CYTOLOGICAL ASPECTS OF SEASONAL CHANGES IN THE MESOPHYLL

CHLORENCHYMA CELLS OF <u>PINUS CONTORTA</u> Dougl. ex Loud ssp

<u>LATIFOLIA</u> (Engelm, ex Wats) IN RELATION TO FROST HARDINESS

by

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ABSTRACT

The needle chlorenchyma of Pinus contorta Dougl. ex Loud spp. Lati-
folia (Engelm. ex Wats) was fixed on site in the sub-alpine forests of
the southern interior plateau of British Columbia, in order to conduct
comparative observations by light and electron microscopy on the chloren-
chymal cytology in summer and winter. Material fixed in the growing sea-
son (summer) demonstrated a chlorenchymal ultrastructure similar to that
of other mature higher plant chlorenchymal tissues. Material fixed in
mid winter (usually at below freezing tempertures) revealed extensive
changes in the structural and positional aspects of most cytoplasmic com-
ponents. Some of the most dramatic changes in the chlorenchyma cytoplasm
in the winter state are as follows: chloroplasts became irregularly shaped
and clumped together usually in certain specific areas of the cell; close as-
sociations form between the outer membranes of adjacent chloroplasts,these
areas may lead to the apparent fusion of some chloroplasts. The cyto-
plasm became massively vacuolated, particularly in areas removed from the
nucleus or the chloroplast clumps. The extent of this vacuolation can be
deduced from the use of the adjective "foamy" to describe the extent and
degree of these vacuolated cytoplasmic areas. Associated with the cyto-
plasmic winter vacuoles were highly osmiophilic bodies. A distinct sea-
sonal cycle of cytoplasmic oil reserves was also noted. The reaction of
the winter chlorenchymal cytoplasm to extentive manipulation in fixative
osmotic potential was remarkably conservative. This observation allows
inferences to be made about the permeability characteristics of winter
cytoplasmic membranes.

In order to verify the observed winter changes, chemical fixation studies were supplemented by observations on material collected, transported, stored, sectioned and observed by light microscopy in the frozen and unthawed, stained or fixed state. This work was conducted with specially designed transport devices and a cryomicrotome and cryomicroscope. Observations were also conducted in summer and winter on the chlorenchyma of Pinus albicaulis Engelm and Tsuga mertensiana (Bong.) Corr. collected near timberline at the Whistler mountain ski resort ninety miles north of Vancouver. Observations from the cryomicroscopy of Pinus contorta needles indicate that the seasonal changes are not artifacts of chemical fixation procedures. Observations on the needles of the two conifer species collected at Whistler mountain indicate that the detailed seasonal observations on Pinus contorta may also apply to the needle chlorenchyma of other conifer species.

A possible spring transitional stage between the winter and summer ultrastructural appearance of Pinus contorta chlorenchyma is discussed. Related experiments were conducted to indicate what effects the needle age of field trees had on winter-summer comparisons, and what effect drought stress and artificial hardening and freezing had on chlorenchymal ultrastructure. The drought and hardening-freezing experiments were conducted on laboratory grown trees in controlled environment chambers. No clear relationship between frost hardiness and drought stress was established. Experiments on the artificial inducement of frost hardiness in seedling trees were not considered successful.

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INTRODUCTION

All non-tropical continental life on this planet must make some deg-
ree of adaption to at least the seasonal presence of low temperatures which
would cause the cellular water of metabolically active cells to freeze. In
the majority of higher organisms in a growing and metabolically active
state, the freezing of water within the cytoplasm is fatal (Burke et al.,
1976 and Tumanov, 1967). Two broad classes of adaptive mechanisms appear
to have evolved to prevent the freezing of cell water. The first mechanism
is termed the principle of frost avoidance. In this principle, the freez-
ing of cellular water is prevented by a number of means, some of which are:
the maintenance of internal tissue temperatures at a high enough level to
avoid freezing (as in the case of homeothermic animals); the build up of
cryoprotective substances to high enough levels within cell tissues (as
in the case of some insect larvae and tree buds) to prevent ice formation;
by possessing a survival phase in a life cycle characterized by the re-
duction of water content in the cells to level low enough to prevent in-
tracellular ice crystalization (such as the seeds or spores of annual plants);
or by creating conditions which allow cell water to super-cool. These
frost avoidance mechanisms have been described in detail by Levitt (1972).

Woody higher plants maintain their competitive advantage (in part) by
maintaining and building upon large living tissue masses from year to year.
In temperate, high latitude and altitude zone species, this requires the
survival of a significant portion of the living cell mass through the frost

season. Such organisms are examples of the second major type of adaptive mechanism to frost, that of frost tolerance. Frost tolerant plants enable vital cells to survive the winter season by preparing cells structurally and chemically in such a manner that they are able to tolerate ice formation in their tissues. The vital cells are able to survive frost related stress by controlled extra-cytoplasmic ice formation with free cell water being released from dehydrating and plasmolyzing cells.

There is a general agreement in recent literature with regard to physical stress factors related to the formation of ice in living tissues which must be tolerated by frost hardy plants (see reviews by Mazur, 1970 and Weiser, 1970). These factors are:

1)   The concentration and in some cases possibly the precipitation or gelling of cell solutes due to a greatly reduced solvent volume.

2)   Changes in ionic strength, pH, and osmotic equilibrium in the cell interior and exterior.

3)   The collapse and severe plasmolysis of the cell as cell water moves to specific ice nucleation sites.

4)   The macromolecular correlation of the above three points would result in the loss of membrane semipermeability and the denaturing of cell proteins whose structure was dependent on cell water and/or the normal equilibrium of cell solutes.

The above stress factors are internal stresses to the cell caused by the freezing of cellular water outside the plasma membrane. In attempting

to understand the frost hardiness processes of woody plants, explanations of how the cell tolerates and ameliorates the above stresses are required.

The ability to survive frost related stresses is a seasonally acquired property in woody plants. Tissues in extremely hardy plants cannot tolerate even a few degrees of frost during the active growth phase of the plant, but can tolerate temperatures as low as $-196^{\circ}C$ when they are prepared for seasonal freezing (Sakai, 1966 and 1973; and Krasavtsev, 1973).

The general factors involved in the process of developing maximum hardiness levels is fairly well understood and this process has been divided into several phases by Tumanov (1967) and Weiser (1970). These phases can be generalized into two main stages. The first stage involves the detection of the impending cold season by shorter photoperiods and cooler daily minimum temperatures and possibly other factors; the cessation of tissue growth, coupled with an increase in certain types of biochemical activity. The cessation of tissue growth is considered (particularly by Russian authors (Tumanov et al., 1973)) to be a specialized, frost hardiness dependent form of plant dormancy.

The detection mechanism is translated into a complete succession of growth and differentiation in the plant tissue. This translation may be by a hormonal factor (Irving and Lanphear, 1967).

Although stage one is a dormancy stage in terms of growth cessation,

i.e., cell division and differentiation into cells of specialized function; cells developing frost hardiness may become more active metabolically than they were during parts of the growing season (Siminovitch et al., 1968). This activity is initiated by a marked increase in ribosomal RNA (Gusta and Weiser, 1972) and certain proteins (Clements, 1938; Siminovitch et al., 1968; Pomeroy et al., 1970; and Gusta and Weiser, 1972). Other cell constituents also increase, some of which are: soluble sugars (Parker, 1956; Heber, 1959 and Pomeroy et al., 1970), lipids, particularly phospholipids (Siminovitch et al., 1975) and unsaturated fatty acids (Willemot et al., 1977). In contrast, the starch content of conifer needles appears to decline with the approach of maximum frost hardiness (Clements, 1938; Parker, 1956 and Little, 1970).

The biochemical changes appear to be highly variable in different plant organs and between different plant species or even varieties (Smith, 1968). Despite the extent of cellular activity, the hardiness of plants in stage one is still relatively low (Weiser, 1970).

Stage one of frost hardiness development is of indeterminate length. But because cellular biochemical transformations are involved, there is probably a minimum time period involved. In the natural state, the biochemical transformations of stage one seem to occur over several weeks (Siminovitch et al.; 1968 and Weiser, 1970).

Stage two of frost hardiness development occurs over a relatively short time (several days or less, Sakai, 1966), and does not require or elicit any substantial metabolic response from the living cell. Instead,

stage two appears to involve a mechanical accommodation of the cytoplasm to increasing amounts of cellular dehydration due to external ice formation. Stage two requires reduction of tissue temperatures by incremental steps below freezing with precisely definable temperature drops before maximum hardiness levels are obtained. Thus this stage requires exposure to moderate freezing temperatures to enable a potentially hardy cell to survive extremely low temperatures. This process has been extensively studied by Sakai (1966 and 1973) and Krasavtsev (1973). As an example of this stage of frost acclimation, Sakai demonstrated the necessity of exposing a twig (after the completion of stage one) to several hours at -30°C before the twig was able to tolerate -196°C (Sakai, 1973).

While stage one is an active metabolic stage, stage two appears to involve physical changes in that the progressive exposure to lower temperatures may involve physical deformations and rearrangements of cytoplasmic components in such a manner as to allow ice tolerance (Weiser, 1970). Weiser points out that some enzymic activity and metabolic control may still be operating during stage two, indicating that stages one and two are not completely separable in time.

The cytological manifestation of stage two is the plasmolysis of the cell caused by dehydration as water is lost to the extracellular ice nucleation sites (see Scarth and Levitt (1937) for a review of cryomicroscopic studies of frozen cells). The Russian authors believe the ice plasmolysis is so extensive that it caused plasmodesmatal cytoplasmic strands to break and withdraw from the cell wall (Genkel et al., 1971).

The exact location of ice is a matter of controversy and appears to occur in the extracellular spaces if present, or in the intramural space around the plasmolyzed protoplast (Scarth and Levitt, 1937). There is general agreement that ice formation within the cytoplasm is fatal in frost hardy cells (Tumanov, 1967; Weiser, 1970; Sakai, 1973 and Burke et al., 1976). But it is debatable whether ice formation can occur in the main vacuole without causing serious damage (Parker, 1963).

Ultrastructural localization of ice has been attempted but it has been confined to tissue cooled at unnaturally high cooling rates (Sakai and Otsuka, 1967), or in animal tissue with no natural frost resistance mechanisms, and again at unnaturally high rates of cooling (Rapatz et al., 1963 and Nei, 1976).

It has been determined by extensive colorimetry studies that in hardy plants at below freezing temperatures, an equilibrium is maintained between cytoplasmic free water and extracellular ice nucleation sites (Weiser, 1970). This equilibrium would shift in direct proportion to changes in temperature; i.e. as the temperature decreases, free water leaves the protoplast and migrates to the ice nucleation sites; and as the temperature rises, the ice melts and the protoplast begins to rehydrate. There are two important physical properties of a hardy plant cell that relates to this equilibrium between the ice phase and the cytoplasmic free water phase.

Firstly, the hardy protoplast must release water as it is thermodynamically required to prevent intercellular freezing (Weiser, 1970). This implies that resistant cells are permeable to water (Scarth and Levitt,

1937 and McKenzie, 1974).

Secondly, the water must be released slowly so that ice formation is not a sudden explosive occurrence but a slow and controlled event. This appears to be the result of an increase in cryoprotective substances, such as sugars and hydrophilic polymers that will release free water only gradually at progressively lower temperatures (Weiser, 1970). The physical state of some of these hydrophilic polymers may be in the gel form (Parker, 1958 and Tumanov, 1967).

Cytological studies on frost resistant cells in the literature are often confusing and contradictory when compared with each other due to the large variety of organisms studied (which may possess differing frost resistant mechanisms), and there is also a frequent failure in the literature to correlate cytological interpretations with the degree of frost hardiness in the observed cells, or with the external environmental temperatures to which they have been exposed.

One example of cytological differences between different species involves the tendency for chloroplasts to swell and disperse randomly within the entire protoplast in winter wheat (Chein and Wu, 1965),whereas in _Pinus_ species , they tend to clump together in specific areas of the cytoplasm (Holzer, 1958). Similarly, the main cell vacuole becomes subdivided into numerous small vacuoles in cambial and secondary woody tissue of several plants (Siminovitch _et al._, 1968; Robards _et al._, 1969; Mia, 1970 and 1972; Murmanis, 1970 and Itoh, 1971). Yet in conifer needles, the main vacuole remains intact throughout the winter (Lewis and Tuttle, 1923; Holzer, 1958, Parker

and Philpott, 1961 and 1963; Harris 1971. and Chabot and Chabot, 1975).

One basic cytological feature which appears to be applicable to most recent cytological work is the concept of cytoplasmic augmentation which was discussed by Scarth and Levitt (1937), but refined and placed into a more modern context by Siminovitch et al. (1968). Cytoplasmic augmentation refers to the increased volume and density of the cytoplasm (at the light microscopy level) of many frost hardy cells in winter. Siminovitch et al. (1968) suggests that the observed increases of certain biochemical compounds in stage one of frost hardiness development may account for this observation. Examples of the proliferation of biochemical components of cytoplasmic structures would be the very substantial stage one increase of membrane phospholipids (Siminovitch et al., 1975).

An historical cytological dispute that has only recently been settled was concerned with the fate of the conifer chloroplasts. Haberlandt (1876) noticed a clumping of conifer chloroplasts after frost exposure. Holzer (1958) confirmed these observation in studies conducted on Pinus species. In contrast, Lewis and Tuttle (1923) and Zacharawa (1929) proposed that the chloroplasts were destroyed during the winter in conifers. In Picea glauca, Lewis and Tuttle (1923) observed the clumping followed by disintegration of chloroplasts with progressively severe frosts. This idea of winter chloroplast destruction was widely cited in the literature, particularly in relation to the chloroplasts in the needles of evergreen conifers. Some papers in support of the concept have appeared quite recently (Gerhold, 1959; and Perry and Baldwin, 1966).

A substantial body of literature has been written on frost resistance in plants. Allen and Herman (1971) estimated that by 1971, more than 4,000 papers on the subject had been written. Review papers on the subject include Maxinov (1929); Scarth and Levitt (1937); Clements (1938); Levitt (1941); Parker (1963); Tumanov (1967); Olien (1967); Mazur (1970); Allen and Hermann (1971); Meryman (1971),Litvan (1972); Tumanov et al., (1973) and Burke et al., (1976). Symposia and books on cryobiology which contained sections directly related to plant frost hardiness include works edited by: Levitt (1956), Meryman (1966) and Troshin (1967). Levitt (1972) has attempted to standardize nomenclature and define the major parameters involved in plant environmental stresses, especially those related to frost hardiness and/or sensitivity. The work is an excellent and important reference on the topic of plant hardiness.

The present study has demonstrated that a fundamental positional and structural rearrangement of cytoplasmic components in pine chlorenchyma occurs during winter. Observations were made using both light and electron microscopes. This work was originally based solely on observations on Pinus contorta ssp latifolia, but it was expanded to include observations on seasonal changes in Pinus albicaulis and Tsuga mertensiana. Similar changes were observed in all three species.

