In 1967, Doi et al. proposed the hypothesis that 'yellows type diseases' are caused by mycoplasma. Since then more than 60 yellows diseases have been associated with mycoplasma-like bodies (MLB) (Maramorosch, 1973). The majority of these associations are based upon electron microscopic observations of mycoplasma-like bodies in situ. However, little is known about the in situ development of MLB. Hull (1971) pointed out that information is lacking about the relative abundance of MLB in different cell types, at different stages of development, and in perennial plants at different times of the year. Therefore, a developmental cytopathological study of X-disease in Prunus persica Batsch. and P. avium L. and Albino disease of P. avium was initiated using techniques of light and electron microscopy.

Three X-diseased P. persica trees were located in an orchard with a history of X-disease problems at The Dalles, Oregon. A
symptomless control tree was located in the same orchard and a healthy control tree was established in a screen house at Corvallis, Oregon. Two albino infected P. avium trees were located in an orchard at Medford, Oregon, with control trees as stated above. Leaves and peduncles of healthy and diseased trees were sampled sequentially during the 1972 and 1973 growing seasons by dicing the distal 2-5 mm of midvein directly in 4% glutaraldehyde. The trees at The Dalles were sampled every other week while those at Medford were sampled once a month. The samples were subsequently fixed in osmium, dehydrated, embedded, sectioned, and observed. Comparisons were made between diseased and healthy phloem parenchyma cells and sieve tube elements.

Preliminary indications suggest that there are structures present during the early ontogeny of sieve tube elements and phloem parenchyma cells that may be confused with MLB. Structures resembling MLB were found in healthy, virus-indexed controls as well as diseased trees. The structures are unit membrane bound, contain ribosomes and fibrils similar to nucleic acid strands. They are spherical to irregularly ellipsoidal in shape and 200-450 nm in diameter. In cross and longitudinal serial sections they are found to be in close association with endoplasmic reticulum and the nuclear envelope. Observations also show the consistent presence of true mycoplasma-like bodies in mature sieve tube elements of diseased
tissue during June, July, and August and the consistent absence of
such structures in immature sieve tube elements, phloem parenchyma
cells, and in comparable cells of healthy controls. Criteria are set
forth for distinguishing between true MLB and the structures in
healthy, virus-indexed plant tissue.

The presence in healthy, virus-indexed controls of structures
similar to or the same as MLB points out the need for more research
in this area. A more complete study of a MLB-associated disease
is needed. The ontogeny of healthy, virus-free phloem parenchyma
cells and sieve tube elements needs to be studied in detail so that
valid comparisons can be made between healthy and diseased material
not only at chronological stages but at comparable physiological
stages of growth and development.
The Developmental Cytopathology of \textit{Prunus persica} Batsch. and \textit{P. avium} L.
Infected with X-disease and Albino Disease Respectively

by

Edwin R. Florance

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Sincere gratitude is extended to Dr. H. Ronald Cameron for his support, patience, and invaluable counsel during the course of this study.

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To Diane and Blaine
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THE DEVELOPMENTAL CYTOPATHOLOGY OF PRUNUS PERSICA BATSCH. AND P. AVIUM L. INFECTED WITH X-DISEASE AND ALBINO DISEASE RESPECTIVELY

INTRODUCTION

Since Doi, Teranaka, Yora, and Asuyama (1967) proposed the hypothesis that yellows-type diseases are caused by mycoplasma, more than 60 'yellows diseases' have been associated with mycoplasma-like bodies (MLB) (Maramorosch, 1973). These associations are based mostly upon electron microscopic observations of MLB in situ. However, little is known about the in situ development of MLB. Information is lacking about the relative abundance of MLB in different cell types of the plant, at different stages of development, or in perennial plants at different times of the year (Hull, 1971). As a result of this lack of knowledge, a study was initiated on the developmental cytopathology of X-disease in Prunus persica Batsch. and Albino of P. avium L.

X-disease in peach was chosen because 1) peach is a perennial, 2) X-disease is reportedly a mycoplasma-caused disease (see Literature Review), 3) comparisons could be made between a known mycoplasma-caused disease and a disease of unknown but suspected MLB etiology, i.e., Albino, 4) a good source of healthy and naturally infected material was available, and 5) X-disease is of significant economic importance. Albino was chosen because 1) it was unstudied
cytologically, 2) the disease agent was unknown, 3) based on comparisons between symptoms, transmission characteristics, and purification characteristics of X-disease and Albino it was felt that Albino had an excellent potential of being associated with some prokaryotic organism, and 4) economic importance in Oregon.

X-disease causes considerable economic loss to peach and cherry growers each year. Fruit on diseased trees is unmarketable and young trees often die within 1 to 3 years after the appearance of initial symptoms. Older trees may remain in a state of decline for several years but ultimately they either die or become so unproductive they require removal. Peach trees do not become uniformly affected during the first year. Symptoms may appear on some branches while others will appear normal. This variability in symptom expression may continue for several years, resulting in a tree with two or three completely diseased scaffolds and one or two normal appearing scaffolds. X-disease is widely distributed in fruit growing areas throughout the United States (except in South Carolina, Georgia, Arkansas, and Texas) and in southern Canada.

Albino is of less economic importance since it only infects sweet cherries and appears to be restricted to the Rogue River Valley of Oregon. However, 25 to 30 years ago Albino virtually eliminated commercial cherry production in the Rogue River area. Therefore, if spread from that area should occur, the economic
results could be disastrous since the disease is so devastating.

Albino expresses the same type of variability in symptom expression as X-disease and infected trees usually die in 1 to 4 years.
LITERATURE REVIEW

X-Disease

Since the literature on X-disease was thoroughly reviewed by Gilmer and Blodgett (1973), only the literature that deals with possible mycoplasma etiology of X-disease will be covered. All of the evidence published to date is based upon visualization of MLB in situ by electron microscopy. However, recently Nyland has treated peach trees with tetracycline and observed remission of symptoms (personal communication). Nasu et al. (1970) reported MLB in cells of Colladonus montanus Van Duzee and Apium graveolens L. (celery), a vector and herbaceous holding plant respectively, infected with X-disease. Nasu et al. collected EM samples from "foliage growing points and root tips." Although they studied the development of X-disease in A. graveolens L. sequentially, they present no evidence of comparison between diseased material (symptoms appeared 26 days after inoculation) and healthy material of comparable physiological age (nor did they publish micrographs of MLB in cells of peach trees). MacBeath et al. (1972) published a paper in which they showed electron micrographs of MLB's associated with X-disease in Prunus persica Batsch. Leaves of peach were collected in October 1969 from infected and healthy trees and the distal 1.5 mm of the midvein was sampled. Granett and Gilmer (1971) reported the
association of MLB with wild *Prunus virginiana* L. naturally infected with the eastern strain of X-disease, *P. avium* L. (Windsor sweet cherry) showing little cherry symptoms, and in *P. avium* L. (mazzard cherry seedlings) inoculated with diseased buds. Leaves showing symptoms were collected during the month of August. The authors do not specify the area of the leaf sampled, stating that "1- to 2-mm sections of leaf vein were fixed in glutaraldehyde and osmium." Neither sequential nor developmental studies were undertaken by the latter workers.

**Albino**

*Albino* of *Prunus avium* L. was originally discovered in Ashland, Oregon by Zeller, Milbrath, and Cordy in 1937. It affects most cherry varieties on either mazzard or mahaleb rootstocks. Zeller et al. (1944) reported that in numerous tests peach varieties inoculated with buds from Albino infected sweet cherries expressed no fruit or leaf symptoms and indexing from inoculated peach to sweet cherry indicated that the agent of Albino was not present in peach. This is the only criterion for separating Albino from X-disease since all the other characteristics (i.e., symptoms, transmission, etc.) appear to be similar. The agent of Albino has never been purified, it is not mechanically transmissible although it is easily transmitted by budding
or grafting, and Albino has never been studied cytologically. (For a complete description of symptoms and disease characteristics, see Cameron, 1973.)

