Mechanism of pathogenesis. In A review of the Dutch elm disease. Can. Dept. Forestry, Forest Entomol. Pathol. Branch, Bi-monthly Prog. Rept. 20 (4): 4-5.

Gothoskar, S.S., Scheffer, R.P. Walker, J.C., and Stahman, M.A. 1953. The role of pectic enzymes in Fusarium wilt of tomato. Phytopathology, 43: 535-536.

Greenidge, K.N.H. 1955. Studies in the physiology of forest trees. III. The effect of drastic interruption of conducting tissues on moisture movement. Amer. J. Botany, 42: 582-587.

Greenidge, K.N.H. 1957. Ascent of sap. Ann. Rev. Plant Physiol. 8: 237-256.

Hawker, L.E. 1957. The physiology of reproduction in fungi. Cambridge University Press, London, New York.

Himelick, E.B. and Neely, D. 1962. Root grafting of city-planted American elms. Plant Disease Reptr. 46: 86-87.

Holmes, F.W. 1954. The Dutch elm disease as investigated by the use of tissue culture, antibiotics, and pectic enzymes. Ph. D. Thesis, Cornell University, Ithaca, N.Y.

Jensen, W.A. 1956. The cytochemical localization of acid phosphatase in root tip cells. Amer. J. Botany, 43: 50-54.

Jensen, W.A. 1962. Botanical histochemistry, principles and practice. W.H. Freeman, & Co., San Francisco, London.

Johansen, D.A. 1940. Plant microtechnique. McGraw-Hill Book Co., New York.

Kenten, R.H. 1955. The oxidation of indole-3-acetic acid by waxpod bean root systems and peroxidase systems. Biochem. J. 59: 110-121.

Kerling, L.C.P. 1955. Reactions of elm wood to attacks of Ophiostoma ulmi. Acta Botan. Neerl. 4: 398-403.

Kramer, P.J. 1959. Transpiration and water economy of plants. In Plant Physiology: A treatise. Vol. II. Plants in relation to water and solutes. Edited by F.C. Steward. Academic Press, New York, London.

Lison, L. 1960. Histochimie et cytochimie animales, principes et méthodes. Vol. II. Gauthier-Villars, Paris.

Ludwig, R.A. 1952. Studies on the physiology of hadromycotic wilting in the tomato plant. Macdonald College, McGill University, Tech. Bull. 20, 38 pp.

Mace, M.E. 1963. Histochemical localization of phenols in healthy and diseased banana roots. Physiol. Plantarum, 16: 915-925.

Mace, M.E. 1964. Peroxidases and their relation to vascular browning in banana roots. Phytopathology, 54: 1033.

Mace, M.E., and Wilson, E.M. 1964. Phenol oxidases and their relation to vascular browning in Fusarium-infected banana roots. Phytopathology, 54: 840-842.

Neely, D., and Himelick, E.B. 1963. Root graft transmission of Dutch elm disease in municipalities. Plant Disease Reptr 47: 83-85.

Nelson, R. 1950. Verticillium wilt of peppermint (Mentha piperita L.). Mich. State Col. Agr. Exp. Sta. Techn. Bull. 221.

Ouellette, G.B. 1960. Studies of host and pathogen in relation to the infection process of the Dutch elm disease caused by Ceratocystis ulmi (Buism.) C. Moreau. Ph. D. Thesis, Cornell University, Ithaca, N.Y.

Ouellette, G.B. 1962. Morphological characteristics of Ceratocystis ulmi (Buism.) C. Moreau in American elm trees. Can. J. Botany, 40: 1463-1466.

Ouellette, G.B. 1962. Studies on the infection process of Ceratocystis ulmi (Buism.) C. Moreau in American elm trees. Can. J. Botany, 40: 1567-1575.

Ouellette, G.B., and Gagnon, C. 1960. Formation of microendospores in Ceratocystis ulmi (Buism.) C. Moreau. Can. J. Botany, 38: 235-241.

Pearse, A.G.E. 1960. Histochemistry, technical and applied. Churchill Press, London, and Little Brown & Co., Boston.

Pierson, C.F., Gothoskar, S.S., Walker, J.C., and Stahman, M.A. 1955. Histological studies on the role of pectic enzymes in the development of Fusarium wilt symptoms in tomato. Phytopathology, 45: 524-527.

Pomerleau, R. 1947. Means of inoculation of the Dutch elm disease by Hylurgopinus rufipes Eich. Can. J. Research, C. 25: 102-104.

Pomerleau, R. 1961. Noms français des maladies des arbres au Canada. 41ième Rapport annuel Soc. Prot. Plants Qué., Suppl., Public. 263. Min. Agr. Prov. Qué. pp. 109-160.