Studies were also conducted on the artificial frost hardening of pine seedlings in controlled environmental conditions, although these experiments were largely unsuccessful. The possibility of a relationship between drought exposure and frost resistance was also investigated.

Besides direct comparisons between summer and winter cytoplasmic states, the summer chlorenchyma of needles of varying ages was also studied in order to ascertain that factors not related to seasonal environmental features, such as senescence, were not involved with the summer-winter comparisons. A cytological condition that may represent a spring or an intermediate condition between the summer cytoplasmic state and the winter cytoplasm was also identified.

Technical difficulties were experienced in obtaining adequate ultrastructual fixations of pine chlorenchyma. The modifications of standard ultrastructural techniques developed in this work to achieve adequate fixations should assist future works on conifer tissue.

Observations on chemically fixed and prepared material were supplemented with observations on material in unfixed, unstained, frozen state. The details of this procedure and the construction of a very simple cryotome and cryomicroscope to conduct these observations is also described.

MATERIALS AND METHODS

I. Light and Transmission Electron Microscopy Preparation Procedures

Young trees (five to fifteen years of age) of Pinus contorta Dougl.
ex Loud ssp latifolia Engelm.,(identified per Critchfield, 1957), were
chosen for this project. The trees from which needles were studied
were collected in two locations: Allison Pass located on the coastal
divide in the northern Cascade mountains, and the Sunday Summit plateau
region about twenty-four miles to the north-east. Both sites are appro-
ximately four thousand feet above sea level. The Allison Pass site, be-
cause of coastal influences, has a more moderate mean average tempera-
ture and considerably more annual precipitation than the Sunday Summit lo-
cation. Both sites have a pronounced dry period which usually last from
late July to early October. In both areas, the selected trees (presumab-
ly seeded from adjacent mature trees) are in sunlit clearings, and grow
in well drained glacial gravel soils. In the Allison Pass region stands
of Pinus contorta of various ages are mixed with Abies amabilis. The Sun-
day Summit site is surrounded by an apparently pure stand of Pinus contor-
ta trees approximately thirty years old.

Five trees at each site were permanently labelled and used for all
experiments. Only needles from the upper branches of the south side
of the tree were processed for microscopic observations. Needles that
showed no chlorotic areas were used, and most fixations were carried out
within an hour before or after sunrise. In all cases, the first 5 mm

of tissue from the tip of each needle was discarded and only the next
10 mm portion was used in fixations.

For winter fixations tissues were fixed in the primary fixative
of glutaraldehyde formalin solution at -4°C (when the air temperature
was below the freezing point).

All needles were cut transversely into sections 0.5 mm thick in a
pool of primary fixative solution. They were then transferred to vials
containing similar fixative at the temperatures mentioned above and
fixed for two to twelve hours. The winter fixation temperatures were
maintained by a salt-water-ice bath and the summer fixation temperature
was regulated by a water bath. Specific details of the time and condi-
tions of each fixation are given in Table I.

Two primary fixative solutions were used. The most used primary
fixative is made up by mixing freshly made 25% formaldehyde (from para-
formaldehyde) and 25% pure glutaraldehyde (Electron Microscopy Sciences,
Fort Washington PA) with a 0.1 M Sodium Cacodylate buffer at pH 6.8.
The final content of the fixative solution contains: 0.5% formaldehyde,
2.0% glutaraldehyde and the following additives: 4% sucrose, 0.0001%
Kodak Photoflo, and 0.01% $CaCl_2$. The osmotic pressure of this fixative
is approximately 700 mOsm. The second primary fixative (the acrolein
fixative), was a solution of 2% acrolein and 2% glutaraldehyde buffered
in the same manner as the standard fixative. In contrast to the first
method, the acrolein fixations were carried out at room temperature for

Table I   Collecting Times and Conditions of _P. contorta_ Needles

Season	Year	Month	Day	Time of Day	Location & Air Temp. (°C)		Fixation Used	Fixation Temp. (°C)	Needle Ages (Months)* 0 = June 1st
					Allison Pass	Sunday Summit			
Summer	1972	Aug.	20	Dawn	12	12	S	4	3, 15
Fall	1972	Nov.	26	Dawn	-4	-	S	5	6
Winter		Jan.	5	Dawn	-14	-16	S	-4	8, 20, 32, 56
				1:00 PM	-8	-8	S	-4	8
		Feb.	9	Dawn	-11 Some of this Material collected for freezer storage in laboratory	-15	S	-4	9
Spring	1973	Mar.	30	Dawn	-3	-6	S	-4 & +4	10
				2:00 PM	-	5	S	4	10,22
		Apr.	27	Dawn	2	5	S	4	11
Summer	1973	June	6	Dawn	6		S	4	0, 12 36
			29	Dawn	7	8	S & A	4	1, 37, 61
Summer	1973	July	21	Dawn	-	14	S & A	4	2, 14

Key to Symbols:

S   Standard fixation (Glut. & Para.)   A   Acrolein fixation   *   Approximate

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$1\frac{1}{2}$ hours.

After primary fixation, the material was taken through two changes in the buffer while the temperature was raised slowly to room temperature. All material was post-fixed in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate buffer with no additive. Winter material was post-fixed for two to six hours and material from other seasons was post-fixed for sixteen hours, (post fixations were carried out at room temperature).

Material was washed twice in distilled water, and dehydrated in a graded methanol series. Methanol was replaced by acetone over several changes. Graded acetone and Spurr's resin(1969) mixtures were used for infiltration of plastic. It is important that the last mixture with the high concentration of plastic be left overnight. After two changes in pure plastic of two hours each, material was transferred into fresh embedding media in a aluminum boats and polymerized in a vacuum oven for 2 hours at 50°C and 12 hours at 70°C.

Technical difficulties were experienced with trimming and sectioning. It was necessary to trim blocks for sectioning with the hypodermis either dissected from the tissue or as far removed as possible from the top of the pyramid in order to prevent tearing of the tissue. Final trimming of the blocks was carried out using glass knives to obtain smooth surfaces on the block face and sides. It was found that the ex-

treme hardness of the contents of the main cellular vacuoles often ⸱⸱⸱
caused the shattering of sections during microtoming.

The best results were obtained by using glass knives and cutting
only one section on each area of the knife edge. Silver-gold sections
(800-1000 A thick) for electron microscopy could be obtained readily,
while 2 - 5μm sections for light microscopy were much more difficult
to obtain. All sections were cut on a Reichert OmU-3 ultra-microtome,
and ultra-thin sections were collected on copper grids coated with
Formvar films. These sections were stained in uranyl acetate for forty-
five minutes followed by twenty minutes in lead citrate (Reynolds,
1963). They were examined with a Zeiss EM 9A or 9S electron microscope.

For light microscopy, 5μm sections were mounted onto glass slides
and stained for 5 minutes in toluidine blue (0.05% ag). Fresh mater-
ial was cut into 15μm to 20μm sections on a Richert CO_2 gas expansion
cryotome. Sections were immediately fixed with 5% formaldehyde for
a further 10 minutes in ethanolic Sudan black B. Tannin staining was
carried out by placing longitudinally-cut needles in the stain-form-
aldehyde mixture of Johansen (1940) (see appendix A) and then cryo-
sectioned by method outlined above. Photomicrographs were taken with
a Zeiss photomicroscope.

II. Scanning Electron Microscopy Procedures

Mature one year old needles were collected from three and four year old saplings grown out doors for two years prior to sampling at the University of British Columbia.

Needles were placed in petri dishes containing 5% freshly prepared formalin in the buffer and additive solution used with the primary fixative solution for transmission electron microscopy. The tip and bottom 1 cm of the needles were discarded and the remaining central portion of the needle was cut longitudinally into two parts. Each longitudinal part of the needle was then cut transversely into two or three segments about 1 cm in length.

After a one hour primary fixation, needle segments were placed in 1% osmium tetroxide in distilled water at $4^{o}C$. The osmium tetroxide solution was then changed after 24 hours to fresh fixative and left for another 24 hours at $4^{o}C$. Needle segments were then washed in distilled water for thirty minutes, four times.

Needles were then dried by critical point method. Dried needle segments were then held at each end with forceps and bent until they broke in half. The broken segments were then cut 2 mm below the breakage point and mounted on a specimen holder with silver amalgam with the broken face facing up (the transverse face of the needle). Tissue was then coated with gold and observed on a Cambridge scanning electron miscroscope.

III. Experimental Fixations and Procedures

Three fixative solutions were used to study the response of winter tissue ultrasturcture to osmotic stress and to determine whether the unsual cytoplasmic appearance of the winter tissue may be partially due to fixation artifacts. These solutions were as follows:

1. A primary fixation solution containing the same fixative as described previously (1% glutaraldehyde and 0.5% formaldehyde), but without any additional chemicals. The osmotic pressure of this solution was approximately 400% mOsm.

2. A mixture of 33% sea water and 66% buffer solution containing the same glutaraldehyde and formaldehyde concentrations as in the standard primary fixative. The osmotic pressure of this fixation solution was greater than 1050 mOsm.

3. A non-electrolyte solution consisting of the standard primary fixative solution but with the addition of 10% instead of 4% sucrose. The osmotic pressure of this solution was 800 mOsm.

Some nine-month-old long shoots complete with needles were removed from trees at the collecting sites in winter at an air temperature of -11°C. The shoots were maintained at -5°C or lower while they were brought to Vancouver by placing them in jars capped with cheese cloth in a brine ice bath. In the laboratory, they were stored in a commercial freezer at -18°C. The needles can be stored in this way for up to two months before being fixed for electron microscopy in a manner similar to the winter fixations in the field.

Material kept in the freezer and summer needles were place in liquid nitrogen until all boiling stopped and then fixed by the same procedure used for summer needles in the field.

To determine whether freezer stored long shoots were viable, the shoots were removed from the freezer, wrapped in aluminum foil and placed in a refrigerator for 12 hours at 4°C to slowly thaw. The base of the long shoot was then freshly cut and placed in distilled water. The cuttings were placed either in a growth chamber with a 16 hr. or 24 hr. light cycle and a temperature cycle ranging from 4 to 12° C, or outdoors (during the spring) with an approximate temperature fluctuation range of 0°C to 15°C. The choice of placing shoots outdoors was made only when a growth chamber with the light and temperature ranges described above was not available. After one week of light exposure and above freezing temperatures, needles were removed from the shoot at the end of a light cycle and free hand sections were cut.

Sections were immersed in IKI solution for several minutes and observed. The presence of highly refractile chloroplasts containing large dark starch bodies was interpreted to indicate viable material.

In order to verify that the cytoplasmic appearance of the winter cells was not an artifact of chemical fixation or of hydrating or thawing the tissue, a procedure was developed for the observation of frozen cells from the field which had never been thawed. The procedure and necessary modifications to the standard cryotome and light microscope are outlined in Appendix II. See also plate XXIV.

IV. Experiments on Seedlings Raised in the Growth Chamber

Cones from the collecting sites were opened by scorching them with a Bunsen burner. The seeds were stratified for three weeks on moist filter paper in the dark at $4^{o}C$, and then planted in sandy soil in 2 gallon pots. Growth was accelerated by placing the plants in a growth chamber under continuous illumination until the field equivalent of two to three year old plants bearing mature needles were obtained. Seedlings with mature needles were then placed in a 12 hr. light and 12 hr. darkness regime and a 12 hr. temperature cycle of $20^{o}C$ followed by $12^{o}C$ in a growth chamber.

To determine the effect of drought on the mesophyll cell ultrastructure, two plants were deprived of water for twenty-five days in the growth chamber. During this time, they were subject to a 16/18 hour temperature cycle of $25^{o}C$ and $15^{o}C$. At the end of this period, needles were fixed according to the general procedure for summer fixation.

Frost hardiness was introduced artificially by placing two plants (grown from seed in 2 gallon pots outdoors) that had just completed a cycle of long shoot and needle elongation in a growth chamber for four weeks. An eight hour photoperiod and temperature of $10^{o}C$ were used for the first week. The same conditions were maintained during subsequent weeks except that the temperature was lowered to , and continuously kept at $4^{o}C$. After this period, the plants were taken out of the growth chamber and place in a commercial refrigerator at $-3^{o}C$ for 2, 3 and 4 hours

every other day for six days. Care was taken to move the seedlings to
the refrigerator only during the light cycle of the growth period. One
day in the chamber at 4 ^{O}C was allowed between each day of the frost ex-
posure treatment. The plants were then placed in the refrigerator for a
final six hour treatment and transferred to a commercial freezer in an
insulated box. There was no lighting in the refrigerator or the freezer.
The plants were placed in the freezer for eight hours at -18°C. The
needles were then removed and fixed at below freezing temperatures fol-
lowing the normal winter fixation procedure. During both the refriger-
ator and freezer treatments, the appliances were opened every thirty
minutes for a few seconds to allow gas exchange to occur. This caused
only a brief temperature fluctuation in the refrigerator and none in
the freezer. The plants received no water during the last week in the
4°C regime and during subsequent hardening at lower temperatures.

Two plants, one from the drought experiment which was not watered
for 30 days and a well-watered tree from the growth chamber under the
12 hr. temperature and light cycle were taken through the hardening re-
gime described above. The final stage of hardening involved placing the
plants in a commercial freezer at -18°C for eight hours followed by fix-
ation in a similar manner to that described above. Some of the needles
of these trees were raised to room temperature after the freezer treat-
ment and fixed as described in the procedure for the fixation of summer
needles.

RESULTS

I. Structure of Needle Tissues and Chlorenchyma Cells.

The needle is a complex structure anatomically. The general nomen-
clature and arrangement of cells and tissues is described with reference
to figure 1, which is a dark field photograph of a portion of an unstained
transverse section of a fresh needle.

A thick cuticle (C and arrowhead), covers a layer of small epidermal
cells (E), which are cuboidal. The thin-walled hypodermal cells of the
so called "water-layer" (WL, Shaw, 1914), lie immediately beneath the epi-
dermis. The second hypodermal layer consisting of larger elongated fiber
cells (FH), running parallel to the axis of the needle. In Pinus albical-
is needles, there is no "water-layer" and the sclerenchmatous hypodermal
layers are several cells thick.

The mesophyll chlorenchyma (M) lies between the dermal layers and
the endodermis (En). In Pinus contorta ssp. latifolia specimens collected
for this work, there are two resin ducts (Rd) located entirely within
the mesophyll. Both appear to run the length of the needle between the
endodermis and the "corners"* of the needle. The resin ducts were sur-
rounded by a layer of thin-walled secretory cells (the secretory epithe-
lium (SE)), which is in turn surrounded by a ring of fiber cells (FC)
often several cells thick.

*"corners"-With respect to the half-circle transectional appearance of the
        needles of this species; the term would mean the surface area
        where the flat side (adaxial surface) meets the curved side
        (abaxial surface) of the needle.

There are usually two layers of chlorenchyma cells on the adaxial and abaxial sides of the needle in the median regions. Most sections in this work were cut from this region for light and electron microscopy, more specifically, from the area outlined by the rectangle in figure 1. This is a region on either side of the median longitudinal plane in the abaxial side of the needle.