**Mycoplasmas**

Mycoplasmas are highly pleomorphic, membrane bound, prokaryotes ranging from 0.5 to 1.0 μ in diameter with minimum viable units as small as 125-250 mm in diameter. They contain ribosomes, nucleic acid strands and sometimes small vacuoles (Allen et al., 1970; Anderson, 1969). Originally, mycoplasmas were referred to as pleuropneumonia-like organisms (PPLO) after the etiologic agent of contagious bovine pleuropneumonia. As additional similar organisms were isolated from other animals, they also came to be classified as PPLO (Hayflick, 1969). From the time of the original work on contagious bovine pleuropneumonia by Nocard and Roux in 1898 until 1966, considerable confusion existed about the nomenclature of such organisms. However, in 1966 the International Committee on Nomenclature of Bacteria, Subcommittee on the Taxonomy of Mycoplasmatales recommended that the general principles of classification proposed by Edward and Freundt (1956) be formally recognized and further extended. This subcommittee then established the following classification system: Kingdom, Protista; Class, Mollicutes; Order, Mycoplasmatales; Family, Mycoplasmataceae; Genus, Mycoplasma.
Mycoplasmas have been implicated in many animal diseases (Hayflick, 1969). Yet several species have been isolated that are nonpathogenic. According to Hayflick, mycoplasmas have been isolated from the urethras of clinically normal men and women and five species are regarded as nonpathogenic and part of the normal microbial flora of the human oral cavity and urogenital tract.

In brief, animal mycoplasmologists have found that mycoplasmas are ubiquitous organisms. They have been able to grow them on cell-free media, have demonstrated a sterol requirement, a resistance to penicillin, an inhibition by tetracycline and that there is no reversion to or derivation from a bacterial parent (for reviews, see Hampton, 1972; Hayflick, 1969; Razin, 1969).

A group of plant diseases referred to as 'yellows diseases', the type specimen being Aster Yellows (Kunkel, 1926), long were thought to be caused by a virus. However, Doi et al. (1967) using electron microscopy reported the presence of mycoplasma-like bodies (MLB) in the sieve tube elements and phloem parenchyma cells of young leaves and shoots from mulberry trees infected with dwarf disease, and a consistent absence of such structures in healthy plants.

Repeated electron microscopic examination of thin sections of young leaves and shoots from mulberry tree infected naturally or artificially with dwarf disease, have failed to show any such uniform particles, spherical or elongated, as have been described for plant viruses in the past. Presence of specific, pleomorphic bodies, however, have been demonstrated consistently in the sieve tubes and occasionally also in the phloem-parenchyma cells. These bodies are spherical to irregularly
ellipsoidal in shape and 80 to 800 nm in diameter. They possess a two-layered limiting membrane of about 8 nm in thickness, instead of cell wall. The smaller bodies, 100-250 nm in diameter, are nearly round, and generally filled with ribosome-like granules of about 13 nm in diameter. Sometimes net-strands similar to those found in the nuclear regions of other bacteria were located in the less electron dense area. The larger bodies are occupied by large central vacuole surrounded with ribosome-like granules at the periphery. Frequently structures similar to nuclear net-strands are observed inside the vacuolated area.

The gross morphology and fine structure of these bodies seem to be similar to the description of either the cells of Mycoplasma species (Pleuropneumonia-like organisms) or agents of Psittacosis-Lymphogranuloma-Trachoma group as given by Domeruth et al. (1964), and others, though any agents of such groups have as yet been reported from plants, so far as we know (Doi et al. 1967, p. 259).

These findings established the hypothesis that MLB may be the causal agents of 'yellows diseases'. To date, this hypothesis has not been proven as routine culture and subsequent fulfillment of Koch's postulates have not been published. However, research since 1967 has followed the precedent set by Doi et al. (1967) and to date more than 60 plant diseases have been associated with MLB (Maramorosch, 1973). The majority of these associations are based upon the visualization of MLB in situ by electron microscopy (Maramorosch, Granodos, and Hirumi, 1970). The procedures to establish such an association are as follows: 1) when symptoms appear, young leaves are collected from diseased and healthy plants that have grown for an equivalent amount of time, 2) vascular tissue from these leaves is prepared for electron microscopy, and 3) subsequent comparisons are made between
healthy and diseased sieve tube elements and phloem parenchyma cells. If MLB's are observed in diseased sieve tube elements, an association is established.

Since the causal agent of 'yellows diseases' is reportedly confined to the sieve tube elements and phloem parenchyma cells (Davis and Whitcomb, 1971; Doi et al., 1967; Maramorosch et al., 1970), it is necessary to thoroughly understand the normal ontogeny of these cells before attempting to interpret ultrastructural events in diseased tissue.

Comparison Between Sieve Tube Element and Phloem Parenchyma Cell Development and Mycoplasma-like Bodies in situ

Since Hartig (1837) discovered the sieve tube element 135 years ago, the structure has been extensively examined by numerous workers (see Esau and Cheadle, 1965; Esau, 1969; Eschrich, 1970; Weatherley and Johnson, 1968). Such research has shown that a unique series of changes occur during differentiation of sieve tube elements. These changes result in the degradation or complete loss of many of the original organelles. The cytoplasm of the young sieve tube element resembles the cytoplasm of any young phloem parenchyma cell. However, during differentiation there is a disintegration of the nucleus, slime bodies, and tonoplast. The mitochondria undergo degenerative changes and assume, with endoplasmic reticulum, a
parietal position. Although the major degenerative stages have been examined thoroughly, work is still needed to refine our knowledge of the changes that are occurring in the fine structure of sieve tube elements between these stages, e.g., changes in endoplasmic reticulum from the daughter cell stage until it assumes a parietal position, the appearance of mitochondrial structure during degradation, and the changes of various vesicles that appear and disappear during differentiation. Further research on phloem parenchyma cell development is also needed to formulate a complete understanding of structural changes that occur not only during differentiation but subsequent to maturity and prior to cell death.

Comparisons between papers dealing with phloem development and papers dealing with the occurrence of MLB in immature sieve tube elements and phloem parenchyma cells show the existence of structures that have been interpreted as MLB by plant pathologists and as vesicles in cisternae of endoplasmic reticulum (ER) by developmental cytologists. Table I is a summary of the publications on sieve tube element and/or phloem parenchyma cell development that contain electron micrographs showing structures similar to MLB. Table II is a summary of the publications on MLB in situ that show structures either similar to or identical with the structures shown in the figures of the papers cited in Table I.
Table I. List of publications from literature on phloem development that show structures similar to reported MLB.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Experimental organism</th>
<th>Fixative</th>
<th>Cell type</th>
<th>Figure in cited publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kollman &amp; Schumacher (1961)</td>
<td>Metasequoia glyptostroboides</td>
<td>K$_2$Cr$_2$O$_7$</td>
<td>PP</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OsO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Falk &amp; Sitte (1963)</td>
<td>Elodea sp.</td>
<td></td>
<td>PP</td>
<td>12b &amp; c</td>
</tr>
<tr>
<td>Srivastava &amp; O'Brien (1965)</td>
<td>Pinus strobus L.</td>
<td>Acrolein Osmium</td>
<td>SC</td>
<td>2</td>
</tr>
<tr>
<td>Behnke &amp; Dorr (1967)</td>
<td>Dioscorea sp.</td>
<td>Glut</td>
<td>STE</td>
<td>5</td>
</tr>
<tr>
<td>Buvat (1968)</td>
<td>Hordeum sativum Pers.</td>
<td>Osmium</td>
<td>STE</td>
<td>3</td>
</tr>
<tr>
<td>Behnke (1969)</td>
<td>Dioscorea macroura Harms</td>
<td>Glut Osmium</td>
<td>STE</td>
<td>11b</td>
</tr>
<tr>
<td>Esau &amp; Hoefert (1971)</td>
<td>Tetragonia expansa Murr.</td>
<td>Glut/Para Osmium</td>
<td>VasP</td>
<td>3, 4, 10, 14</td>
</tr>
<tr>
<td>Esau &amp; Hoefert (1972)</td>
<td>Beta vulgaris L.</td>
<td>Glut/Para Osmium</td>
<td>CC</td>
<td>1-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PP</td>
<td>25-35</td>
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</tbody>
</table>