Postlethwait, S.N., and Rogers, B. 1958. Tracing the path of the transpiration stream in trees by the use of radioactive isotope. Amer. J. Botany, 45: 753-757.

Pope, S.E. 1943. Some studies on the Dutch elm disease and the causal organism. Ph.D. Thesis, Cornell University, Ithaca, N.Y.

Reeve, R.M. 1951. Histochemical tests for polyphenols in plant tissues. Stain Technol. 26: 91-96.

Reeve, R.M. 1959. Histological and histochemical changes in developing and ripening peaches. I. The catechol tannins. Amer. J. Botany, 46: 210-217.

Reeve, R.M. 1959a. Histological and histochemical changes in developing and ripening peaches. II. The cell walls and pectins. Amer. J. Botany, 46: 241-248.

Reeve, R.M. 1959b. Histological and histochemical changes in developing and ripening peaches. III. Catechol tannin content per cell. Amer. J. Botany, 46: 645-650.

Reeve, R.M. 1959c. A specific hydroxylamine-ferric chloride reaction for histochemical localization of pectin. Stain Technol. 34: 209-211.

Rosinski, M.A., and Campana, R.J. 1964. Chemical analysis of the cell wall of Ceratocystis ulmi. Mycologia, 56: 738-744.

Rutenberg, A.M., Rutenberg, S.H., Monis, B., Teague, R., and Seligman, A.M. 1958. Histochemical demonstration of β-D-galactosidase in the rat. J. Histochem. Cytochem. 6: 122-129.

Saaltink, G.J., and Dimond, A.E. 1964. Nature of plugging material in xylem and its relation to rate of water flow in Fusarium-infected tomato stems. Phytopathology, 54: 1137-1140.

Sass, J.E. 1951. Botanical microtechnique. (2nd. Ed.) Iowa State College Press, Ames, Iowa.

Schwarz, M.B. 1922. Das Zweigsterben der Ulmen, Trauerweiden und Pfirsichbäume. Mededeelingen uit het Phytopathologisch Laboratorium "Willie Commelin Scholten" Baarn. 5: 7-32. Utrecht. Translated by L.D. Kelsey, Bartlett Research Laboratory Bull. 1: 5-25. (1928)

Siegel, S.M. 1953. On the biosynthesis of lignin. Physiol. Plantarum, 6: 134-139.

Vanden Born, W.H. 1963. Histochemical studies of enzyme distribution in shoot tips of white spruce (Picea glauca (Moench) Voss). Can. J. Botany, 41: 1509-1527.

Van Fleet, D.S. 1959. Analysis of the histochemical localization of peroxidase related to the differentiation of plant tissues. Can. J. Botany, 37: 449-459.

Verrall, A.F., and Graham, T.W. 1935. The transmission of Ceratostomella ulmi through root grafts, Phytopathology, 25: 1039-1040.

Waggoner, P.E., and Dimond, A.E. 1955. Production and role of extracellular pectic enzymes of Fusarium oxysporum f. lycopersici. Phytopathology, 45: 79-87.

Watari, H., Kearney, E.D., and Singer, T.P. 1963. Studies on the respiratory chain linked reduced nicotinamide adenine dinucleotide dehydrogenase. IV. Transformation of the dehydrogenase into cytochrome c reductase-diaphorase by the action of acid-ethanol. J. Biol. Chem. 238: 4063-4073.

Westenberg, J. 1932. Uit de historic van de Graphium-ziekte in de Iepen. Tijdschr. over Plantenziekten, 38: 61-66.

Wilson, C.L. 1965. Ceratocystis ulmi in elm wood. Phytopathology, 55: 477.

Wollenweber, H.W. 1927. Das Ulmensterben und sein Erreger, Graphium ulmi, Schwarz. Sonderabsdruck aus dem Nachriechtenplatt fürden deutschen Pflanzenchutzdienst. No. 10. Translated by L.D. Kelsey, Bartlett Research Laboratory Bull. 1: 26-31. (1928).

Wood, R.K.S. 1960. Pectic and cellulolytic enzymes in plant diseases. Ann. Rev. Plant Physiol. 11: 299-322.

Zalokar, M. 1959. Growth and differentiation of Neurospora hyphae. Amer. J. Botany, 46: 602-610.

Zentmeyer, G.A. 1942. Toxin formation and chemotherapy in relation to Dutch elm disease. Phytopathology, 32: 20 (Abstr.).

Zentmeyer, G.S. 1942a. Toxin formation by Ceratostomella ulmi. Science, 95: 512-513.

Zentmeyer, G.A., Horsfall, J.G., and Wallace, P.P. 1946. Dutch elm disease and its chemotherapy. Conn. Agr. Exp. Sta. Bull. 498: 1-70.