The chlorenchyma cells are characterized by their crenulated appearance. This feature, which is a distinctive characteristic of most species of Pinus, is the result of numerous invaginations of the primary cell wall. These structures have been assigned various names[*], but in this work the term 'trabiculae' (Bold, 1957) will be used.

The chlorenchyma cells in the corners (lateral portions) of the needle and about the resin ducts are circular or ovoid in shape in transverse sections while the cells in the medial region examined in this work as described above are rectangular in transectional profile. In the circular or ovoid cells, the trabiculae are short and invaginate with uniform spacing from the cell walls whose planes are normal to the plane of a needle transection. The cell walls lying parallel to the plane of needle transections have no trabiculae.

The three dimentional shape of chlorenchymal cells in the median abaxial and adaxial mesophyll is shown in figure 2. The generalized model was reconstructed from the study of serial sections and later confirmed

[*] See Esau (1965) and Mirov (1967)

by evidence from scanning electron microscopy. The three dimensional shape of the cell approximates the geometric shape described as a right angled parallelepiped. This shape has six sides with opposite sides being parallel to each other and having the same area. Three differing surfaces or sides of the cell are shown in figure 2 and are labelled wall surfaces A, B and C. Each wall surface will have an identical opposite surface not shown in figure 2. These surfaces are also termed surfaces A, B and C in this work.

The wall surfaces labelled A in figure 2 corresponds to the wall surfaces of a cell in the plane of needle transection. Note the peripheral parts of the cell face A contain clefts in the cell surface caused by the invagination of trabiculae from the cell wall faces labelled B and C. No trabiculae were observed in pine chlorenchyma on the cell faces in the transverse plane of the needle (face A). A cell very similar to the diagram in figure 2 is shown labelled A in figures 1 and 8.

The wall surfaces labelled "B" in figure 2 lies in the radial longitudinal planes of the needle. In the median chlorenchyma, these walls have a short* trabiculae invaginating from their surface. The wall surfaces labelled "C" lie in the tangential planes of the needle. In the outermost chlorenchymal mesophyll tier of cells, one cell face "C" is appressed to the hypodermal sclerenchyma and conversely, one cell face "C"

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*   Short and long trabiculae refer to the relative distance the trabicular wall structure penetrates the cell interior.

attaches to the endodermis in the innermost tier of needle chlorenchymal cells. Throughout this work, the differing wall surfaces of the chlorenchymal cell will be refered to as face A - the two cell walls lying in the transverse plane of the needle; face B - the two cell walls lying in the radial planes of the needle; and face C - the end walls of the cell.

The trabiculum appears to be basically a shelf-like ingrowth of the cell wall perpendicular to the cell surface. For almost the entire length of the invagination, the primary walls are appressed to each other on either side of a middle lamella. At the end of each trabiculum, the wall folds back on itself similar to a 'hairpin turn' (figure 3). The middle lamella often separates at the tip of the trabiculum forming a small extracellular space. The study of serial sections indicates that this extracellular space is often continuous with the large extracellular air spaces about the cell.

The arrangement of the chlorenchymal air spaces and the chlorenchyma cells in Pinus contorta is in a specific manner. The air spaces occur next to the cell surfaces A and extend as narrow unbroken passages from the hypodermis to the endodermis in the transverse plane of the needle. The chlorenchyma cells are arranged in the same manner so that cell surfaces B and C are contiguous with other mesophyllic, hypodermal or endodermal cells. Thus in needle longitudinal sections, the mesophyll cells form sheets of single cells extending from the endodermis to the hypodermis alternating with hollow passages of extracellular spaces. This

organization breaks down only under the rows of stomata which run longi-

tudinally almost the length of the needle.   Beneath the stomata are sub-

stomatal air spaces which form longitudinal connections between the chlor-

enchymal air spaces.

When fixed and critical point dried needle segments were broken

open for scanning electron microscopy in the transverse plane of the nee-

dle, the mesophyll chlorenchyma broke apart along the trasverse air spaces.

This exposed an intact sheet of chlorenchyma cells lying in the trans-

verse plane of the needle with very little damage to the exposed trans-

verse cell wall faces.   Figure 7 indicates that there were few areas of

attachment between the cell walls of separate transverse sheets of meso-

phyll cells.

When the mesophyll chlorenchyma fractured in the longitudinal plane

of the needle, the mesophyll chlorenchyma cells usually broke apart at

the junction between the end wall of the adjacent cells.   In this case,

intact cell walls were not usually observed.   Instead, fragments of cell

walls remained attached to adjacent cells or pieces of the cell walls includ-

ing remnants of the protoplast were broken off (figure 8).   In thin sect-

ions of the junctional regions between the end walls of adjacent mesophyll

cells, the middle lamella and numerous primary pit fields are observed.

Thus the scanning electron microscopic observations appears to indicate

that in the transverse plane of the needle, the needle mesophyll consists

of single tier or sheet of mesophyll cells extending from the endodermis

to the hypodermis. Within each transverse tier, individual cells are firmly attached to each other with middle lamella between adjacent end walls and radial cell walls. Primary pit fields and plasmodesmata in the end walls of mesophyll cells provide continuous cytoplasmic connections between the endodermis to the hypodermis through the transverse mesophyll cell sheets.

The complex trabicular infoldings of the cell wall of the chlorenchyma result in sectional profiles which are difficult to interpret. Figures 3 to 6 of plate II show how sections of the generalized cell in figure 2 would look if sectioned in several different planes. When the needle is sectioned transversely, the chlorenchyma cells are cut in the plane of face A (figure 2). Sections in this plane show the cell in the transverse plane of the needle and the shelf-like trabiculae are viewed from the side (i.e., at right angles to the wall from which they invaginate, figure 3).

If cells are sectioned in planes which extend in the same direction as the longitudinal axis of the needle, it can be seen that the chlorenchyma cells are not nearly as thick in the needle's longitudinal plane as they are wide or long in the transverse plane (figures 4 to 6).

When the cell is sectioned in the same plane as the needle's surface (i.e., a tangential cut), the cell will be cut in the same plane as the surface C in figure 2. If this section is cut near the cell surface, then the trabiculae will be cut in the section plane and the section would have a profile similar to that shown in figure 4 which could

be confused as three separate cells (the trabicular walls are identical with the appearance of the walls of two immediately adjacent cells). If the section is cut deeper into the cell and approaching the equatorial plane, the section may be below the point of maximal trabicular invagination and the section would have a profile similar to that shown in figure 5 in which no parts of the trabiculae are seen.

When the chlorenchyma cell is sectioned in the radial planes of the needle, the section would be parallel to the plane of wall surface B of figure 2. Figure 6 represents a section in this plane close to surface B to make it appear as if the short trabiculae dissect the cell into two portions.

The two long trabiculae which invaginate from face C in figure 2 can be viewed in two different planes as shown in figures 3 and 4. When viewed in these two sectional planes, it can be seen that the two trabiculae divide top areas of the cell interior into three large lobes. The open side of two of the lobes is partially restricted to the cell interior by the small trabiculae invaginating into the cell from surface B. In figure 2, the cell is shown to be subdivided into six lobes by six long and shor-trabiculae.

The shape of mesophyll chlorenchyma cell walls in Pinus albicaulis needles collected at Whistler mountain were very similar to those described for Pinus contorta. Sections of mesophyll from Tsuga mertensiana

collected from Whistler mountain revealed a mesophyll chlorenchyma consisting of cylindrical cells with rounded ends. The long axis of the cells were arranged radially from the endodermis to the hypodermis. No evidence of trabicular infolding were found in chlorenchyma of this conifer species. The relationship between mesophyll cells and the intercellular air spaces was not as uniform as in the Tsuga mesophyll. The air spaces formed a more anastomosing system about individual cells rather than being largely confined to the transverse plane of the needle. As in Pinus contorta, the primary pit fields, plasmodesmata and the areas of wall to wall contact between mesophyll cells and mesophyll endodermal cells or mesophyll hypodermal cells was largely confined to the end walls of the cylindrical cells.

Below the mesophyll in Pinus contorta was a well defined endodermis consisting of a single layer of closely packed cells which are ovoid in transection, (figure 1). Within the endodermal layer and surrounding the vascular bundles (VB) is the transfusion tissue, which is uniquely gymnospermous (Esau, 1965). It consists of irregularly shaped transfusion tracheids (TT), with secondary walls containing numerous bordered pits, and large living transfusion parenchymal cells (TP) which are circular to oval in transection, and have thick primary cell walls (figure 1).

II. The Cytology of the Chlorenchyma in the Summer Condition

A. Light Microscopy

A frozen section of summer chlorenchyma prepared by the method outlined in Appendix I.I is shown in figure 10. Frozen sections of summer and winter material have a very granular and opaque appearance but several cytoplasmic features can be identified. The cytoplasm appears as a thin peripheral band containing chloroplasts (arrowheads), and the nucleus (N) is centrally located within the cell.

When cryosections are allowed to thaw, the granularity largely disappears and cytoplasmic objects are more distinct. Figure 11 is a thawed section at higher magnification. Chloroplasts (arrowheads) can be seen to be regularly spaced along the cell wall and the sides of the trabiculum (T). The sperical appearance of the chloroplasts in frozen and then thawed cryosections appears to be an artifact of cryosectioning.

The cell in figure 10 has an unusual shape due to the U-shaped indentation in its upper surface which is the substomatal space that connects the air spaces of the needle in the longitudinal plane. The guard cells (Gd) of the stomata are also shown in this figure.

Fresh cryosections treated with either ferrous sulfate based stain

fixative for tannin (Johansen, 1940), the nitrous acid staining procedure of Reeve (1951) or one percent osmic acid resulted in the intense specific staining of the tannins within the main vacuole. The intensity of the staining of the main cell vacuole when the Johansen procedure is used is illustrated in figure 12. In order to avoid confusion with other vacuolar structures to be discussed later, this tannin-filled vacuole will be called the tannin vacuole (TV). The tannin vacuole in figure 12 outlines clearly the thin band of unstained parietal cytoplasm, (cytoplasm between arrowheads). A nucleus is located close to the tip of a long trabiculum; this is a typical location of the nucleus in the summer cell.

When formalin fixed cryosections in the summer condition were stained with Sudan Black B, many small oil droplets (O) appeared in the cytoplasm. The largest of these were about two microns in diameter (arrows, figure 13) and their size extended down to the limit of resolution (arrows, figure 14). In the endodermis and the transfusion parenchyma, numerous and considerably larger oil droplets (arrowhead, figure 13) can be seen. .

No cryosections were cut of other conifer species mentioned in this work.

B.   Ultrastructural Changes in the Chlorenchyma Related to the Aging

of the Needle

Although the elucidation of the process of senesence in pine chlo-
renchyma was not the object of this investigation, it is essential that
any major changes due to aging in the summer needle be characterized so
that these features will not be confused with changes related to season-
al dependent factors. For this purpose, summer and winter needles from
current season needle flush to five years of age were examined.

Three general cytological conditions dependent on needle age were
identified. In the first condition, the chlorenchyma has a character-
istics which reflect a state of high metabolic and synthetic activity.
The ground cytoplasm is filled with numerous polysomes and a large amount
of RER, particularly near the nucleus (figure 22). Dictyosomes were
frequently seen in sections and were surrounded by numerous vesicles. In
many cases, numerous dictyosome vesicles are located in the area between
the dictyosomes and the plasma membrane (figure 29). In other cases, a
large number of the dictyosome associated vesicles appear in regions
where vacuolization occurs (figure 26). Mitochondria were large and
extensively lobed and had numerous cristae. Tannin is present at a very
early stage of needle elongation but in young expanding needles, it is
often clumped into irregular aggregates and does not occupy the full vol-
ume of the main vacuole (figure 20).

It was concluded that the first condition observed in young expand-

ing current season needles in June and July represented cells character-
ized by features usually associated with active metabolism. In this
work, this cell condition will be referred to as the type I cell type.

In late July, the young needles had completed elongation and the
cytoplasm had transformed into the second cytoplasmic condition which
will be referred to as the type II cell. This condition of needle cyto-
logy is observed in the current season needles during late July and Au-
gust. When second, third and fifth year needles are examined, the major-
ity of cells appear to also belong to this condition. It appears to be
characterized by the cell being committed to, and active in photosynthesis
and respiration. The cytoplasm is dominated by large numbers of chloro-
plasts and mitochondria, and contains few other organelles. Other fea-
tures of this cell type are:

    a.   Dictyosomes were rarely seen and usually associated with few
        surrounding vesicles.

    b.   Ribosomes occured in the ground cytoplasm.

    c.   Only small fragments of RER were seen, usually near the nucleus.

    d.   The nucleus had extensive areas of heterochromatin.

The third condition of summer chlorenchyma cells consists of what
appeared to be old or damaged cells to a variety of degrees. Some of
these are probably caused by preparative precedures for electron micros-
copy. These cells are characterized by organelles that are damaged and
poorly fixed, or by scarcity of organelles indicating the low level of

cessasion of cellular functions. Cells of this type had some or most of
the following characteristics:

a. Large quantities of osmiophilic material which tend to obscure
the cytoplasmic detail. The osmiophilic material is usually
tannin-like or lipoidal in appearance. Figure 70 illustrates
part of a cell of this type. The cytoplasm contains a large
osmiophilic oil-like body (O), and poorly discernable chloroplasts.
Despite electron density and lack of membranous detail, the cy-
toplasm is probably still functional as evidenced by the pre-
sence of starch grains within the chloroplasts. However, many
cells of this type did not contain the amount of fine structural
detail shown in this figure and starch grains were absent.

b. Tonoplast and/or plasma membrane fragmented or absent.

c. Very poor detail and/or with apparent mechanical damage to the
cell, i.e., swollen organelles, ruptured cytoplasmic membranes.
This could be a physical phenomenon due to changes in osmotic
pressure and membrane permeability within these older cells.

Type III cells were not observed in young maturing needles (first
summer), but the frequency of their occurence increases with needle age.
Approximately twenty-five percent of the chlorenchyma cells in five year
old needles were of this type.

Most needles of one year old or older have occasional chlorotic
patches scattered along the length of the needle. These chlorotic pat-

ches increase in size and frequency with respect to the age of the nee-
dle. Small chlorotic (less than 1 mm) patches do not usually spread
along the needle axis but extend around the needle as a ring or a girdle.
Larger chlorotic patches, however, not only extend completely about the
needle but also spread to a lengh of a centimeter of more along the
needle axis.

Type III cells usually occurred in large groups and appeared to be
more extensive within the transverse plane of the needle than they are
in the plane of the longitudinal needle axis. They show the same basic
pattern of orientation as the macroscopic chlorotic areas.

Summer fixation problems increased in proportion with needle age and
adequate fixations of three and five year old needles was only achieved with
the acrolein-glutaraldehyde primary fixatives (figures 69,70 and 71 as ex-
amples). One problem associated with the use of acrolein based fixative
on pine chlorenchyma is that the cytoplasm becomes considerably more os-
miophilic than with the formalin-glutaraldehyde fixatives.

Predominently green needles were observed on young open grown trees
that were as old as twelve years. Attempts to fix such needles were un-
sccessful. However, starch grains and chloroplasts were observed in cryo-
sections of primary fixed (acrolein-glutaraldehyde) material at the light
microscopy level.