*a* Abbreviations: Glut = Glutaraldehyde, PP = Phloem parenchyma, STE = Sieve tube element, CC = Companion cell, SC = Sieve cell
Table II. List of publications from literature on MLB in situ that show structures similar to structures reported to be cisternae of ER.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Experimental organism</th>
<th>Fixative</th>
<th>Cell type</th>
<th>Figure in cited publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lombardo et al. (1970)</td>
<td><em>Trifolium repens</em> L.</td>
<td>Glut</td>
<td>PP</td>
<td>3, 6, 7, 9</td>
</tr>
<tr>
<td>Pellegrini &amp; Gerola (1970)</td>
<td><em>Oryza sativa</em> L.</td>
<td>Glut</td>
<td>PP</td>
<td>1, 2, 3</td>
</tr>
<tr>
<td>Amici &amp; Favoli (1972)</td>
<td><em>Oryza sativa</em></td>
<td>Glut</td>
<td>PP</td>
<td>1, 2</td>
</tr>
<tr>
<td>Cousin et al. (1972)</td>
<td><em>Nicotiana tabacum</em> L.</td>
<td>Glut</td>
<td>STE</td>
<td>2, 5, 6</td>
</tr>
</tbody>
</table>
In all cases there is a close association of the structures to ER. With the exception of Kollman and Schumacher (1961), Figure 3, all other figures show ribosomes inside the structures. All figures appear to show spherical to pleomorphic structures bound by a single unit membrane. The structures occur singly or in groups surrounded by a second membrane, the ER. Fibrils may or may not be present within the structures. Where size can be calculated it falls within the range given for known mycoplasmas. Information, presently available in the literature, does not specify whether the observed structures are a normal feature in differentiating phloem parenchyma cells and sieve tube elements, independent organisms in situ, or abnormalities resulting from infection. With the exception of Esau and Hoeffert (1972), few researchers of phloem development consider the aspect of disease infection and vice versa; few researchers working on the in situ presence of MLB consider the entire development of sieve tube element and phloem parenchyma cell.
MATERIALS AND METHODS

Peach Materials

In 1971, a survey of the Albert D. Francois Mill Creek orchard located at The Dalles, Oregon revealed a good source of X-diseased material. Based on symptomatology, four peach trees (P. persica Batsch.) were marked for experimental study. Tree #1 appeared mildly affected, tree #2 moderately affected, tree #3 severely affected, and tree #4 represented a healthy control. The Dalles control tree was judged to be healthy on the basis that it was symptomless and tested negative for latent Prunus viruses by indexing to P. serrulata Lindl. (Shiro-fugen). A healthy, virus-free, indexed, control tree was located in a screenhouse at the Oregon State University Botany and Plant Pathology Research Facility. This tree was judged to be healthy based on the following criteria:

1) It was propagated from bud wood obtained from the Inter-regional Deciduous Tree Fruit Repository at Prosser, Washington (Fridlund, 1962, 1972). The Repository's main function is to develop and maintain virus-free deciduous fruit trees (Fridlund, 1962).

2) While at the repository the tree was intensively indexed on 10 Prunus virus indicator plants (Fridlund, 1973).

3) The tree has been maintained in a screenhouse throughout its
history in order to prevent transmission of insect carried
viruses or mycoplasma into it.

4) At Oregon State the tree has been reindexed on all 10 indicator
hosts and has tested negative in yearly indexing on *P. serrulata*
(Shiro-fugen).

5) The tree is symptomless.

In addition to the controls listed above, healthy, virus-indexed
material was collected from trees located in screenhouses and from
a heat treated, virus-indexed tree at the repository. Heat treatment
eliminates the causal agents of yellows-type diseases (Hilderbrand,
1953; Kunkel, 1941; Stoddard, 1947) (Table III, summary of controls).

Sampling of experimental plants for light and electron micros-
copy was started on March 8, 1972 by collecting small twigs and
forcing bud-break in the greenhouse at 20°C. Subsequently, samples
were taken from leaves at the time of natural bud break, April 4,
1972, and continued on a biweekly basis for the remainder of the
growing season. The distal 2-5 mm of midvein was diced directly in
4% glutaraldehyde buffered to pH 7.0 with 0.125 M sodium phosphate
buffer. Fixation was carried out for 1-2 hr at room temperature
followed by 10 hr at 4°C under vacuum. The tissue was then washed
for 2-4 hr in sodium phosphate buffer and post-fixed for 6-8 hr in
2% sodium phosphate buffered osmium tetroxide at 4°C under 380-
508 mm Hg (15-20 inches).
Table III. Summary list of controls.

1. Orchard controls at The Dalles and Medford, Oregon (one peach and one cherry tree respectively).

2. Virus-indexed controls under screen at Corvallis; *P. persica* cultivar Elberta tree E-64 and *P. avium* cultivar Bing tree W-201. These trees were obtained from the Interregional Deciduous Tree Fruit Repository at Prosser, Washington.

3. Tissue samples collected from healthy, virus-indexed trees at the Repository; IR3-5 and IR559-1.


Note: Code numbers are identification numbers used by Dr. H. R. Cameron at OSU and Dr. P. R. Fridlund at Prosser, Washington to locate specific trees.
Samples were dehydrated at room temperature by three 10-min washes in 50% acetone/dist H₂O, 6-8 hr in 70% acetone/dist H₂O saturated with uranyl acetate, three 10-min washes in 100% acetone and two 15-min washes in propylene oxide. The 70% acetone/uranyl acetate solution was made by adding excess uranyl acetate to a small amount (∼10-20 ml) of 70% acetone/dist H₂O solution and placing on a magnetic stirrer for 15 min. The amount to be made varies as per the number of samples. After stirring, the solution is double filtered through Eaton-Dikeman filter paper grade 613. Samples were continually rotated during the 6-8 hr in 70% acetone.

After dehydration, samples were infiltrated with plastic by placing them in a 75% propylene oxide/plastic mixture for 30-60 min followed by 50% and 75% plastic/propylene oxide mixtures ∼12 hr each. The plastic mixture was made from two stock plastic solutions as follows: solution A consisted of 31 ml Epon 812 and 50 ml of Dodecenyl Succinic Anhydride (DDSA); solution B consisted of 50 ml Epon 812 and 45 ml Nadic Muramic Anhydride (NMA). Each solution was mixed thoroughly and stored in a refrigerator. The final plastic mixture was made by adding one part solution A to three parts solution B, catalyst, Benzyl Dimethyl Amine (DBMA), at a concentration of 1% of the volume, and mixing thoroughly. The plastic mixture used during infiltration contained all components except catalyst. The
samples were placed on a rotator during each step of the infiltration process.

The samples were embedded by placing them in the complete plastic mixture without catalyst after the last infiltration step (i.e., 75% plastic/25% propylene oxide) for 6-8 hr at room temperature under vacuum (380-500 mm Hg, ≈ 15-20 inches). This was followed by another change of complete catalyzed plastic. At this stage the tissue was transferred to flat embedding molds, placed under vacuum (380-500 mm Hg), and allowed to polymerize at 37°C for ≈ 12 hr. The temperature was then increased to 60°C and the samples polymerized for another 12-24 hr.

Pieces of tissue were cut from the flat embedding disks and glued on 00 size, plastic-filled, gelatine capsules. A Sorvall "Porter-blum" MT-2 ultramicrotome and a diamond knife were used for sectioning. Cross and longitudinal thick sections (1 μ) were mounted on glass microscope slides, allowed to dry at least 5 min on a slide warmer, stained 1-5 min with 0.5% Toluidine Blue (Feder and Obrien, 1968), rinsed with distilled water, dried, covered with a No. 1, 22-mm sq. cover slip and examined by light microscopy. These sections were used for orientation of the tissue prior to thin sectioning, location of specific cell types (i.e., sieve tube elements), and coordination of structures visualized at the light microscope level with those visualized by the electron microscope. Serial cross and
longitudinal thin sections 60-90 nm were picked up from the water-filled knife trough using colloidion (1% in amyl acetate) covered metal or plastic washers (1/2 in. outside diameter). These sections were subsequently transferred to 100 mesh parallel line, copper grids using a dissecting microscope rigged with special apparatus (Rickson, personal comm.). It is important to sonicate the grids in detergent followed by two rinses in acetone ~ 5 min each before mounting. This facilitates the membranes adhering to the grids during the transfer process. The sections were then post-stained for 10 min in lead citrate pH 12.0 (Reynolds, 1963), examined, and photographed with a Phillips EM-300 electron microscope operating at 60 KV. It was decided in advance that 50% of the observation time would be used in examining healthy tissue and 50% examining diseased material.