C. Observations on Specific Organelles: Electron Microscopy

All organelles in summer pine chlorenchyma appear to be very similar to those described for the chlorenchyma of other higher plants. Only types I and II cells will be described since these are the cells which are of functional significance to the tree.

The plasma membrane is well defined in types I and II cells. At the fixative osmolarity used for electron microscopy, it was usually tightly appressed to the cell walls and the walls of the trabiculae. However, the high osmolarity of the formalin fixative used to stain tannins for light microscopy often caused plasmolysis of the protoplast. (figure 12). Plasmodesmata were observed frequently when sections were cut near the ends of the cell (surface C, and the corner areas near its junction of wall surface C and B in figure 2).

The tonoplast is also well defined and remains intact in all types I and II cells observed. Tannin material appeared to shrink and separated somewhat from the tonoplast forming a gap. This is evidence for membrane continuity since strands are clearly seen linking the two structures. Such a phenomenon also appears to cause the convolution in the profile of the tonoplast (TO, open arrows, figure 23). The size of the gap between tonoplast and tannin appears to be fixation dependent, varying considerably from one fixation to the next (see figures 22, 23 and 26). Acrolein fixatives did not appear to form a gap between the tonoplast and the tannin material. In some sections, the tonoplast is observed to interdigi-

tate extensively into the adjacent cytoplasm (open arrows, figure 27).
When the tannin vacuole is sectioned obliquely, the interdigitating
tonoplast forms complex membrane patterns (open arrows, figure 28).

The tonoplast is also most susceptible to damage caused by needle
subdivision, or due to osmotic changes during fixation. When the tono-
plast does rupture, the cytoplasm appears filled with tannin, and cell-
ular fine structure is obscured. The sensitivity of the tonoplast dur-
ing fixations appears to be dependent on the osmolarity of the fixative.
Fixatives with a high osmotic pressure cause the tannin material to ap-
pear to contract and separate from the tonoplast (forming the gap pre-
viously mentioned). Fixative with osmotic pressures below that used
for this work tended to rupture the tonoplast which resulted in the
dispersion of tannin throughout the cytoplasm and the destruction of
cytoplasmic fine structure.

In very young (2mm) needles, the main vacuole is largely electron-
transparent (figure 20) and tannin deposits, if any, occur as scattered
clumps. However, well before needle elongation is completed, the main
vacuole becomes filled with tannin, and the main vacuole is then, as stated
previously, referred to as the tannin vacuole.

In the cell of the mature summer chlorenchyma, the tannin vacuole
is a dominant feature and occupies most of the cell volume. The tannin
material has a very uniform granular appearance, but occasionally a
fibrillar fine structure was also seen along with the granular texture.

In type II and type III cells particularly, the appearance of the tannin
is modified by minute fracturing and knife chatter artifacts formed dur-
ing sectioning. An example of this type of artifact is shown in the tan-
nin vacuole of figure 70. The tannin material is exceptionally difficult
to cut and this difficulty appears to increase with needle age. Osmio-
philic material with the texture of tannin was not seen outside of the
main vacuole in types I and II cells in summer fixations.

In some older needles (three to five years), the homogeneity of the
tannin in the tannin vacuole was often replaced by the presence of large
and small inclusions. The inclusions were consistently very osmiophilic
and smooth in texture and are thus described as a lipoidal type (figure 70). In
this cell, the tonoplast could not be resolved and thus the lipid mat-
erial (O) could be within a cytoplasmic vacuole or within the tannin va-
cuole.

In types I and II cells, a variety of cytoplasmic vesicles and vacu-
oles are seen within the peripheral cytoplasm of the cell in addition
to the main tannin vacuole. These cytoplasmic vacuoles may contain small
osmiophilic bodies (OB) and/or material that is possibly of membranous
origin (figures 22, 23 and 25). The osmiophilic bodies discussed through-
out this work are structures of unknown chemical composition. They are
identified on the basis of: size, (usually less than 1 μm in diameter),
texture (very homogeneous and "lipid-like") and staining intensity, (they
are the most osmiophilic structures in most sections). Compare the ap-

pearance of osmiophilic bodies with tannin material in figures 22, 23, 24 and 25, and with the oil body in figure 29. In this work, cytoplasmic vacuoles are differentiated from the tannin vacuole on the basis of the former being much smaller than the tannin vacuole (less than 5 μm in diameter) and containing electron transparent lumens, or membranous material or material that stains similar to that of the osmiophilic bodies. The cytoplasmic vacuoles were not observed to contain material that had staining and textural properties similar to tannin.

The number of osmiophilic bodies tends to increase in late summer in needles of all ages. Most osmiophilic bodies are found within cytoplasmic vacuoles (figure 22), but some are found in the cytoplasm without any apparent bounding membranes (figure 23, cf. upper and lower osmiophilic bodies).

As previously noted, dictyosomes were very numerous in type I cells. In elongating needles, dictyosomes appear associated with the trabicular regions of the cell wall. These organelles appear to often have their maturing face towards the plasma membrane. Dictyosomal vesicles also often occupied the space between the cell wall and the dictyosomes. These vesicles generally were the smallest in size when located near the maturing face, the largest in the proximity of the cell wall (figure 27). Dictyosomes are also associated with the cytoplasmic vacuoles described previously (figure 25). A transitional series of vesicles may occur between dictyosomal faces and the cytoplasmic vacuoles (figure 25). In type II cells of

the mature summer tissue, the number of dictysomes is greatly reduced as compared with expanding needles. But in mature needles, the close association between dictyosome vesicles and the cytoplasmic vacuoles (which do not contain osmiophilic bodies) is still observed (figure 26).

In the summer needle, the chloroplasts of the chlorenchyma have the characteristic discoidal shape seen in higher plants with one side of the plastid flattened against the plasma membrane (figures 19, 20 and 21). The shape and uniform distribution of the chloroplasts along the cell wall and the trabiculae remain constant regardless of needle age in type I and type II cells.

The chloroplast envelope is distinct and consists of two sharply contrasted membranes (figure 21). The grana were made up of not more than five or six stacked thylakoids in all cell types. The granal thylakoids are of very different sizes, thus the resulting stacks of thylakoids have very irregular edges. Partitions are often elongated, resulting in very low and broad profiles of granna (figure 21). The greatly elongated grana shown in figure 21 is made up of two to three stacked thylakoids. This type of grana is more common in chloroplasts from older needles, however, exceptions were noted. The chloroplasts of the three year old cell in figure 71 show well developed granal stacks similar in height to those seen in younger cells.

Starch grains were usually found in summer chloroplasts of type I

and type II cells and often in type III cells in which the chloroplasts remain intact. The starch grains in the chloroplasts in this work (figure 21 for example) are usually small because all summer material was fixed at dawn. Afternoon fixations of summer tissue were not conducted because starch in cells fixed in this manner would obscure much of the internal structures of the plastids and greatly distorted their shape and the surrounding cytoplasm.

Prominent plastoglobuli are a feature of the chlorenchymal chloroplast of Pinus contorta. In very young type I cells, the plastoglobuli are small (arrows, figure 20), but in type II cells, they are larger but remain at about the same relative number per chloroplast profile. After an increase in plastoglobuli size from type I to type II cells, plastoglobuli size then appears to be independent of the age of the cell in types II and III cells.

The variation of chloroplast structure from cell to cell or within one cell is greater in older needles. The chloroplast in figure 71 for example has fewer plastoglobuli and more extensive thylakoids than is typical for a three year old type II cell.

The structure of chlorenchymal mitochondria in summer also varies with needle age. In type I cells, mitochondria appeared to be cup-shaped or multi-lobed when sectioned in the plane parallel to and just below the cell wall (figures 27 and 28). When transverse sections of the cell are taken with respect to the cell wall, the mitochondria are not so ex-

tensively lobed in appearance and are usually ovoid in sectional profile
(figure 19). The degree of lobing of the mitochondria is greatly dimin-
ished in type II cells. In this cell type, sectional profiles of mito-
chondria usually appear ovoid in all planes of sectioning. Summer mi-
tochondria in both types I and II cells have extensive tubular cristae.

The nucleus in the summer chlorenchyma is usually located in the
central region of the cell, typically near the ends of one or more of
the long trabiculae. In this position, the nucleus is nearly surrounded
by the tannin vacuole. Immediately about the nucleus is a region of
perinuclear cytoplasm. The perinuclear cytoplasm is connected by sever-
al broad cytoplasmic strands through the tannin vacuole to the peripher-
al cytoplasm near the trabiculae and/or the cell wall (figure 9 shows
two such connections). In type II cells, this area of cytoplasm is the
only area where RER and cytoplasmic ribosomes were consistantly noted.
Sections from this area of cytoplasm also appeared to have a higher pro-
bability of containing profiles of a dictyosome than other parts of the
cell.

Small oil bodies up to three to four μm in diameter were also a
common occurence in this and other area of cytoplasm of type I and types
II cells (figure 32).

In general, the nucleoli appear to be circular in sectional profile.
The nucleolus is predominently composed of pars granulosae material sur-

rounded by an unusual heterochromatin-free electron transparent ring

of nucleoplasm (figure 30). In type I cells, as many as three nucleo-

lar profiles were observed (figure 31) in sections. The number of nu-

cleolar profiles appears to be usually one and occasionally two per

section in type II cells (figure 32). In third and fifth year needles,

nucleoli were rarely observed.

The nucleus of all cell types contains dense areas of heterochroma-

tin, between which are areas of clear nucleoplasm consisting of scatter-

ed granular material and very fine fibrillar material. The extent of

the heterochromatin regions in the nucleus appears to slightly increase

in older needles (cf. figures 32 and 69).

D.   Experiments on Summer Needles

It has been already noted that the chloroplasts in cryosectioned summer needles swell into spherical shapes (figure 11). When these needles were kept in a freezer at $-18^{O}C$ or placed into liquid nitrogen and then fixed for electron microscopy at $4^{O}C$, images similar to that shown in figure 72 are obtained. An area of the cytoplasm containing what appears to be plastoglobuli and starch grains probably represents what may have been a chloroplast. No clear cut membrane boundaries can be seen. The mixture of tannin material in this cell region may indicate the breakdown of the tonoplast.

Summer tissue frozen to $-18^{O}C$ and then fixed in the winter procedure at $-4^{O}C$ (without thawing) also was severely disrupted but more cytoplasmic membranes remain visible. Following this treatment, chloroplasts were greatly swollen with the envelope often ruptured. Although they still contained thylakoids, most of them were extensively dilated. In these cells, the tonoplast had apparently ruptured and tannin material was present within the cytoplasm.

The various osmotic extremes used in some of the winter fixations were also carried out on summer tissues as control experiments. Extensive plasmolysis of the protoplast was noted with the fixative containing 10% sucrose. In this fixation, the cytoplasm appeared crushed or compacted against the tannin vacuole and was very osmiophilic. The major cell organelles however could be observed and they appeared to

be structurally intact. Cytoplasmic organelles were swollen and com-
pletely disrupted by fixative made up in either sea water or distilled
water. In both cases, tannin material was again released throughout
the cytoplasm and the tonoplast was not observed.

III. Autumnal Changes in the Ultrastructure of the Chlorenchyma

Fixations of material between August 20 and November 26, 1972 were not satisfactory. Material fixed on August 20th revealed basically a summer condition within the cells. There are, however, some slight modifications compared to earlier summer fixations: the electron density, size and number of the oil droplets increased above that seen in the early and mid-summer fixations; the chloroplasts maintained their even distribution within the cell but appeared to be located slightly away from the plasma membrane. Material fixed in late November had the general ultrasturctural appearance of the winter condition.

IV. The Chlorenchyma in the Winter Condition

A. Light Microscopy

At the light microscopy level, changes in the nature and position of cytoplasmic structures were observed in the winter condition. A photograph of an unthawed section of winter tissue prepared by the procedure outlined in Appendix II is shown in figure 33. The band of peripheral granular cytoplasm (between arrows) is several times greater, in width, than of comparable tissue in the summer condition. Very little detail is discernable inside the cytoplasm and the chloroplasts lining the cell wall, (which characterized summer tissue prepared by the same preparative procedures), cannot be clearly visualized (e.g., cf. figure 7). The only recognizable structures are the nuclei and the clear tannin vacuoles which appear similar in size and location to observations made on these structures in chemically fixed material.

Mechanically, this unfixed frozen winter tissue was more susceptible to damage caused by the stresses of cryo- or freehand sectioning. Sections tended to crumble or the main vacuoles often fell out as intact units when the sections were cut. If the sections are allowed to thaw while being observed, the tannin vacuoles seem to retain their shape, while the cytoplasmic areas appear to liquify with the granular material within it moving about rapidly in fluid convection currents.

Figure 34 shows a section of several cells from wintering pine chlorenchyma that have been stained by the Johansen's tannin stain solution as described in Appendix J. The wide band of cytoplasm is in marked contrast to the densely stained tannin vacuoles.

Using 1 μm sections of plastic-embedded material stained with toluidine blue and safranin, it was possible to observe the structure of the winter chlorenchyma cytoplasm in more detail (figure 35). The most remarkable feature is the clumping of the chloroplasts. These clumps appeared to be compartmented in areas bounded by the trabiculae, the cell walls and by the tannin vacuole. Close examination of each clump of chloroplasts indicated it to be composed of densly stained internal membrane systems with a discoidal shape similar to that of the summer chloroplast. Surrounding each membrane system is a medium density homogeneous area which is probably corresponding to the stromal region of the chloroplast. It is the swollen stromal areas of the chloroplasts which are responsible for the generally circular or irregular profiles of these clumped chloroplasts. In some chloroplasts, the internal thylakoid system appears to be twice as large as the adjacent chloroplasts.

The nucleus in the cell of winter material occupied a different position. In marked contrast to the summer state, it does not protrude into the tannin vacuole. Instead, it is completely located within the peripheral cytoplasm (figures 33, 34 and 48). The winter nucleus is variable in shape, but is usually ovoidal in sectional profile with the major axis parallel to the proximal cell wall. The winter nucleus is

usually found in a specific part of the peripheral cytoplasm. This area is the same cellular region where the larger chloroplast clumps are found.

The broad band of peripheral cytoplasm in figure 35 appears as a network of clear vacuoles and narrow strands of cytoplasm. So extensive is this cytoplasmic vacuolar system that it could be suitably described as 'sponge-like'. Small dense granules can often be seen in the cytoplasm near the cell wall (open arrowhead, figure 35).

Cryosections stained with Sudan Black B reveal oil droplets usually ranging between 5 and 10 μm in diameter (figure 36). They are mainly in the perinuclear region. The volume of the largest sudanophilic droplets is approximately ten times the average size of those observed in the summer condition while larger oil bodies are found near the nucleus. Smaller droplets, often the size of those in summer condition are usually found scattered throughout the cytoplasm. Oil droplets are also more extensive in the endodermis and transfusion parenchyma than in summer.