When pertinent sets of serial photographs were obtained, the photographic images from the negatives were enlarged onto 8 x 10 DuPont Cronar Ortho Litho Type S sheet film. This results in a positive image on a transparent film base (Brown and Arnott, 1971). These transparencies were aligned and stacked using techniques outlined by Brown and Arnott. Once stacked and aligned, stereo pair photographs were taken for subsequent study and recording of three-dimensional structure.
Cherry Materials

Three trees (P. avium L.) infected with albino were located in an orchard at 2178 Pioneer Road, Talent, Oregon during the summer of 1971. Talent is a small community 7 miles south of Medford, Oregon. Controls were a healthy appearing tree in the same orchard, a healthy, virus-indexed tree maintained under screen at the OSU Botany and Plant Pathology Research Facility, and all of the peach material both healthy and X-diseased. Sampling of cherry trees started on April 3, 1972 and continued on a monthly basis until September. The tissue samples were handled as per the procedures given for the peach tissue. Cytological data obtained from healthy and albino diseased cherry (P. avium) tissue were compared to those obtained from peach (P. persica) tissue by comparing electron photomicrographs of specific cell types.
RESULTS

Control Tissue

Vesicles resembling mycoplasma-like bodies (MLB) were present in healthy, virus-indexed, heat treated control trees of *P. persica* (Figures 1-4, 6 and 7). The structures occurred in 1) phloem parenchyma cells and immature sieve tube elements of the midvein in leaves collected prior to bud-break and forced in the greenhouse (March 8, 1972), 2) leaves emerging naturally from buds of trees in the field (samples taken at bud-break April 4, 1972), and 3) all samples taken through June 6, 1972.

The bodies occur singly and in close association with endoplasmic reticulum (ER) (Figures 1, 4 and 7), in groups which appear to be localized in expanded ER cisternae (Figures 2, 4 and 6), in cisternae created by the expansion of the outer nuclear membrane (ONM) (Figure 2), and range from 0.2-0.4 μ in diameter and up to 0.7 μ long. In some cases, ribosomes (r) arranged similarly to those found on typical rough ER line the inner side of the bounding membrane (Figure 4B, arrow). However, in other cases the ribosomes appear scattered throughout the body (Figure 2B, arrow) and/or both. Fibrils (f), which have been interpreted to be nucleic acid strands, can be seen in some of the bodies (Figure 1). The bounding membrane is a single unit membrane (UM) (Figure 1, insert).
Figure 1. Phloem parenchyma daughter cell in healthy, virus-indexed Prunus persica tree E-64. Note structures resembling mycoplasma-like bodies (MLB). The structures contain ribosomes and fibrils (f). 34,500 X. Scale is 1 mm = 29 nm. Insert shows the unit membrane structure of the membrane bounding the bodies. 71,000 X. Scale is 1 mm = 14 nm. Sample collected April 4, 1972.
Figure 2A. Immature sieve tube element in leaf from tree E-64. Note the vesicles in the expanded ER cisternae and the vesicles contained within the cisternae formed by the outer nuclear membrane (ONM). 20,600 X. Scale is 1 mm = 48 nm. Sample collected April 4, 1972.

Figure 2B. A portion of expanded ER cisternae from the cell in Figure 2A (box). Note how the ribosomes (r) appear scattered throughout the vesicle. 54,400 X. Scale is 1 mm = 18 nm. Sample collected April 4, 1972.
Figure 3. Immature sieve tube element in leaf of tree E-64. Note variation in fixation between bodies labelled MLB in Figure 3 and those labelled MLB in Figure 1. Also overall variation in fixation between Figure 3 and Figure 2A. 24,500 X. Scale is 1 mm = 40 nm. Sample collected April 4, 1972.
Figure 4A-D. Serial sections of expanded endoplasmic reticulum (ER) cisternae observed in phloem parenchyma cells and immature sieve tube elements of tree E-64. Note the vesicles in the expanded cisternae and their similarity to mycoplasma-like bodies. The serial sections show that the bodies labelled 1 and 2 in Figure 2B arise from invaginations (i) of the ER surrounding the cisternae (Figure 2C). Note other invaginations (i) in Figure 2D and the ribosome (r) studded membrane of the vesicle in Figure 2B (arrow). 30,400 X. 1 mm = 34 nm. Sample collected April 4, 1972.
The bodies appear in both cross and longitudinal serial sections (Figure 4A, B, C and D). They arise from an invagination (i) of ER as shown in serial sections (Figure 4B, C and D, arrows). In Figure 4B, two bodies are labeled by numbers 1 and 2 so that these bodies can be followed in subsequent figures (Figure 4C and D). Body 1 in Figure 4B appears to be unattached to the membrane surrounding the entire cisterna and free within it. However, Figure 4C shows that body 1 is actually attached to the cisternal membrane and is arising as a result of the invagination of this membrane. In Figure 4D, body 1 is still closely associated with the cisternal membrane but the attachment is not as prominent as in Figure 4C. The same analysis can be made of body number 2. The structure of other bodies can also be followed through the serial sections enabling one to determine the interconnections between bodies and the three-dimensional shape and size.

The bodies shown in Figures 1-4 are not found in serial sections of mature sieve tube elements (STE). Mature STE's contained P-protein (PP) (Figure 5D), remnants of sieve tube element plastids (i.e., starch accumulations, st) (Figure 5C), aggregations of ER (Esau and Hoefert, 1971) (Figure 5A), and secondary vacuolations (SV) (Mahlberg, 1972) (Figure 5D).

Variation in the appearance of the vesicles occurs in cells of the same tissue sample. Figures 2A and 3 show two immature sieve
Figure 5A-D. Serial sections of mature sieve tube element from tree E-64, *P. persica*. Note secondary vacuolation (SV), phloem protein (pp), starch (st), and the lack of expanded ER cisternae. 14,800 X. 1 mm = 67 mm. Sample collected April 4, 1972.

Figure 5E. Sieve tube element from tree E-64, *P. persica*. Same cell as that shown in Figure 5D enlarged to approximately the same magnification as Figures 4A-D for comparison of starch accumulations to vesicles in expanded endoplasmic reticulum cisternae. Note the difference in appearance of starch accumulations i.e., electron density verses the ground cytoplasm appearance of the vesicles in Figure 4A-D, the lack of ribosomes, and lack of bounding unit membrane around starch accumulations. 26,400X. Scale is 1 mm = 38 nm.
Figure 6. Vesicles in expanded ER cisternae in an immature sieve tube element of heat treated *P. persica* (IR 757-1) obtained from the Interregional Deciduous Tree Fruit Repository at Prosser, Washington. *65,300 X*. Scale is 1 mm = 15 nm. Sample collected March 25, 1973.
Figure 7. Longitudinal sections of maturing sieve tube element. Note vesicles in expanded ER cisternae (arrow). 8,250 X. 1 mm = 118 nm. Sample collected April 18, 1972.
tube elements in the same vein. In Figure 2A, the vesicles appear clear and distinct with the contents appearing very similar to the ground cytoplasm surrounding them. However, in Figure 3, the vesicles are electron dense and the internal structure is not visible.

**Diseased Tissue**

Vesicles in expanded ER cisternae and in cisternae formed by the outer nuclear membrane, as shown in Figures 1-4, were also observed in immature phloem parenchyma cells and sieve tube elements of albino diseased *P. avium*, and X-diseased *P. avium* and *P. persica*. However, in addition MLB were observed in mature sieve tube element of samples taken from diseased trees during June, July, and August. The bodies were present in mature sieve tube elements of both leaf (Figures 10 and 11) and peduncle (Figures 8 and 9). No virus particles were found and comparable structures were not found in control trees at the same stage of development, i.e., mature sieve tube elements. The structures ranged from 0.15 to 1.0 µ in diameter with a mean diameter of 0.41 µ at a 99% confidence level that the mean lies between 0.34-0.47 µ. Length ranged from 0.51-1.8 µ with a mean length of 0.96 µ at a 99% confidence level that the mean lies between 0.82-1.1 µ. They vary from spherical (sph) to tubular (tu) in shape (Figures 18 and 19), are bound by a unit membrane, and usually contain a dense nucleoid area.
Figure 8A-D. Cross serial sections of sieve tube element in albino infected *P. avium*. Note the mycoplasma-like bodies packing the sieve tube and the morphology of the structures. 20,500 X. 1 mm = 55 nm. Sample collected July 25, 1972.
Figure 9A-E. Cross serial sections of sieve tube element in 12-year-old Bing, *P. avium* infected with X-disease. Note the mycoplasma-like bodies. Four bodies are numbered and marked to facilitate following them through the sections. Note that body #2 appears to be completely contained within the five sections whereas body #3 is not. 19,200 X. 1 mm = 53 nm. Sample collected July 17, 1973.
Figure 10A & B. X-diseased *P. persica* from California. Note the mycoplasma-like bodies in both figures (arrows). 19,200 X, 1 mm = 53 nm and 54,400 X, 1 mm = 18 nm respectively. Sample collected July 31, 1973.
Figure 11A, B & C. Longitudinal serial sections of sieve tube element in albino infected *P. avium* leaf. Note spherical morphology of mycoplasma-like bodies. 6,600 X. 1 mm = 152 nm. Sample collected July 25, 1972.
Figure 12. Cross section of peduncle from albino infected \textit{P. avium}.
Note how sieve tube element number 1 appears packed with 
mycoplasma-like bodies, numbers 2 and 3 are less packed and 4 and 5 contain only a few. 11,200 X. 
1 mm = 89 nm. Sample collected June 30, 1972.
In Figure 9, four bodies are numbered and marked for the convenience of following the structures through the serial sections. It can be seen that body number 2 begins in section 9A and ends in section 9E. Similar analyses can be made of the other numbered bodies. These structures can be compared to the structures in Figures 14 and 15, which appear in six sections and the structure in Figure 17 (arrows) which appears in eight sections. Using this procedure and figuring the range of section thickness as 60-90 nm, a diameter range of 0.30-0.72 µ is obtained. This range fits within the range obtained by measurement.

The tubular and spherical forms are exemplified in the stereo pair photographs of Figures 18A, B, C and D. It should be noted that the diameter of the tubular forms ranges from 0.15 to 0.20 µ, which is at the small end of the range given earlier for diameter and probably accounts for the wide range in diameter -- whereas the spherical bodies have a diameter which coincides more closely to the mean. The tubular forms appear to be attached at points along the cell wall where stacked endoplasmic reticulum occurs. For example, in stereo pair 18A the tubular structure in the lower left hand corner (arrow) of the picture can be traced to the cell wall. If one then examines section 16D (arrow) it can be seen that a connection appears to exist. Also, in stereo pair 18B (arrow) a connection is visible which can be seen in section 17D. In each case this connection
Figure 13A-C. Cross serial sections through a sieve plate in albino infected *P. avium* peduncle. Note extensive filamen- 
tation (fil) in the sieve tube element on the right. 20,500 X. 1 mm = 49 nm. Sample collected June 30, 1972.
Figure 14A-F. Cross serial sections of sieve tube element in albino infected *P. avium* peduncle. Note that the body numbered 1 can be followed through all six sections and in section C, a filament (arrow) can be seen projecting into the sieve plate pore. 47,600 X.

1 mm = 21 nm. Sample collected June 30, 1972.
Figure 15A-F. Cross serial sections of sieve tube element in albino infected *P. avium* peduncle. Note that the body numbered 2 in section C appears to contain a unit membrane bound inclusion (arrow). However, in section D the inclusion can be seen to be an invagination of the outer membrane. 47,600 X. 1 mm = 21 nm. Sample collected June 30, 1972.
Figure 16A-H. Cross serial sections of sieve tube element in albino infected *P. avium* leaf. Note the electron dense fibrils contained within the microplasma-like bodies, tubular (tu) and spherical (sph) morphology, and the connection of the tubular MLB in section D (arrow). 20,700 X. 1 mm = 48 nm. Sample collected July 25, 1972. (See stereo pairs of these sections in Figure 18A.)
Figure 17A-J. Cross serial sections of sieve tube elements in albino infected *P. avium* leaf. Note tubular and spherical morphology. Figure 17K is a composite photograph made by stacking transparencies of the serial sections A through J. Note that the tubular branched structure in Figure K (arrow) is made up of the structures marked by arrows in sections C through J. In sections A-J, the magnification is 11,300 X, scale is 1 mm = 88 nm. In K it is 62,900 X, scale is 1 mm = 16 nm. Sample collected July 25, 1972. (See stereo pairs of these sections in Figure 18B, C and D.)
Figure 18A. Stereo pair made from the serial sections in Figure 16. Note the apparent connection of the structures numbered 1 and 2 and the three-dimensional structure of the mycoplasma-like structures. 20,700 X. 1 mm = 48 nm.

Figure 18B. Stereo pair of a structure in the serial sections of Figure 17A-J. Note the connection and morphology. 35,500 X. 1 mm = 28 nm. tu = tubular; sph = spherical

Figure 18C & D. Stereo pairs of serial sections in Figure 17A-J. Figure D is a reciprocal arrangement of the sections in C. Note the spherical structure in C (arrow) is located over several tubular forms and the tubular structure with the constrictions is located above the branched tubular form, whereas in stereo pair D they are the opposite. 22,600 X. 1 mm = 44 nm.
appears in only one section and therefore is a very small point of attachment, i.e., less than the thickness of a single thin section. None of the spherical structures are attached; however, some intermediate forms between tubular and spherical do occur and appear to have an attachment (Figure 18A, arrows). The tubular forms also appear to branch. The structure marked by the arrow in Figure 17K is a composite structure of the components marked by arrows in serial sections 17A-J. Notice the Y shaped branch in the structure. This structure also can be seen in stereo pairs 18C and D. Figures 18C and D represent reciprocal arrangements of the ten serial sections in Figure 17A-J. The spherical structure in stereo pair 18C (arrow) is located over tubular forms. In stereo pair 18D, the same structure can be seen under the tubular forms. There appears to be no interconnection between the spherical MLB and the adjacent tubular MLB. The same spherical MLB can be traced through sections 17A-C and no evidence of interconnection is visible.

Some sieve tube elements are completely packed with bodies (Figures 9 and 11), while other sieve tube elements contain few to many (Figure 12). Serial sections show that the bodies can obtain a filamentous form (Figure 13), especially when located at or near the sieve plate. Figure 13 shows how extensive this filamentation can be. In the serial sections of Figure 14 a filament can be seen to project from the body numbered 1 in Figure 14C into the sieve plate pore.
In Figure 14D the filament can be traced through the pore and in Figure 14E a portion of the filament can be seen on one side of the sieve plate and the spherical part of the body on the other side. Serial sections also have shown what appears to be a unit membrane bound inclusion in some bodies, for example, body number 2 in Figure 15C. It is, in fact, an invagination of the bounding unit membrane (see arrow at body number 2, Figure 15D).

Comparison of Vesicles in ER Cisternae Found in Healthy Tissue to Mycoplasma-like Structures Found in Albino Diseased Tissue

The vesicles that resemble MLB observed in healthy control trees were always found in immature sieve tube element and phloem parenchyma cells. In some cases, the cells were beginning cytoplasmic degradation (Figures 2 and 3), but in no cases were MLB observed in mature sieve tube elements of healthy material (Figure 5). The vacuoles formed by the process of secondary vacuolation are the only structures in mature healthy sieve tube elements that even remotely resemble MLB (Figure 5). In the diseased material the MLB were only observed in mature sieve tube elements (Figures 8, 9 and 19B). On occasion, phloem protein and remnants of various cell organelles were observed (Figures 10 and 11). Vesicles in expanded ER cisternae are the only structures that show a ribosome
Figure 19A. Expanded ER cisternae containing vesicles that resemble mycoplasma-like bodies in healthy, virus-indexed *P. persica* sieve tube element. 43,500 X. 1 mm = 23 nm.