B. Observations on the Winter Condition: Electron Microscopy

The electron microscopic observations of the winter chlorenchyma
confirm the protoplasmic structure observed in light microscopy. Fig-
ures 37 to 40 represent views of the protoplasmic structure in winter
chlorenchyma cells obtained from various planes of sectioning. These
figures are low magnification micrographs showing sectional profiles
of nearly entire cells. They reveal how the position of the chloroplast
clumps is related to the nature of the cell wall.

Figure 37 shows several cells sectioned in a plane similar to the
illustration in figure 5. There are several small chloroplast clumps
shown in the cytoplasm adjacent to the cell walls corresponding to the
cell wall face B in figure 2. From these faces of the cell wall, the
small trabiculae invaginate. Also shown in this section are the cell
walls corresponding to face A in figure 2. The cytoplasm next to these
walls is marked with asterisks in figure 37. Note that chloroplast
clumps are absent in the cytoplasm adjacent to these faces of the cell
wall.

Figure 39 represents portions of two cells sectioned in a plane
similar to the illustration in figure 3. A short trabiculum is shown
invaginating from the wall face B in each cell. Adjacent to the tra-
biculae are small chloroplast clumps and in the lower cell, a large oil
body is also adjacent to the trabiculum. To the left of figure 39 is
an area corresponding to the end region of the lower cell; i.e., adja-

cent to the cell face labelled C in figure 2. Note the chloroplast
clumps in this area.

Nearly an entire cell section is shown in figure 38. This section
is in the sectional plane of the cell similar to the illustration in
figure 6. Note the presence of large numbers of chloroplasts near the
ends of the cell (to the left and right) near the cell walls corres-
ponding to face C in figure 2. Note the relatively few chloroplasts in
the cytoplasm adjacent to the cell walls corresponding to the cell wall
faces labelled A in figure 2.

Figure 40 is a section of several cells in the sectional plane of
the cell similar to that illustrated in figure 4. In this plane, the
size of the chloroplast clumps located in the cytoplasm adjacent to the
end walls of the cell becomes apparent.

Studies of the winter mesophyll chlorenchyma in various planes of
sectioning reveal that the distribution of chloroplasts is related to
the nature of the adjacent cell wall. Very few chloroplasts are found
adjacent to the cell faces labelled A in figure 2 where no trabiculae
invaginate. Small chloroplast clumps (usually two to ten organelles)
are found in the cytoplasm adjacent to the cell wall faces labelled B
in figure 2 where the short trabiculae occur. Large chloroplast clumps
composed of up to twenty or thirty chloroplasts are found in the cyto-
plasm adjacent to the cell wall faces labelled C in figure 2. Long tra-

biculae invaginate from this part of the cell wall.

In addition to the clumping phenomena, the winter chloroplasts have
structural differences from summer chloroplasts. At low magnification,
the chloroplasts appear bounded by a distinct, dark boundary. At higher
magnifications, the nature of the chloroplast envelope can be somewhat
resolved (figure 47, CE). The two bounding membranes of the chloroplast
envelope, easily distinguished in summer chloroplasts, are consistently
indistinct in all winter chloroplasts fixed by various methods. Dila-
tion or swelling of the inter-membrane space of the chloroplast envelope
was never observed in the winter observations. It appears therefore,
that the two envelope membranes are consistently parallel to each other.
Furthermore, envelopes of adjoining chloroplasts appear to be extensively
in contact with one another (see figures 42, 45 and 46). A triple-mem-
brane appearance of the contact regions between the chloroplasts can be
seen in places (figure 46), characterized by a thick central membrane and
two thinner outer membranes. This triple layered membrane system pro-
bably represents the partial fusion area between clumped chloroplasts
and can often be seen at lower magnifications (arrows, figure 42). When
the regions of chloroplast adherence are cut tangentially to the membrane
surface, the contact region appears as a homogeneous dense band (figure 43).

As noted at the light microscopic level, the thylakoid systems of the
winter chloroplasts retain the discoidal shape of the summer chloroplast.
The arrangement and size of the stromal and granal thylakoid membranes

also remain distinct and similar to the summer condition (figure 47).

However, in contrast to the summer condition, two and sometimes three

complete thylakoid systems are found within a single envelope of winter

chloroplasts. Occasionally, two chloroplasts appeared to be joined by

a contriction or isthmus of stroma (figure 44). When two or more thy-

lakoid systems are found within a common envelope, thay may be arranged

at various angles to each other (asterisks, figure 43). In all cases,

however, the thylakoid systems are not in continuity and are always

separated from each other by an area of stroma (figure 43).

The stroma is very extensive in winter chloroplasts and, in general,

this expanse of stroma accounts for nearly half the sectional area of

the chloroplast (figures 39, 43 and 47). Winter chloroplasts that have

only one internal membrane system within the envelope appear to be about

fifty percent greater in volume than summer chloroplasts. This volume-

tric increase is due to an increase in the width of winter chloroplasts

without a corresponding decrease in length (i.e., the chloroplasts swell

substantially in winter).

Plastoglobuli have, in general, been noted in summer material to in-

crease slightly in size and numbers with increasing needle age. This

relationship also appears to be occuring in winter with older winter chlo-

roplasts containing larger and slightly more plastoglobuli (cf. figures

43 and 44). This relationship has many specific exceptions however, as

was noted in the summer observations. In winter, the plastoglobuli are

randomly distributed within the chloroplast in the swollen areas of the

stroma as well as in the stroma immediately around the thylakoids (figures 42, 45 and 47).

The structural and positional transformations of the chloroplasts represent the most dramatic changes in the winter protoplasts, but other protoplasmic structures also undergo seasonal changes. The matrix of the tannin vacuole appeared to contract in winter material. This contraction is associated with an approximate hundred percent volumetric increase in the peripheral cytoplasm (figure 34, 35, 36 and 45). The tannin-cytoplasmic border when viewed in sections of entire cells is more rounded and conforms less to the contours of the cell walls (figures 37, 38, 39 and 48). The texture of the tannin material is similar to that observed in the summer and yielded similar shatter artifacts when sectioned. Occasionally, small blebs of tannin material appear to be separated from the main deposit (arrowhead(s), figures 39 and 40). The tonoplast, presumably about the tannin material, could not be clearly visualized in winter fixations.

In winter, the cytoplasm is confined to the cell periphery between the tannin vacuole and the cell wall and no cytoplasmic strands as described for summer condition were observed. The winter peripheral cytoplasm is of irregular width; the widest areas are usually located in the pockets formed by the long trabiculae at the end of the cell (figures 38, 39 and 40). The narrowest portions of cytoplasm occur along the cell walls corresponding to faces A of figure 2 (figures 37 and 38).

In the narrower cytoplasmic regions, the cytoplasm is dominated by massive cytoplasmic vacuolation. These vacuolized areas can be described as appearing 'foamy' or 'sponge-like' and contain little cytoplasmic matrix and very few organelles (see asterisks, figure 48).

Large numbers of vacuoles are found about and between the chloroplast clumps in the end regions of the cell (asterisks, figures 41 and 50) and between areas where cytoplasmic organelles occur and the plasma membrane (figures 49 and 60). Vacuoles are not common, however, in the cytoplasm immediately adjacent to the nucleus and the chloroplast clumps. The non-vacuolated cytoplasm in these areas contains the occasional polysomes and other cell organelles (GC, figures 41, 42 and 57).

Another prominent feature of the cytoplasm is the presence of very large numbers of osmiophilic bodies. The osmiophilic bodies are usually found in the areas that abound with vacuoles and in some cases, the osmiophilic material constitutes the bulk of the vacuolar material (OB, figure 49). The osmiophilic material may also form a thin layer lining the interior face of the cytoplasmic vacuole (CV and arrowheads, figure 49). In some sections, almost every cytoplasmic vacuole appears to contain an osmiophilic body (figures 51 and 52), while in other sections, there are few osmiophilic bodies associated with the cytoplasmic vacuoles (figures 50 and 51).

In the regions of the cell where the majority of the chloroplast clumps

occur and where the cytoplasm is not highly vacuolated, the osmiophilic bodies are found in vacuoles arranged in a file or tier adjacent to the plasma membrane (figures 51 and 52). It is interesting to note that the cytoplasmic vacuoles between the chloroplasts and the tannin material (not shown in figure 51) contain very few osmiophilic bodies (double ended arrow). It seems that the cytoplasmic vacuoles appear to consistently have their long axis parallel to each other (figure 51). Configurations such as this appear to indicate the shrinkage of tannin material causing the stretching of the cytoplasmic vacuoles.

Lipid inclusions appear to build up significantly in winter (figure 36). Figure 39 shows a large oil body (O) about 5 to 7 μm in diameter, many of the oil bodies are located in the peri-nuclear cytoplasm (figures 55 and 58). The size, number and distribution of the large oil inclusions noted in winter tissue at the ultrastructural level agree very well with the size, number and distribution of sudanophilic bodies seen at the light microscopy level.

The majority of mitochondria in the winter chlorenchymal cytoplasm are loosely associated with the chloroplast clumps (figure 37). Mitochondria are also occasionally localized near the large oil inclusions (figure 58) or are randomly distributed throughout the vacuolated cytoplasm (figure 48). The mitochondria are smaller than in summer needle chlorenchyma and are predominently circular in sectional profiles indicating a spherical shape. As with winter chloroplasts, the two membranes of the

mitochondrial envelope are very indistinct and usually appear as a single dense line at low magnification (figure 53). There is, however, no sign of fusion between mitochondria. When mitochondria come in close proximity to one another or to chloroplasts, a distinct gap was visible between their envelopes (e.g. figures 43 and 58, arrows). Although some mitochondria still have clear and extensive tubular cristae in winter (figure 58), the majority of mitochondria appear to have more poorly developed cristae than those found in the organelles in summer (figure 53).

Dictyosomes were not common in mature mesophyll chlorenchyma type II cells in both summer and winter fixations, but those present in the winter condition were characterized as having very few dictyosomal vesicles associated with them (figure 54). The few winter dictyosomes observed were found close to the nucleus or the chloroplast clumps.

As noted previously, the nucleus changes its position within the cell from a central location partially surrounded by the tannin vacuole (in summer) to a more peripheral location near the cell walls during winter. In summer, the nucleus appeared consistently circular in sectional profile while in winter, the most common sectional shape was ovoid (figure 57) with the long axis parallel to the cell wall (figure 48).

The two layers of membranes of the winter cell nucleus are distinct

and parallel with each other and there is no evidence of swelling (fig-
ure 56). Numerous nuclear pores are present (figure 56) and contain an
amorphous dense material. Comparison between the structure of the win-
ter nucleus at the light microscopy and electron microscopy levels is
illustrated by figures 55 and 57. The darkly stained heterochromatic
regions shown at the light microscopy level in figure 55 can be seen to
be considerably smaller and evenly spaced within the nucleoplasm in con-
trast to the counterpart regions in summer nuclei (figure 32). At the
ultrastructural level (figure 57), the darker chromatin regions of the
nucleus as described at the light microscopy level have a fine granul-
ar texture and are considerably more diffuse than the heterochromatin
of the spring or summer nucleus. In the lightly stained nucleoplasmic
regions, fine fibrillar material can be seen (figure 56).

One or two circular nucleoli were usually noticed in sectional pro-
files of winter nuclei. The nucleoli in winter condition were struct-
urally similar to those found in the summer tissue (figure 56). The un-
usual less dense nucleoplasmic area about the nucleolus seen in the sum-
mer tissue (figure 30) was not observed in winter fixations.

C.   Winter Material Fixed with Differing Fixative Solutions

When winter chlorenchyma cells were first examined, it was thought that the winter cytoplasmic conformation was at least partly induced by osmotic pressure damage caused during fixative procedures. A series of fixative solutions of varying osmolarities was then made and applied to winter material. The results of these experiments indicated that the winter material failed to vary significantly in structural appearance with any of the fixative solutions used. These experiments were then expanded to include not only extreme variations in fixative osmolarity, but also the use of electrolyte or nonelectrolyte additives in fixation media.

An unbuffered hypotonic fixative solution was made by using one percent glutaraldehyde and 0.5% formalin in distilled water. This fixative solution was below 400 mOsm. Tissue fixed in this fixative could not be distinguished from winter tissue prepared by the standard winter fixation procedures.

A very hypertonic fixative solution was also prepared using sea water and 2% glutaraldehyde and 0.5% formalin as a primary fixative without any buffer or sucrose in the solution. The osmolarity of this hypertonic electrolyte solution was much greater than 1,050 mOsm. This solution caused some noticeable but minor changes in the ultrastructure of the winter chlorenchyma.

In figure 60, it can be seen that the chloroplasts are elongated, but the stromal material is somewhat reduced. In general, the areas of contact between the envelopes of adjacent chloroplasts are also reduced (figure 60). The osmiophilic bodies in the cytoplasm appear to change their shape from circular forms to a stellate profile (figure 61). The major cytoplasmic features that are characteristic of the winter fixations were unaffected by sea water electrolytes in the fixative solution.

A third type of fixative, the non-electrolyte fixative solution was made up of 10% sucrose in addition to the standard fixative solution. No identifiable changes in winter chlorehchymal ultrasturcture was noted with this fixative.

Control studies with the above fixative solutions were conducted on summer tissue from the field collecting sites or from mature needles of seedlings grown under optimal growth conditions in a growth chamber. The fixative solution without buffer and the solution containing sea water as an additive caused complete disruption of the summer cytoplasm. The fixative solution containing ten percent sucrose as an additive resulted in an inferior fixation to material fixed with the standard summer fixative solution largely due to extensive plasmolysis of the protoplast.

D.   Experiments with Winter Needles Frozen and Thawed Under Field

and Laboratory Conditions

It was found that needles collected in mid winter maintained their

viability for at least two months when stored in a commercial deep freeze

at -18°C. Viability was tested by the cytological methods outlined in

the material and methods section. Attempts to store needles in the free-

zer section of a refrigerator whose freezer temperature varied at least

between -3°C and -8°C were not successful. After one month in the free-

zer compartment of the refrigerator, no needles could be termed viable.

Material collected in mid winter at -11°C and transported to Van-

couver at slightly below freezing temperatures failed to survive immer-

sion into liquid nitrogen. When material stored in the chest freezer

for several weeks to four months was placed into liquid nitrogen and

then had the viability test conducted on the needles, the results were

positive.

Some of the chest freezer stored material and also the chest free-

zer material which had been in liquid nitrogen was warmed to about -4°C

and fixed for electron microscopy by the standard winter procedure. The

usual winter state of the winter chlorenchymal cytoplasm previously des-

cribed was observed. The only changes were in material that had been in

liquid nitrogen. Figure 62 shows that in this material the osmiophilic

bodies have two distinct regions, a light outer area and a more electron

dense inner core.

When mid winter material collected in the field was placed in a
growth chamber under summer temperature and light regimes and then ex-
amined by light microscopy, starch appeared in the chloroplasts and the
chloroplast clumps become loose aggregates within four days. After
eight days in the growth chamber, the chloroplasts were evenly distri-
buted along the cell and trabicular walls when tissue was examined by
light microscopy. After about two weeks in the growth chamber, the nee-
dles started to become chlorotic at the needle tips and then died in
several days.

In winter, the needles of Pinus contorta growing in the collect-
ing areas had changed from a summer green colour to yellow with only
a little pale green in the needle's coloration. This colour change
was most pronounced in needles with full sun exposures (the only needles
used in this work). A similar but less extensive colour change was not-
ed in the winter foliage of Pinus albicaulis and Chamecyparis nootkaten-
sis growing on Whistler mountain. A chlorotic coloration was also noted
in specimens of P. contorta ssp. contorta growing naturally in the "Her-
on Swamp" area of the University of British Columbia endowment lands in
mid winter. Marked winter colour changes were not noticed in the foli-
age of other conifer species growing in the collecting areas. It is
interesting to note that after several days in the summer conditions of
the growth chamber, the needles of previously frozen Pinus contorta ssp
latifolia foliage began to turn to the summer green colour.

The above experiments and observations indicate that after summer
conditions in a growth chamber for several days, the arrangement of the
chloroplasts at least begins to revert to the summer condition in pre-
viously frozen needles. Two fixations conducted in the field on March
the third, 1973 demonstrated that the winter cytoplasmic state is stable
at above freezing temperatures for at least several hours. Needles fix-
ed at dawn on this date were fixed at temperatures of between $-3^{\circ}C$ and
$-6^{\circ}C$. This fixation showed the normal winter ultrastructure for the
chlorenchyma.

Later in the day, a second fixation was conducted between two and
four p.m. when the air temperature was $+4$ to $+6^{\circ}C$. Due to the bright
sunshine and lack of wind, the temperature of the micro-environment about
the sun exposed needles which were fixed was probably considerably higher
than the ambient air temperature. The needles were fixed in solutions
in equilibrium with the air temperature. The afternoon fixed needles de-
monstrated a similar appearance to needles fixed at dawn except for the
appearance of small grains of starch in nearly all of the chloroplasts.
Normally, starch grains were seen only very rarely in winter fixations
conducted at dawn. On January the fifth, 1973, another afternoon fixa-
tion was conducted. This was a sunny day in which the temperature never
went above $-8^{\circ}C$. It is possible that the temperature in the micro envir-
onment of the needles was near or above freezing. No starch grains were
observed in the chloroplasts of material from this fixation.

Winter fixations of three conifer species at the collecting sites all demonstrated a similar unique winter cytoplasmic state. Winter at these collecting sites is characterized by near continuous below freezing temperatures. In early January, 1973, a fixation of <u>Pinus</u> <u>contorta</u> ssp. <u>contorta</u> growing naturally in the "Heron Swamp" region of the University of British Columbia endowment lands was fixed at $+3^{\circ}C$ and again a week later when the air temperature was $-2^{\circ}C$. Air temperatures in the area for the winter to the date of fixation contained several days of mild frosts and no temperature below $-6^{\circ}C$. The results of these fixations were poor but the cytoplasm of the needle cells of these trees resembled the summer state except for extensive oil droplets and some osmiophilic bodies. There was no extensive vacuolation of the cytoplasm and the chloroplasts were not clumped and contained small starch grains. The chloroplasts were removed from the cell wall by a narrow region of cytoplasm but the flat face of the plastids was orientated parallel to the cell wall. The position of the plastids was very similar to that shown in figure 76.

V. Vernal Changes in the Ultrastructure of the Chlorenchyma

Fixations were conducted in March and April in an effort to dem-
onstrate any possible cytological transition phase from the winter to
the summer condition. Tissues fixed on March the third (table 1) show-
ed the winter condition despite the fact that some of the material fixed
on this date was fixed at above freezing temperatures. Material fixed
on June the sixth (table 1) showed the summer condition despite light
frost in the area the night before.

The fixations conducted in the field on material collected on Ap-
ril the twenty-seventh, however, revealed a unique ultrastructural con-
dition which can be interpreted as an intermediate stage between the
summer and winter conditions. The chloroplasts were no longer in clumps
as noted in the winter fixations. Most chloroplast were dispersed about
the peripheral cytoplasm. These chloroplasts were located near or appress-
ed to the plasma membrane with the major sectional axis usually parallel
to the cell wall or trabiculae (figures 63 and 67). The remaining chlor-
oplasts were associated in small groups, (figure 65).

In the spring fixation, the distribution of chloroplasts in the
peripheral cytoplasm about the cell wall and trabiculae was not uniform
as observed in summer fixations. In the cytoplasm near the ends of the
cell near the long trabiculae, the chloroplast density was greater than
observed in summer. In other areas of the cells, particularly in the
central regions of the cell lying in the transverse plane of the needle

(central part of face A, figure 2), chloroplasts were rare, In regions
where the chloroplast density was high, chloroplasts usually formed a
uniform tier along the plasma membrane with several additional chloropl-
asts lying deeper in the cytoplasm (figure 63).

Some of the chloroplasts distributed near the plasma membrane were
discoid in shape with the side proximal to the plasma membrane being
more flattened (figure 66). These chloroplasts appeared to be very sim-
ilar to summer chloroplasts. Most of the chloroplasts distributed furth-
er from the plasma membrane and nearly all of the chloroplasts associa-
ted in small groups appeared irregular in shape (figures 65 and 67).

The irregular sectional appearance of some of the spring chloropl-
asts is similar to the winter chloroplasts. However, in the spring fix-
ation, the irregularly shaped chloroplasts do not appear to have the
extensive stromal areas as seen in the winter chloroplasts (figure 63).
The irregular shape of the spring chloroplasts appears to be due rather
to the twisting of the internal membrane systems. No spring chloroplasts
were observed to have more than one internal thlakoid membrane system
(figure 57).

Most spring chloroplasts had plastoglobuli that appeared generally
smaller and slightly less numerous than in the winter condition. How-
ever, in nearly every section studied, there were certain chloroplasts
that were noted for the very large masses of plastoglobuli, which appear
in dense clusters, (figure 65).

In regions of the spring chlorenchyma where the chloroplast density was high, the chloroplasts lining the plasma membrane and those lying deeper in the cytoplasm were often in very close proximity. High magnification studies of these areas revealed a consistent 75nm to 150 nm wide space between the outer membranes of adjacent chloroplast (figures 63, 64 (high magnification of boxed area in figure 63), and open arrows in figure 65). There is no apparent contact between the outer membranes of the chloroplast envelopes of adjacent chloroplasts. Small starch grains were noticed in many chloroplasts in the spring fixation (figures 62 and 64).

The tannin vacuoles remained partially contracted and generally circular in sectional profile and the tonoplast could not be resolved. Thus the tannin vacuoles appeared similar to the winter state. The peripheral cytoplasm still occupied a greater volume of the total cell than that usually observed in the summer, but not as great as that observed in the winter condition.

The condition of the ground cytoplasm however, appeared more similar to the summer condition than the winter state (figure 67). Cytoplasmic vacuoles were present but were widely scattered and not associated with osmiophilic bodies. The occasional osmiophilic body was found scattered in the cytoplasm and near the cell wall (open arrows, figure 67). The majority of osmiophilic bodies present in the spring cytoplasm had a different morphology than that observed in the winter condition. The osmiophilic bodies were often very large (figure 64,

arrowheads) or consisted of localized aggregates of smaller bodies (figure 68 (0')).

The mitochondria were generally located close to the chloroplasts in the spring fixations (figure 63 and 67). The mitochondria had ovoid to irregular sectional profiles (figures 63 and 67) and appeared very similar to the description of summer mitochondria in type II cells. Dictyosomes were not common and rarely seen in the spring fixation.

The nuclei in the spring fixation were located either near the ends of the cells in the peripheral cytoplasm (similar to the winter position) or in a more central location in the cell usually at the end of a long trabiculum (figure 66). When located in a more central location, the nucleus was located in an indentation into the tannin vacuole similar to what was observed in the summer fixations. All spring nuclei had a generally circular sectional profile. The nuclear membrane and the internal structure of the nucleus appear very similar to summer nuclei (figure 66). Nucleoli were observed in the spring fixation but detailed observations of their structure were not made.

The number of oil bodies within the cytoplasm was greater in spring than any other season. Oil bodies were located near the nucleus (figure 66) and also extensively throughout the rest of the cytoplasm (figures 67 and 68).

VI. Experiments on Pine Seedlings in Controlled Environments

A. Artificial Frost Hardening: Ultrastructural Observations

Tumanov (1967) and Saiki (1973) elaborate on their earlier studies which established the conditions necessary for the artificial induction of frost hardiness in certain hardy woody plants. Seedlings were grown under optimum conditions until they reached the equivalent of two or three years of field growth. The seedlings were artificially hardened following the procedure outlined in materials and methods. All seedlings survived freezing conditions when artificially hardened but sustained considerable damage.

Ultrastructural observations on needle chlorenchyma revealed some features characteristic of the winter condition. The tannin vacuole had contracted to a similar extent. Some osmiophilic bodies were found but not to the same extent as in winter cytoplasm. These bodies were also associated with the extensive cytoplasmic vacuolar membrane systems within the cytoplasm (figure 73). The cytoplasmic vacuoles were usually larger than those observed in the winter condition. The chloroplasts were not aligned along the cell walls. They were instead scattered in the cytoplasm. They are often irregular in shape and many grouped into clusters of between two and five chloroplasts. The outer envelope membranes in the clumped chloroplasts were in close contact (figure 74). These small chloroplast groups are not equivalent to the extensive clumping chlaracteristic of the

winter condition. The nucleus appeared to be similar to the summer con-
dition, and no significant build-up of large perinuclear lipid bodies
was observed.

Long term damage was observed when the seedlings were returned to
summer conditions in the growth chamber. The youngest and the oldest
needles became chlorotic, particularly at the tips and bases within a
week after freezing. Many of the apical buds failed to open. Figure
75 shows a seedling (A) which was given the hardening treatment. The
seedling was still alive four months later, although more than half of
the needles on the tree were chlorotic and the youngest needles had
yellowed (light areas shown by arrow) and later died. The youngest
needles were shown in figure 71.

Tree B in figure 75 was not given any hardening treatments (photo-
periodic and low temperatures) before being placed in the freezer. After
being placed in the freezer, this tree quickly died. Ultrastructural
observations on the needle chlorenchyma of the control seedling (tree B)
which did not receive pre-freezing hardening treatments revealed that
the chlorenchyma fine structure was destroyed.

B. Summer Condition Trees Exposed to Drought: Ultrastructural Observations

The nature of the cellular ultrastructure of the needles was briefly investigated to see if there is any correlation between the drought exposure condition and frost-hardiness states. The tannin material of drought exposed summer needles seems to be reduced in volume as in winter conditions, coinciding with a considerable increase in cytoplasmic volume (figure 76). The chloroplasts however, remained evenly distributed along the cell wall but had moved slightly away from it (figure 76). Chloroplasts with constrictions similar to those observed in some cases in winter (figure 44) were occasionally observed (figure 77). Approximately the same number of osmiophilic bodies were present as in the late summer fixations under field conditions. There was development of cytoplasmic vacuolar systems, (these were more extensive than in normal summer fixations but not nearly as extensive as those seen in the winter condition, figure 76). The bulk of the ground cytoplasm in the drought condition consisted of lightly staining homogeneous material with very few ribosomes or other inclusions. Mitochondria were few and circular in profile, and small oil droplets were seen. The nucleus appeared similar to that seen in the summer condition.

One seedling tree that had been exposed to long day photoperiods and warm growth chamber temperatures while not being watered for thirty days was then watered once and placed in the artificial hardening regime described previously. This seedling was given the same freezer frost exposure as described previously for seedling A. The drought exposed

seedling survived the frost exposure but subsequent observation revealed

it had received about the same amount of damage as tree A. Fixations

on the needles of this tree were not successful.

DISCUSSION

I. Discussion of Technical Problems and Procedures

A major portion of this work was spent on developing preparative techniques for electron microscopy which would generate reproducible ultrastructural observations. It is hoped that the reporting and discussion of the technical problems dealt with in this work will assist future studies on conifer tissue.

The fixation of mature conifer chlorenchyma for ultrastructural studies is very difficult during the growing season. Testimony to this is shown by the fact that nearly all published ultrastructural studies on mature conifer chlorenchyma make reference to fixation problems; (Parker and Philpott, 1961 and 1963; Harris 1971; Campbell, 1972, and Chabot and Chabot, 1975). Most of the above authors speculate that the prolems stem from the interference with fixation chemistry by the resinous and tanniniferous material in the mesophyll tissues or in the mesophyll chlorenchymal cells.

A major initial problem was found to be related to the method of cutting and subdividing the needle for fixation. In the early stages of this work, the flat surface of the needle (adaxial side) was placed on a plastic petri dish, flooded with fixative solution and subdivided

into small transverse segments. In this procedure, the razor blade used to cut the needle did not initially slice and penetrate the epidermal-hypodermal layer effectively. This resulted in the crushing and mechanical damage to the delicate mesophyll chlorenchyma situated between the tough but flexible epidermis-hypodermis, and the tissues of the endodermis and the vascular tissue.

The problem was aleviated by the use of higher quality stainless steel razor blades and cleaning them in an ultra-sonicator in acetone rather than by hand. The latter treatment removed oil films from the blades without damaging the knife edge. Turning the needle on its side at right angles to the flat side of the needle also helped to prevent mesophyll damage. The flat surface of the needle (adaxial side) was now at right angles to the petri dish surface so that the strong and supportive epidermal-hypodermal layers were at right angles to the razor blade, thus supporting the chlorenchyma from the knife pressure rather than crushing.

The addition of formalin to the primary fixative solution resulted in a rapid and detectable hardening of the chlorenchyma so that transverse sections of the needle could be further subdivided after about one hour of primary fixation. The general hardening of the mesophyll lessened the problem of the knife crushing the material.

The best results were obtained when the secondary subdivision was

carried out by dissection and removing as much of the endodermis and hypodermal-epidermal layers as possible from the mesophyll. The removal of these comparatively impermeable layers was found to facilitate the infiltration of the fixative and embedding media. Esau (1965) noted that the pine needle surface is characterized by a thick cuticle and that endodermal cells may be lignified and contain suberin. Thus the dissection of these tissues from the mesophyll probably freed an additional one or two facets of the tissue block for fixative, dehydration, and embedding media penetration. The 0.5 mm to 1.0 mm tissue pieces still required sixteen hours of post-fixation in osmium tetroxide at room temperature to achieve an uniform osmification of the tissue block. Harris (1971) also advocated similar long secondary fixation periods. The removal of sclerenchymous cells also aided in the trimming and sectioning of the plastic embedded material.

With respect to the quality of ultrastructural preservation, fixations with glutaraldehyde as the sole primary fixative were found to be inferior to a mixture of glutaraldehyde and freshly prepared formalin. Harris (1971) did achieve adequate fixations with glutaraldehyde as the only primary fixative, but Campbell (1972) and Chabot and Chabot (1975) both used a mixture of the two aldehydes as primary fixative on conifer tissue. Srivastava and O'Brien (1966) suggest that the resins in Pinus cambial tissue bind and precipitate glutaraldehyde which may disrupt the fixation of the tissue.