Figure 19B. Mature sieve tube element from albino infected *P. avium* showing mycoplasma-like bodies. 25,600 X. 1 mm = 39 nm. Compare the differences and similarities between the vesicles in Figure 19A to mycoplasma-like bodies in Figure 19B.
studded membrane (Figures 9 and 19A). However, this depends upon whether they arise from invaginations of rough or smooth ER. In general, ribosomes are more abundant and distinct in vesicles of expanded ER cisternae (Figure 19A) than in MLB (Figure 19B). Vesicles in expanded ER cisternae usually are closely associated with or surrounded by a second membrane, the ER (Figure 19A), whereas MLB are not. The internal contents of vesicles in expanded ER cisternae usually have a very close resemblance to ground cytoplasm (Figures 1, 2 and 19A). However, in some cases the vesicles may appear very electron dense (Figure 3). The contents of MLB appear different than ground cytoplasm (Figure 19B). Areas resembling nucleoids are rare in the vesicles (Figure 19A), whereas in MLB nucleoids are conspicuous (Figures 16 and 19B). Although fibrils do appear in the vesicles (Figure 1), they are less distinct than in MLB (Figures 16 and 19B). The fibrils in MLB are more abundant per given body, larger, and very distinct (Figure 16). Vesicles in expanded ER cisternae have a smaller size range than MLB with more irregular shapes (Figures 4 and 19A). Even though MLB have the property of plasticity, as demonstrated by the formation of filamentous forms at sieve plates, they tend to have a more definite shape and a larger size range. The greatest difference between vesicles in expanded ER cisternae and MLB is the fact that in serial sections the vesicles can be seen to arise from invaginations of the ER (Figure 4) (see Table IV for summary of major differences).
Table IV. Summary list of differences between vesicles in ER cisternae found in sieve tube elements and phloem parenchyma cells of healthy tissue and MLB found in sieve tube elements of diseased tissue.

<table>
<thead>
<tr>
<th>Vesicles in expanded ER cisternae</th>
<th>Mycoplasma-like bodies (MLB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Found in immature STE &amp; PPC</td>
<td>Observed only in mature STE</td>
</tr>
<tr>
<td>2. Ribosome studded membrane, ribosomes abundant</td>
<td>Ribosomes scattered and sparse</td>
</tr>
<tr>
<td>3. Usually associated with or surrounded by a second membrane (the ER)</td>
<td>Not found in cisternae of expanded ER</td>
</tr>
<tr>
<td>4. Contents usually resemble ground cytoplasm; however sometimes appear electron dense</td>
<td>Contents do not closely resemble ground cytoplasm</td>
</tr>
<tr>
<td>5. Nucleoids mostly absent</td>
<td>Nucleoids dense and abundant</td>
</tr>
<tr>
<td>6. Fibrils less distinct</td>
<td>Fibrils abundant and distinct</td>
</tr>
<tr>
<td>7. Less definite form; greater variation in size; irregular shapes present</td>
<td>More definite shape, size, and form; few if any irregular shapes present; filamentous form at sieve plates</td>
</tr>
<tr>
<td>8. In serial sections can be seen to arise from invaginations of the ER</td>
<td>Not invaginations of ER</td>
</tr>
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</table>
DISCUSSION

Healthy Material

The procedures for establishing an association between MLB and a given 'yellows disease' by electron microscopy are outlined on p. 8 of the Literature Review. An important point that has not been considered in many studies is that comparisons of diseased and healthy tissue at a given point in time may not be valid. The affects of the disease may be seen rather than the causal agent. The MLB associated diseases are prime suspects for this type of error. The symptoms of 'yellows' diseases are vierscence of flower petals, precocious growth of some flower parts, and breaking of dormancy of axillary buds with subsequent proliferation of lateral branches. These symptoms resemble juvenile stages of normal plant development. Therefore, a diseased plant is at one physiological stage in its growth and development while a comparable healthy plant may be at another stage. It is reasoned that these differences would also be apparent at the cellular level. Sieve tube elements of diseased tissue expressing the above symptoms would be at a juvenile stage in ontogeny while sieve tube elements from chronologically equal healthy plants would be at a more mature stage. If there are structures present during the early ontogeny of sieve tube elements that resemble MLB, they may be involved in the interpretations of MLB associated
plant diseases. Consequently, it is important to compare similar physiological stages of development in addition to chronological stages. My data show the importance of such comparisons.

There are structures in immature phloem parenchyma cells and sieve tube elements of peach and cherry trees that can be confused with or misinterpreted as MLB. Ultrastructural studies have revealed a simple structure for mycoplasma. They are highly pleomorphic, membrane bound, contain ribosomes, polyribosomes, nucleic acid strands and sometimes nucleoids (Allen et al., 1970; Anderson, 1969; Razin, 1969). They range from 0.5 to 1.0 \( \mu \) in diameter with minimum viable units as small as 125-250 nm (Razin, 1969). If one compares the structure of the bodies in Figures 1-4 etc. with the description given above, the similarities are obvious. Structures identical to those shown in the figures have been reported to be mycoplasma or MLB and were only found in young sieve tube elements and phloem parenchyma cells of 'yellows diseased' plants (Amici and Favali, 1972; Cousin et al., 1972; Lombardo et al., 1970; Pellegrini and Gerola, 1970; Worley, 1970).

Earlier research on phloem differentiation suggested that the structures in Figures 1-9 may be products of changes that are occurring during sieve tube element and phloem parenchyma cell differentiation. Many researchers allude to the type of structure shown in the figures in written text but very few show pictorial
evidence of such. The structures that are pictured (Behnke, 1969; Behnke and Dorr, 1967; Buvat, 1968; Esau (1969), Kollman and Schumacher, 1961; Srivastava and O'Brien, 1965) do not appear as numerous in any of the published micrographs as they do in the figures in this study. In all cases there is a close association of the vesicles to ER. With the exception of Kollman and Schumacher (1961), all other publications show ribosomes inside the vesicles. All published micrographs show vesicles bound by a single unit membrane. A second membrane, the ER, is often closely associated with the vesicles. Fibrils similar to those in Figure 1 can be seen in Figure 2 of Srivastava and O'Brien (1965) and Figure 5 of Behnke (1969). However, the question of diseased versus healthy tissue was not considered by most of these workers.

It is apparent that structures identical to those shown in Figures 1-19 have been called mycoplasmas. It also is apparent that structures either similar to or identical with those in Figures 1-9 have been observed during developmental studies of phloem differentiation and referred to as vesicles. One must now ask the question whether the observed structures are a normal feature in differentiating phloem parenchyma cells and sieve tube elements of healthy and diseased tissue? First, the structures appear in phloem parenchyma cells of healthy, symptomless, virus-indexed, heat treated trees. Secondly, cross and longitudinal serial sections reveal that the
bodies originate from an infolding or invagination of ER. That the process is invagination is supported by the fact that larger cisternae containing increased numbers of bodies are found in progressively older cells. If the investigation is from rough ER, then the bounding membrane is studded with ribosomes on the inside. Thirdly, the structures appear at very early stages in cellular development, i.e., in daughter cells as shown in Figure 1 and are no longer present at later stages of development, i.e., in mature sieve tube elements (Figure 5). Fourthly, if one compares the overall ultrastructure to the ultrastructure of known mycoplasmas such as *Mycoplasma gallisepticum* or *M. meleagridis*, a durth of nucleoids is noticed. And fifthly, in the study of Albino and X-diseased Prunus avium and *P. persica*, the same type of structures were observed in both diseased and healthy control trees. However, after sieve tube elements had differentiated, MLB appeared in the diseased trees but not in the healthy ones. If the structures observed at early stages of development were some early stage in the development of MLB, then the MLB should have been present in the healthy controls also. Therefore, it is concluded that the structures visualized in Figures 1-9 are a normal feature in differentiating phloem parenchyma cells and immature sieve tube elements. The fact that the bodies are invaginations into cisternae of ER has been confirmed by Dr. Kathrine Esau (personal comm.).
Variation in electron density of the vesicles in expanded ER cisternae is considerable (compare Figures 2 and 3). While structures similar to those shown in Figure 3 have been reported as MLB by Brýšk et al. (1969), nothing similar appears in the literature on sieve tube element development. The cause of the variation in electron density is unknown. However, it is probably due to the variation in fixation upon cells at different physiological stages of development.

In summary, it is important to emphasize that the structures reported in this paper were found in phloem parenchyma cells and sieve tube elements of healthy, virus-indexed, heat treated trees. The cells studied were in early developmental stages. The structures visualized resemble mycoplasma but they are actually a normal part of cellular differentiation. It is suggested that any 'yellows disease' association based solely on the visualization of MLB in immature phloem parenchyma cells and sieve tube elements should be re-evaluated. In the future, maturity of the sieve tube elements must serve as one of the criteria for establishing such associations, i.e., MLB must be observed electron microscopically in mature sieve tube elements.

Diseased Material

Albino disease of P. avium and X-disease of P. persica and
P. avium were long thought to be caused by a virus. However, the causal agent became suspect subsequent to 1967 as a result of research on Aster Yellows and other 'yellows diseases' closely related to albino and X-disease such as pear decline. My research shows there is a definite association of MLB with albino and X-disease. Repeated thin sectioning and EM observation of tissue samples taken sequentially throughout the growing season showed the consistent presence of MLB in mature sieve tube elements of diseased tissue during June, July and August, and the consistent absence of such structures in healthy controls. This, of course, establishes an association but not causality. Proof of causality awaits fulfillment of Koch's Postulates.

The tubular MLB (Figures 16 and 17) bear some resemblance to Spiroplasma recently reported by Davis and Worley (1973) and Davis et al. (1972). Diameter measurements and structural components coincide most closely. However, serial sections show MLB are shorter (i.e., 1.8 μ maximum length compared to 3-15 μ) and have no helical structure. Davis et al. (1972) visualized whole spirals in 0.3 μ thick sections. Since thin sections range from 60-90 nm the sections in Figures 16 and 17 cover between 0.48-0.72 μ and 0.60-0.90 μ of a single cell, yet no spirals are observed in these sections and spirals were not observed in expressed plant sap. Therefore, it is concluded that the tubular forms associated with
albino disease of *P. avium* and X-disease of *P. avium* and *P. persica* are not spiroplasma.

Both tubular and spherical structures are quite different from rickettsia-like structures reported by Goheen et al. (1973) and Nyland et al. (1973). The size of the MLB in albino and X-diseased material is smaller, there is no evidence of a rippled cell wall, and the structures are always found in mature sieve tube elements, whereas rickettsia-like structures are found in vessels.

There is considerable difference in morphology between the MLB observed in albino and X-diseased *P. avium* and *P. persica* from Oregon and the MLB in X-diseased *P. persica* observed by MacBeath et al. (1972) in California. They reported...

... cross and longitudinal serial sections of infected leaf tissue of peach indicate that the MLB associated with peach X-disease in late summer are elongate undulating tubules, some of which are over 5.0 μ in length (p. 935).

Oregon data show: 1) tubules not exceeding ca. 2 μ, 2) that in longitudinal sections the sieve tube elements appear packed with spherical forms (Figure 11) rather than the tubule packed sieve tube elements of MacBeath et al. Figures 1G and H, and 3) in X-diseased *P. persica* collected in California on July 31, 1973, no tubules as long as those reported by MacBeath were observed (Figure 10). The differences may be due to the fact that different developmental stages were observed, climatic variation, and or strainal differences. The
structures in albino also differ morphologically from the MLB reported by Nasu et al. (1970) in the cells of Colladonus montanus and Apium graveolens even though both were infected with X-disease agent. In this case, the structures in albino are larger. Nasu et al. (1970) reported a size range of 200-400 nm compared to 150-1020 nm and up to 1800 nm long for albino. The discrepancies may be caused by different fixation methods, the fact that the X-disease agent was in two entirely different types of tissue, and Nasu et al. present no evidence of serial sectioning so that overall form could not be determined.

Of the various structures reported to be associated with 'yellows type' diseases the structures observed in albino and X-diseased tissue most closely resemble those reported by other researchers as mycoplasma-like bodies or mycoplasma-like organisms. However, they also closely resemble Chlamydia as described by Lepinay et al. (1970, 1971) and Cutlip (1970), and L-forms described by Hayflick (1969). One fact is obvious—the structures do resemble some type of prokaryotic organism.

One feature of the structures associated with albino and X-diseased tissue which has not been reported previously is the connection of the tubular forms at points along the cell wall (Figures 16D and 18 A and B, arrows). The point of attachment appears to always be at a location where there is abundant ER. The connection is small,
less than the thickness of one thin section (60-90 nm), and would most likely not be observed unless serial sectioning is used. This may explain why it has not been reported by other researchers. The presence of the connection raises an interesting point: are the tubular structures that resemble a MLB developing from ER or are the MLB attaching to the ER? Enough information is not available at this time to answer this question.

**Comparisons between Vesicles in ER Cisternae and Mycoplasma-like Bodies**

In 1972, Horne (1972) stated that no normal cell organelles have been found in the cytoplasm of animal or plant cell systems that have the four basic ultrastructural characteristics of mycoplasmas. These are 1) size range, 2) presence of a bounding unit membrane, 3) dimensions of the ribosomes, and 4) presence of slender strands of DNA within the mycoplasma cell cytoplasm. The data presented in the Results section, Control Tissue, represent the first report of normal organelles in phloem parenchyma cells and immature sieve tube elements of healthy, virus-indexed, heat treated plants that resemble mycoplasmas.

This discovery tends to complicate the hypothesis that 'yellows diseases' are associated with mycoplasmas. The hypothesis is complicated because 1) more thorough cytological studies must be
done before an association can be established, 2) researchers need
to compare plants of the same physiological age as well as the same
chronological age, 3) MLB should be observed in mature sieve tube
elements, and 4) if they can only be visualized in immature phloem
parenchyma cells and sieve tube elements, then serial sections must
be cut to determine whether or not the bodies are ER invaginations.

There are differences between vesicles in ER cisternae in
healthy material and MLB in diseased material. The most significant
are 1) vesicles in ER cisternae are observed in immature sieve tube
elements and phloem parenchyma cells whereas MLB are observed in
mature sieve tube elements during later stages of development, and
2) in serial sections, vesicles in ER cisternae can be seen to arise
from invaginations of the ER whereas MLB do not.

An extensive ultrastructural analysis must be completed before
the two types of structures can be identified. Therefore, before any
type of MLB is associated with a particular 'yellows disease' most,
if not all, of the criteria listed in Table IV should be satisfied.

General Discussion

As mentioned in the Introduction, the majority of the associa-
tions between a mycoplasma-like body and a 'yellows type' disease
are based upon electron microscopic observations. However,
evidence for mycoplasma etiology also comes from the remission of
symptoms following tetracycline treatment and attempted culture.
Tetracyclines have been reported to inhibit protein synthesis on 80S as well as 70S ribosomes (Bermek and Matthaei, 1970; Franklin, 1966; Vazquez and Monro, 1967). Therefore, it is possible that they inhibit the affect of the pathogen rather than the pathogen. It is possible that the causal agent may activate the ribosomes and/or ER in mature sieve tube elements causing the production of mycoplasma-like bodies. Once the sieve tube elements become packed with such structures, nutrient flow is reduced which ultimately causes the appearance of disease symptoms. The fact that tetracyclines inhibit 80S ribosomes may account for the fact that treated plants show remission of symptoms and mycoplasma-like bodies are no longer visible after treatment. Presently, this is sheer conjecture and more research is needed to explain the mode of action of tetracyclines in 'yellows type' diseases and in higher plants.

Historically, animal mycoplasma media has been used in attempted culture of plant mycoplasmas. Conflicting reports appear in the literature (Hayflick, 1973) and to date, Koch's Postulates have not been fulfilled. Culture in pure plant sap has not been attempted because techniques for collecting large enough volumes to use for cultural purposes have not been developed. During this study some effort was spent on this endeavor and a technique was worked out (see Appendix). It has been possible to collect up to 200 ml of mixed xylem and phloem sap but as yet mycoplasmas have not been successfully cultured.
Throughout this thesis, I have used the terminology mycoplasma-like body in order to conform with most previous publications. I prefer a broader term, prokaryotic-like structure (PLS) because:

1) There is no direct evidence, aside from structural similarity, that the structures observed in mature sieve tube elements of diseased tissue are related to mycoplasma.