Fixations of older (one or more years of age) summer needles showed

the best results when acrolein was added to the primary glutaraldehyde fixative solution. Campbell (1972) also obtained the best results with acrolein/glutaraldehyde primary fixatives on mature pine needle tissues. Acrolein differed from glutaraldehyde/formalin fixative in that it tended to make the cytoplasm more osmiophilic and also often altered the tannin material substructure when used as a primary fixative with glutaraldehyde (figures 69, 70 and 68). (More studies should be conducted on the use of this fixative for conifer tissues).

Campbell (1972) also used potassium permanganate solution successfully as a fixative on pine needles but only published one figure of chlorenchyma prepared with this chemical. It was a high magnification photomicrograph showing only part of a chloroplast. Parker and Philpott (1961 and 1963) also used permanganate as the sole fixative on Pinus chlorenchyma with poor results. Attempts to use potassium of barium permanganate as a fixative in this project failed because the material in the plastic become so hard that the blocks could not be trimmed or sectioned. A possible reason for this apparent contradiction was the use of barium and potassium permanganate as a secondary fixative in this work after initial aldehyde fixation. The above authors used permanganate only as the sole fixative, a procedure not attempted in this work.

The experimental use of glycol methacrylate (Feder and O'Brien, 1968) and Epon resins for embedding pine needle tissue resulted in the consistent failure of these resins to be properly polymerized within the tissues despite extremely long dehydration and infiltration procedures.

The failure in polymerization appeared specifically to be located in the tannin vacuoles. The embedding resins in the tannin vacuoles remained semi-liquid, while polymerization was adequate in the cytoplasm and extracellular spaces.

Spurr's resin, (Spurr, 1969), gave adequate polymerization only after a long dehydration and infiltration series. Campbell (1972) was apparently successful in using Epon resin while Harris (1971) and Chabot and Chabot (1975) both used Spurr's resin.

Osmium, and to a lesser extend, the plastic resins, react with the contents of the tannin vacuoles of the needle chlorenchyma to form an extremely hard substance which is very difficult to section. This material often visibly shatters glass knives. Good sections for light or electron microscopy could only be cut simply from newly broken and extremely good portions of the knife edge. The fact that osmium is involved with the formation of this very hard material was deduced from the fact that aldehyde fixed material was considerably easier to section.

The types of electron microscopic preparative procedural difficulties described above generally increased with needle age during summer fixations. In winter fixations. however, very few preparative difficulties were encountered. For example, long fixation and embedding procedures could be greatly reduced without detectable changes in ultrastructural preservation. Similarly, it was not necessary to subdivide or dissect tissue blocks as extensively as in the summer fixations. Winter

needles from six months of age to five years of age were fixed during winter but no relationship between needle age and ultrastructural preservation was observed in marked contrast to the observations on summer tissue. In the winter fixations, plastic embedding media other than Spurr's resin and permanganate fixatives were not used.

In an attempt to reduce the possibility of artifacts caused by thawing frozen tissue or by chemical fixation and embedding procedures, the techniques for cryomicroscopy were developed. The techniques employed are based on the principle that an _in situ_ needle in mid-winter at the collecting sites (with temperatures of $-10^{o}C$ to $-15^{o}C$) exists in almost a completely frozen state. The bulk of free water in needle tissue at these temperatures will be crystalized, (Kaku, 1975). The object of the study of frozen tissue was to find an efficient way to collect, transport, store, and process the material for light microscopy while maintaining the tissue temperature between $-10^{o}C$ to $-15^{o}C$.

An important limitation to the low temperature techniques was the difficulty in monitoring temperature at all stages of the preparative procedure: from the field to cryomicroscopy. In lieu of accurate temperature measuring and control systems, the tissue was consistently kept as cold as, or colder than the field temperature. The use of liquid nitrogen as the coolant material at several stages of the preparative procedure resulted in temperature difference of as much as from $-10^{o}C$ at collection sites, to a possible minimum temperature of $-196^{o}C$.

These fluctuations probably would not have affected the structural and positional nature of bulk tissue ice. It is also difficult to envision how the structural components of the cell could significantly rearrange at these temperatures.

Sakai (1966 and 1973) has demonstrated that extremely hardy living woody tissue can survive temperatures as low as $-196^{\circ}C$, provided they had been initially exposed to temperatures of $0^{\circ}C$ to $-40^{\circ}C$ for several hours previous to immersion. He also demonstrated that after the initial frost exposure, various rates of freezing usually did not cause damage. Similar studies have been cited by the Russian authors (Tumanov, 1967).

In this study, freezer stored needles that had been collected in mid winter were dropped into a liquid nitrogen vat and then removed and fixed at $-4^{\circ}C$. Very little change from the normal winter ultrastructure was observed (figure 62). These observations are the basis for the conculsion that the observations from material processed by cryo-technique represent a very close condition of the in situ cytoplasmic arrangement in the frozen cell.

It would have been desirable to do more detailed studies on winter cells in the frozen state, but the construction of a cryomicroscope that could give direct control of stage temperature for freeze-thaw studies is very complex and expensive (Diller and Cravalho, 1971). However, the cryo-

tome, cryomicroscope and handling apparatus used for this study were adapted from simple materials and equipment commonly found in most ultrastructural research laboratories.

The needle chlorenchyma of Pinus contorta ssp. latifolia was chosen as the major experimental material in this work due to several properties which simplified and assisted seasonal studies on frost hardiness. One method of highlighting seasonally related cytological changes is to study organisms which demonstrate extreme cases of the seasonal phenomena. Pinus contorta ssp. latifolia specimens collected in the subalpine forest of Wyoming and Montana were tested by Sakai and Weiser (1973) for the degree of the tree's frost hardiness. These authors have placed P. contorta ssp. latifolia in the most "hardy" catagory of North American woody plants. Their specimens were able to withstand at least -80°C in winter. In this work, Pinus contorta ssp. latifolia was sampled at the western edge of the interior plateau of south western British Columbia at an altitude of four thousand feet. At the sample locations, the coastal influences are reduced and the climate tends to be more of the high latitude continental type. At the sample locations, P. contorta is a major member of the interior plateau subalpine forest (Critchfield, 1957).

Tsuga mertensiana is found in a more moderate climatic zone in the coastal subalpine forests of British Columbia, (Sudworth, 1908). However, specimens used in this work were collected near timberline at five thousand feet in altitude on Whistler mountain which is a site on the coast

range, somewhat removed from the moderating influences of coastal regions.

Pinus albicaulis is often the treeline species of the coastal forests of south western British Columbia and grows in very exposed severe locations (Sudworth, 1908). Trees sampled in this work grew at about 5,700 feet on Whistler mountain and were found in full tree growth habits where other conifers were absent or reduced to low lying krumholtz growth habits.

Sakai and Weiser (1973) did not test the hardiness of Pinus albicaulis, but on the basis of the climate of collection sites, the tree must be very frost resistant. These authors did test Tsuga mertensiana and found it to be only moderately frost hardy, tolerating only $-40^{\circ}C$. However, these authors sampled Tsuga mertensiana close to sea level near Seattle, Washington.

When comparing the results obtained in frost hardiness studies with the results of other studies in the literature, care must be taken to ensure that the hardiness and growth conditions of the organisms being compared are well defined. Burke et al. (1976) cite evidence that frost resistant mechanisms may be different in slightly, moderately and extremely hardy plants. Harris (1971) studied Pinus strobus (an extremely hardy tree, Sakai and Weiser, 1973) but his seasonal observations were conducted on specimens growing well south of the tree's natural range on the campus of the university of Arkansas. Thus Haris's winter observa-

tions may have been very different if conducted in the tree's natural distribution range. The different parts of a woody plant may also differ in the degree of frost hardiness obtained, with exposed parts such as the needles of conifers usually obtaining the maximum hardiness levels (Parker, 1963).

An important concept to be considered in interpreting and comparing frost hardiness observations between different taxonomic plant groups is the possibility of differing frost hardiness mechanisms being employed for a given level of hardiness acquisition. The genus _Pinus_ is believed to have evolved in the higher latitudes of the northern hemisphere (Mirov, 1967) and the frost hardiness of most members of the genus is probably a very ancient trait. The angiospermous plants that dominate the planet today are believed to have been progressively expanding into more hostile high altitude and latitude regions since the Cretaceous time period (Regay, 1977). Thus the acquisition of frost hardiness has probably occurred many times in geologic time and is probably still occurring in genetically and range expanding taxonomic groups. These factors necessitate the use of caution when comparing frost hardiness studies between differing groups of plants, and has probably resulted in the frost resistance mechanisms of plants becoming a very complex phenomenon, particularly on a comparative basis.

The development, maintenance, and loss of frost hardiness is a seasonal part of the continuum of growth, differentiation, and senescence in plant tissues. The study of frost hardiness must take these phenomena

(which overlap seasonal cycles) into consideration. Experimentally, the pine needle is well suited for separating other cellular processes which may confuse frost hardiness studies because of a characteristic rapid growth phase followed by several years of maturity and functional activity, before senescence becomes a dominant factor.

Sudworth (1908) observed green needles of at least eight years of age on young *Pinus contorta* trees. Needles which were green and healthy in appearance up to twelve years in age were observed on young open grown trees of *P. contorta* at the sampling sites. The age of needles on young *Pinus* sp. can be simply determined by counting long shoot internodes from the terminal buds to the internode on the branch or axial trunk on which the needles occur (Dickmann and Klozlowski, 1970). The occurrence of lammas or prolypsis growth forms would invalidate this method of needle age determination, but no examples of these growth forms were observed. Lammas forms of growth have been reported in *Pinus divaricata* (*banksiana*) (Rudolph, 1964), (which is a hybridizing close relative of *P. contorta*, Critchfield, 1957), in the Great Lakes area of eastern North America.

The common occurrence of very old needles on young open grown trees at the sampling sites appears to be dependent on continued high growth rates and insolation during the life of the needle. The open grown, well spaced young trees at the sampling sites had very large annual long shoot growth increments. This resulted in internode distances of usually greater than 20 cm, which when combined with a lack of crowding

and shading between individual trees, gave the crown a very open growth form and lessened the shading of older needles by recent growth resulting in the continued insolation of older needles.

The growth of new needles was observed to start in early June at the collecting sites with the emergence of short shoots and rapid long shoot expansion. By the end of July, that season's needles had reached the length of older mature needles. Mirov (1967) has observed special cases in which needle elongation continues for a year or more in tropical and sub-tropical pines. This growth appears to be due to the activity in the basal meristem of the needle (Esau, 1965). Cell division does not occur in the apical mesophyll regions of fully expanded needles observed in this work. Residual activity in vascular cambia within the stele throughout the life of the needle would also not affect the mesophyll since it appears to solely to propagate vascular tissue (Huber, 1947).

Cytologically, the mesophyll chlorenchyma of P. contorta needles in the period of needle expansion were observed to have the characteristics of cells with high metabolic activity relative to cells of older needles. This cell type was classified as the type I cell form in this work.

After needle expansion was completed, the size and complexity of mitochondria were reduced and the occurence of dictyosomes, dictyosomal vesicles, RER and SER were greatly reduced. This cell type was

termed the type II cell. It is concluded that the type II cell repres-
ents the transformation of the cytoplasm from a state of growth (type
I cell) to a state of mature functional activity (type II cell). The
type II cell was found to be the dominant cell type in the needle chlo-
renchyma in current season needles fixed in July and August and in one,
two, three, and five year old needles fixed between early June and late
August (see table I).

A third cell type was identified and called the type III cell. The
incidence of this type of cell increased in proportion to needle age.
This cell type was interpreted to be a senescent or degenerating cell
type on the basis of cytological properties (Butler and Simon, 1971) and
was characterized in this work only to insure that it would not be con-
fused with seasonal changes.

The cytological stability of the type II cell and the correspond-
ing long needle life span made the needle material very useful for
studying seasonally related phenomena since the same type of tissue can
be sampled before, during and after winter through several yearly cycles.
In contrast, the majority of the cytological studies on the frost hardi-
ness phenomena in woody plants have been conducted on the vascular cam-
bium (e.g. Murmanis, 1970) or secondary stem parenchyma (Siminovitch et
al.,1968) in which individual cells have a life span of one to two
years, during which growth differentiation and senescent processes must
be segregated from cytoplasmic changes solely dependent on seasonal fac-
tors.

II   The Discussion of Seasonal Changes in the Cytoplasm of Needle Chlo-

     renchyma

After establishing what was occurring in the chlorenchyma cells with

regards to growth, maturity and senescence, it was then possible to pro-

ceed with the main objective of this work which involves the study of

the seasonal changes in the cytoplasm of the needle chlorenchyma of P.

contorta.

It has been established that the type II cell was the predominant

cell type observed in all fixations conducted between June 6th and August

10th (table I). The cytological consistency of this cell type has led

to the term "the mature summer state" being applied to describe type II

cells. Fixations conducted during the winter of 1972 to 1973 (November

26th to March 30th) revealed a completely different cytoplasmic state.

Like the summer state, equal ultrastructural consistency is observed in

all fixations of all needle ages examined. The remarkable different cy-

toplasmic state in winter is termed "the winter cytoplasmic state". The

winter state is believed to be the form of the summer type II cell dur-

ing the winter frost hardy state between November 16th, 1972 and March

30th, 1973.

A number of winter state cells, (proportional to needle age) were

believed to be the winter equivalent of type III summer state cells.

The more intensely osmiophilic winter equivalents of type III cells ap-

peared identical to their summer counterparts. The less osmiophilic type

ill cells that had some recognizable organelles and had some of the cyto-
logical properties of the winter state type II cells. These observations
have led to the conclusion that the summer and winter cytoplasmic states
are completely reversible on a seasonal basis. Thus the summer and wint-
er "states" are seasonal transformations of mature functional mesophyll
chlorenchyma cells which have an unusually long life span for cells with-
in the plant kingdom.

In the winter cytoplasmic state, almost every cytoplasmic structure
and organelle has changed structurally or positionally. So different and
unusual is the winter cytoplasm compared to summer fixations and publish-
ed photomicrographs of mature plant tissue that the author believed init-
ially that the winter state was a fixation artifact.

The most dramatic change in the winter chlorenchyma is the structur-
al and positional changes in the winter chloroplasts. The aspect of sea-
sonal change in chloroplasts of winter evergreens has received much atten-
tion in the early literature. Mohl (1837) noticed the seasonal chlorosis
in some winter conifers and suggested that organic acids destroyed
·the pigments in assimilatory cells but the pigment bodies (chloroplasts)
remained intact. Kraus (1874), Haberlandt (1876) and Schimper (1885) no-
ticed the clumping of chloroplasts in the needles of several winter coni-
fers. However, in herbs, non-coniferous deciduous and evergreen woody
plants, the behavior of chloroplasts or plastids was highly variable. In
some species, these authors reported no change, some slight movement from
summer positions, clumping, or chloroplast disappearance. They also re-

ported great variations in chloroplast changes within individual leaves. However, Haberlandt (1876) particularly believed that in most over wintering plants, chloroplasts were not destroyed.