2) They are also structurally similar to other prokaryotes, e.g., chlamydia, rickettsia, and L-forms (Hayflick, 1973).

3) As pointed out by Davis and Worley (1973), the presumed plant yellows disease agents may comprise an entirely new taxon.

4) A rickettsia-like organism has been associated with Pierce's Disease of grapes and Alfalfa Dwarf (Goheen et al., 1973) and Phony Disease of peach (Nyland et al., 1973). However, it is now thought that these may in fact be very small bacteria (Nyland, personal comm.).

5) A Spiroplasma has been associated with Corn Stunt disease (Davis et al., 1973).

It is obvious that a wide variety of structural forms have been associated with a variety of diseases. Until such a time when more precise classifications can be made of the structures associated with various yellows diseases, it is proposed that they all be referred to as prokaryotic-like structures.
SUMMARY AND CONCLUSIONS

The questions investigated in this study were 1) whether there are normal structures present during any stage of sieve tube element and phloem parenchyma cell development that resemble mycoplasma-like bodies, 2) the relative abundance of mycoplasma-like bodies in situ at different stages of sieve tube element development, and 3) the relative abundance in perennial plants at different times of the year.

The data show that there are structures normally present in immature phloem parenchyma cells and sieve tube elements that resemble mycoplasma-like bodies. These structures are vesicles in expanded ER cisternae and have been misinterpreted as mycoplasma. The error appears to be due to insufficient published information on immature sieve tube element development and the lack of developmental studies on 'yellows-type' diseases.

Results show that when mature sieve tube elements are observed, bodies appear in X-diseased and albino infected sieve tube elements. These bodies are not present in equally mature healthy, virus-indexed, heat treated control material. There are significant differences between the structures observed in immature phloem parenchyma cells and sieve tube elements and the structures observed in mature sieve tube elements.
A connection between tubular mycoplasma-like bodies and the stacked ER of the mature sieve tube element has been demonstrated. The point of connection is small, less than the thickness of a single thin section. It would probably not have been observed unless the techniques of serial sectioning and transparency stacking were used.

Finally, the mycoplasma-like bodies observed in association with the diseased material were found to be most abundant during the months of June, July, and August. In albino, the peduncle was found to be an excellent tissue in which to study in situ development of mycoplasma-like bodies.

Biweekly intervals for collection and fixation of tissue are not close enough together to elucidate all the ultrastructural changes that are occurring in phloem parenchyma cells and sieve tube elements. The significance of the connection between tubular prokaryotic-like structures and the stacked ER in mature sieve tube elements could not be ascertained. In the future, tissue samples need to be fixed at very short time intervals so that a sequence of events can be established which would explain the significance. Also, future research should involve a developmental study of the type culture of yellows diseases, i.e., Aster Yellows, cytochemistry of mycoplasma-like bodies in situ, and attempted culture of the prokaryotic-like structures.
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APPENDIX
APPENDIX

Technique for Collecting Plant Sap

Historically, media developed for animal mycoplasmas has been used when attempting to culture suspected plant mycoplasmas. Since, in plants, the intracellular location within a constantly changing phloem sap is a unique environment, some researchers have attempted to simulate this environment by adding sugars found in phloem sap, adjusting osmotic pressure, and/or adding plant extracts to animal mycoplasma media. To date, there have not been any reported attempts to extract whole plant sap and use it as a culture media. The probable reason for this is that techniques for collecting large volumes of sap have not been utilized.

Using a modified pressure bomb built by J. B. Zaerr and D. P. Lavender of the Forest Research Laboratory at Oregon State University, as much as 200 ml of mixed xylem and phloem sap were collected from three 3- to 4-year-old cherry trees (Figure 20A). The following is a detailed outline of the steps for collection.

1. Obtain trees to be used in the pressure bomb. They can be up to 6 ft tall and 2 in. in diameter. For maximum sap yield, the trees should be irrigated the day before and cut early in the morning the day of collection. The foliage should be kept covered with a large plastic bag to keep transpiration to a minimum. Transpiration can also be reduced by keeping
Figure 20A. Pressure bomb used for collecting plant sap. The bomb is 6 ft. long. There is a hole in the pressure plate at the end of the bomb facing away from the camera. The 4 in. to 6 in. of tree trunk seen extending beyond the spacer in Figure 20B projects out of that hole and sap is collected as it flows from the cut end of the tree.

Figure 20B. Detailed illustration of tree being prepared for treatment in pressure bomb.
unused trees in a cold room but extractions should be made as soon as possible.

2. Prepare a tree for extraction in the following manner.
   a. Cut the trunk approximately 6 in. to 8 in. below the lowest branch on the tree.
   b. Screw a screw-eye into the trunk opposite the branch (Figure 20B).
   c. Fit a size 14 rubber stopper, bored to fit tightly, over the trunk. In the spring when the bark is slipping, care must be taken not to tear the bark.
   d. Slide the stopper up the trunk until it comes in contact with the branch and screw-eye (Figure 20B).
   e. Place a radiator clamp around the stopper and tighten until the stopper starts to bulge.
   f. Place an aluminum spacer, bored to the appropriate diameter, next to the end of the stopper (Figure 20B). There should be approximately 4 in. to 6 in. of the trunk extending beyond the spacer.

3. Place the whole tree in the pressure bomb and seal the end tightly. The 4 in. to 6 in. of trunk should protrude from the pressure plate at the end of the bomb.

4. Pressure is applied by an air compressor connected directly to the bomb (Figure 20A).
   a. Place a collection vessel containing 2 to 4 g of insoluble polyvinylpyrrolidone (PVP) under the protruding end of the trunk.
   b. Xylem and phloem sap can be separated by trimming 1 in. to 2 in. of bark from the cut end of the trunk (Zaerr, personal comm.). The xylem sap flows from the end and the phloem sap from the trimmed end of the bark.
c. Apply pressure slowly at first until it reaches 100 psi then shut off the compressor. Sap will begin to flow at about 80 psi and is collected by funneling it directly into the collection vessel.

d. Check to be sure the tree is not being pushed out of the bomb by the pressure. Even if everything is tight, a small amount of movement will occur. If it appears excessive then the pressure should be released, the tree removed, and everything readjusted to give a tighter fit.

e. If everything appears tight, increase the pressure in accordance with the rate of sap flow from the end of the trunk. As rate of flow decreases, increase the pressure until 250 to 300 psi is reached. This usually takes 30 to 45 min.

f. When the extraction is complete, release the pressure and discard the tree.

5. After the collection process is complete, sterilize the sap immediately in the following manner.

   a. Filter through a Büchner funnel to remove PVP and plant debris.

   b. Centrifuge 30 min. at 7000 x g. This removes remaining PVP, bacterial contamination, and facilitates millipore filtration.

   c. Double filter through 0.45 μ and 0.22 μ sterile millipore filters. During the last filtration, filter 5-10 ml aliquots into sterile screw cap culture tubes using an automatic pipetting system.

   d. Tubes can be stored in the refrigerator until ready for use.
Upon exposure to air, phenolic compounds in the sap oxidize rapidly turning it an amber color. This reaction can be prevented by removing the phenols with polyvinylpyrrolidone (PVP). Two grams of insoluble PVP added to the collection vessel was enough to inhibit discoloration of 85 ml of sap. However, it should be noted that both sap collected with PVP and without PVP supported growth when inoculated with known bacterial cultures. The pH of the sap after filtration was 5.2 if collected without PVP and 5.7 to 5.8 with PVP.

This technique of sap collection might be used for culturing all kinds of organisms that inhabit either the phloem or xylem of higher plants. There may be many fastidious bacteria that require some constituent of whole plant sap for growth. In addition to using the technique as a source of sap for culture media, it can also be used to obtain large amounts of inoculum. As pointed out by Hampton (1972), this is a necessary step toward the successful isolation and cultivation of plant mycoplasmas.