Lewis and Tuttle (1923) observed chloroplast clumping about the nucleus in P. glauca in the fall and by early winter, with frosts of increasing severity, the chloroplast clumps disintegrated and disappeared. They used the term "laked" (apparently an old medical term more or less synonynous with lysed) to described this disintegration. Zacharowa (1929) observed the winter break down of chloroplast after an initial clumping in both Pincea and Pinus species, and used the term "zerfallen" (German: fallen apart) to described this process. Holzer (1958) conducted detailed observations of chloroplast clumping in the cytoplasm between the trabiculae in Pinus and in the parietal cytoplasmic regions of the cells in other conifers. His observations appear to be very similar to those reported in this work. Unfortunately, Holzer's work is not known in the general literature.

The controversy over the fate of winter chloroplasts continued when both Gerhold (1959) and Perry et al. (1965) published papers which indicated a continued acceptance of the possibility that the chloroplasts in conifers may break down during winter. Harris (1971) failed to notice any winter clumping in the chloroplasts of P. strobus but as stated earlier, his observations were conducted in a sub-tropical location. Parker and Philpott (1961 and 1963) published ultrastructural photomicrographs of winter chloroplasts in Rhododendron and Pinus with the express purpose of disproving the chloroplast destruction concept. They also reported exten-

sive clumping of the chloroplasts in winter fixations of <u>Pinus</u> needles and bark parenchyma.

In this study, chloroplast clumping was regularly observed in all the winter fixations of the conifers examined. It was also observed later by Chabot and Chabot (1975) in <u>Abies</u>. No evidence for chloroplast destruction or a decrease in their relative numbers was seen in this work, nor it was mentioned by Chabot and Chabot (1975) or by Parker and Philpott (1961 and 1963). It appears from these studies that the destruction theory concerning winter chloroplasts was either an observational error and/or the result of plastid destruction during preparation for observation.

The structural nature of clumping of winter chloroplasts is best revealed with electron microscopy. High magnification studies of the regions of chloroplast contact reveals what may be a tripartite layering of the envelope membranes (figures 45 and 46). This structure is interpreted to indicate a fusion of the outer membranes of each adjacent chloroplast envelopes to form a structure consisting of two attached membranes. The fused outer envelope membranes are believed to make up the central thick component of the tripartite structure. The inner membranes of the envelope of each plastid constitutes the outer two thinner membrane components of the complex. Parker and Philpott (1961 abd 1963) did not discuss the fine structure of the chloroplast envelopes within the chloroplast clumps and their published photomicrographs are of too low a magnification to determine the structure of adjacent envelope membranes.

Considerable interest is now focused on the fusion of living proto-
plasts of different cells from similar organisms or completely different
organisms. Besides removal of the cell wall (if present), an apparently
essential prerequisite to successful fusion is the treatment of the cell by
some mildly toxic material to induce the protoplasts to fuse. Such methods
involved treatment with sodium nitrate(Power and Fleming, 1970) radiation, vi-
ral inoculation, specific antisera (to the membrane proteinous components),
high pH, partial and brief lysozyme digestion, polyethylene glycol and poly-
L-lysine, and probably several more methods have been found (see Burgess
et al. (1974) for references to the above).

Douglas (1974) cites many cases in which calcium is involved in the
process of the membrane fusion in the exocytosis of cytoplasmic vesicles
and the fusion of plasma membranes. Schober et al. (1977) has observed that
the aggregation of isolated chromaffin granules occurs in areas where there
has been a calcium induced area on the granule membrane that is free of
surface particles.

What these treatments and observations appear to have in common is that
the site of action by these treatments is the plasma membrane. Ultrastr-
uctural studies of the adherence zones seem to indicate the areas of fusion
occur where the apposing plasma membranes of adjacent protoplasts come into
very close contact (Burgess et al., 1974). Subsequent to this, the fusion
of protoplasts occur. Similar process may apply to the fusion between Pinus
chloroplasts which lead to the mixing of the stroma of neighboring chloro-
plasts. The existence of separate systems of thylakoids within a common
stroma seems to support this.

Two questions arise out of the observation of chloroplast clumping. The first is what brings the chloroplasts into close contact and the second is what may be happening at the surface of the outer envelope membranes to allow their close association. The cause of clumping and close apposition of outer envelope membranes is believed to be the result of frost plasmolysis and will be discussed later.

The second question concerns what may be happening at the membrane surface. Most membranes in apposition to each other in Eukaryotic cells are characterized by an approximately 40 nm or more gap between surfaces which result in regular parallel spacing of the membranes. Examples of such spacings would be golgi cisternae, algal thylakoid systems, nuclear and chloroplast envelopes etc., and the spacing between animal tissue cells. This gap is probably a balance between adherence promoting forces and mutual repulsive forces on the apposing membrane surfaces that cause this structural arrangement. Repulsive forces are the net negative charges associated with membrane surface proteins (Power et al., 1970), and/or the strong bonding due to the hydrophilic phase of the polar ends of the membrane lipids and the aqueous phase of the cytoplasm. The adherence forces that maintain a regular parallel spacing, and which prevent the membrane surfaces from drifting apart may due to the cross bridges formed by proteinacous bridges between the parallel membranes (Franke et al., 1971).

The chemical, physical and biological treatments described above for the induction of protoplast fusion appear to affect mainly the repulsive forces between cells in order to promote closer membrane association. Rad-

iation, enzymatic digestion and positively charged electrolytes are probably targeted towards membrane outer surface proteins; while the poly-alcohols may affect proteins and/or hydrophilic lipid interactions. Similarly, antisera would be targeted to membrane surface proteins. Calcium may neutralize surface repulsive charges on membrane proteins thus allowing them to clump and open protein free areas. Schober (1977) supports a theory suggest by Ahkong et al. (1975) that before membrane fusion can occur, membrane surface proteins must be displaced so that the lipid bilayers of adjacent membranes can come into close contact.

The common inference from all the above procedures for promoting membrane fusion is that the target site of the agents used is the membrane surface proteins. Membrane surface proteins (ectoproteins) are now known to be largely glycoproteins and are proteins specific to the external plasma membrane surfaces (Rothman and Lenard, 1977). It can be suggested that the glycoproteins of the chloroplast outer enveloped membranes are damaged or altered in some manner during winter.

In this work, one of the few structural changes observed with the sea water electrolyte fixative on water tissue was the marked reduction of chloroplast adhersion zones. This may have resulted from some interaction between the salts in the fixative solution and the physical properties of the membrane surface proteins.

The observations on the fine structure of the envelope membranes of clumped winter chloroplasts in this work have not been reported in the lit-

erature.   Parker and Philpott (1961 and 1963) did not discuss the fine str-

ucture of chloroplast envelopes in their observations on the clumped wint-

er chloroplasts of <u>Pinus</u> <u>strobus</u>. Their published plates of clumped chlo-

roplasts are of quite low magnification.   However , their photographs ind-

icate that close association between outer membranes of adjacent chlo-

roplast may occur.   Chabot and Chabot (1975) mentioned chloroplast clumping

in <u>Abies</u> sp. but did not publish a plate showing clumped chloroplasts or

discuss the fine structure of the envelope membranes of clumped chloroplasts.

Only the chloroplasts exhibit the clumping and close outer membrane association

properties. Mitochondria were often in loose clusters but never were ob-

served to adhere.   Similarly, there was never any adherence between chloro-

plasts and other organelles in winter fixations.   It can be concluded that

clumping of winter chloroplasts and the fusion of their outer membranes in-

dicate that the chloroplasts outer envelope membranes have differing chemi-

cal properties than other organelle membranes during the winter.

It was observed that about ten to thirty percent of all winter chloro-

plasts had two or more thylakoid systems within a common envelope based on

observations on thin sections. This indicates that in the reality of three di-

mensional space, the majority of the winter plastids may be double systems.

Harris (1971) and Chabot and Chabot (1975) did not report any double chloro-

plasts in winter conifer tissue but Parker and Philpott (1963) did mention

that "division like constrictions" were noticed in observations during the

fall on the chloroplasts of <u>Pinus</u> <u>strobus</u> but not during winter.   From these

authors' description of the divisional structures, the plastids they observed may have looked like the chloroplasts in figure 44. It seems reasonable for Parker and Philpott to have concluded that the chloroplast's narrow constrictions similar to those observed in figure 44 could represent pre-divisional structures. However, in this work, the chloroplasts in figrue 44 were atypical, and the appearance of most winter chloroplasts appear to be more likely the result of fusion for the following reasons:

1. Membrane adherence in close association may be a prerequisite for fusion (Ahkong et al., 1975).

2. For division by constriction to occur, one would expect to find contrictions of, or separations in the internal thylakoid system as well as in the envelope membranes. But although thylakoids are visible between plastids constrictions in figure 44 (open arrow), they were not observed to be in the process of separating or pinching apart. Chloroplast thylakoids could also greatly elongate before constriction division. If this were the case, one would expect chloroplast with very long thylakoid systems would have been observed in this work. No such structures were seen.

3. The thylakoid systems of double chloroplasts are all of the same morphology and size as in normal mature chloroplasts. No smaller systems than would be expected from constriction division products were observed.

4. It would be unusual to expect chloroplast divisions to be occuring in the dormant winter state.

5. The thylakoid systems within the common envelopes were oriented at various angles to each other and often had large areas of stro-

ma separating them (figure 43). In post division products, one would expect to find some thylakoid membrane systems partially attached or at least arranged in the same plane.

On the basis of this discussion, it seems more likely that the multiple thylakoid system chloroplasts were the result of fusion between mature plastids.

Apparent fusions between chloroplasts have been observed in a natural population of the hydrophyte Mimosa pudica (Esau, 1972) and in radiation induced mutants (Allen et al., 1972). These authors also cited several other cases where radiation has induced chloroplast fusion in differing plants. Radiation has also been used to fuse plant protoplasts as discussed above. Furthermore, chloroplasts in the radiation exposed prothali of a fern used by Allen et al. were observed to clump together after radiation exposure and before the fusion process. Initially, aggregate chloroplasts in radiation exposed fern prothali consist of many individual, normal, discoidal appearing thylakoid systems. But the individual thylakoid systems appear to eventually fuse into large membrane masses (Allen et al., 1972). This does not occur in winter pine chlorenchyma. The thylakoid systems appear identical to the summer thylakoid systems both in shape and in individual membrane structure. It is interesting to note that the thylakoid membrane of the winter chloroplast are the only winter membranes that are structurally similar to summer membranes.

By the virtue of their transformation from discoidal to approximately spherical shapes, the winter chloroplasts increase substantially in volume.

This conclusion was reached by transposing sectional areas into volumes which indicated that winter chloroplasts (not including the fused chloroplasts) are about one and one half to two times the volume of summer chloroplasts. This volumetric increase was found to be due to the increase in the stromal areas of the chloroplasts. A simple increase in stroma probably also accounts for the change in shape. The stain density of chloroplast stroma in winter remains the same as those of summer chloroplasts. Thus the volumetric increase due to additional stroma may indicate a substantial augmentation of the stromal phase of the chloroplasts. Heber (1959) has noted a considerable increase in soluble protein and sugars in the chloroplasts of hardy plants. The swelling of chloroplasts into spherical forms is observed in nearly all reports on the cytology of frozen plant cells (Heber, 1959 and Chein and Wu 1965). In non hardy species, the swelling also occurs even at slightly above freezing temperatures (Kimball et al., 1973).

In conclusion, dramatic changes in winter chloroplasts in pine chlorenchyma appear to be cytologically due to: their translocation into clumps, in physical properties of the chloroplast envelope membranes, the large increase in stromal volume and the possible augmentation of stromal contents. The thylakoid system appears to retain a similar form to that of the summer chloroplasts.

A definite summer-winter-summer cycle was also established for the mitochondria in the pine chlorenchyma. In summer, the mitochondria appear associated with organelles with which they may have a functional relationship, i.e., chloroplasts and oil bodies. In winter however, the mitochondria are

dispersed in the expanded cytoplasm and in loose clusters about the chloro-
plast clumps. They cannot be said to be consistently associated with any
definite structures such as oil bodies, although this was occasionally ob-
served (figure 58). Chabot and Chabot (1975) also noted a general dispersion
of winter mitochondria in Abies. The shape and volume of winter mitochon-
dria also changes from larger and irregular forms (in summer) to the smaller
and spherical form in winter.

Catesson(1974) also described a seasonal reduction in the mitochondria
of maple secondary cambia. Mitochondria became much simpler and more uniform
(usually ovate) in sectional shape in winter as opposed to larger and more
complex forms in fall. In early spring, he noted an increase in size and
complexity in shape. But there was a return to simple shapes and small size
in late spring and early summer. This second transformation was not observ-
ed in this study.

Mitochondrial membranes become amorphous and indistinct in winter (fig-
ure 53) in both the envelope and the cristae. The obscuring of structure,
the random distribution of mitochondria, and the reduction in volume may be
correlated to the minimal amount of respiration observed in over-wintering
conifers even when they are briefly raised to above freezing temperatures
(Clark, 1961).

Recently, microbodies have been reported in the mesophyll chlorenchyma
of needles up to six months old (November of the year of needle flush) in P.
strobus and P. mariana (Chabot and Chabot, 1974). No microbodies were observed

in P. Contorta. At the low magnifications, (at which most sections were studied), they could have been missed by interpreting them as mitochondria. No attempt was made to identify them by enzymic localization. As Chabot and Chabot (1974) noted the presence of microbodies in fat storing trees is very interesting, but they were only able to cytochemically identify catalase activity. Ching (1970) has demonstrated the presence of glyoxysomes in the oil bodies of germinating megagametophytes in P. ponderosa seeds. The presence of glyoxysome microbodies (if present in mature pine chlorenchyma) should be most apparent (based on the studies in this work) in the spring when it appears that the fat reserves of the chlorenchyma are mobilized.

The conifers appear to have a markedly stage by stage spring-summer growth cycle, with root growth, secondary cambial activity and shoot growth occuring at differing times (Kozlowski and Winget, 1964). The needles that survive winter play an important role in these growth phases. Extant needles supply fixed carbon for root growth initially in early spring. Later, the over wintering needles supply the secondary cambial growth (Gordon and Larson, 1970)and reproductive structures of their respective internodes with photosynthates (Dickmann et al., 1970). In early summer or late spring, one year old needles in particular, supply a significant amount of ^{14}C-labelled carbon to the late spring current needle flush (Loach and Little, 1973). The supply of photosynthates to the expanding needles may supply the fixed carbon necessary to compensate for the negative photosynthesis that occurs in expanding buds and needles (due to very high respiration; Clark, 1961) during the period of maximum current needle growth.

Older needles are not as photosynthetically efficient as current year
needles. However, Clark (1961) has shown that six year old <u>Abies</u> <u>balsamea</u>
needles still produce more than half the net photosynthesis of current
needles and that the one year old and older foliage of <u>Picae</u> <u>glauca</u> pro-
duce about 75% of the net photosynthesis of the tree* during the growing
season. If the shading effects on older needles by younger needles were
removed, the contributions of the older needles may be even higher. The
fact that the older needles retain an ability to expand their normal pho-
tosynthetic capacites has been domonstrated by deliberately destroying
current season needles (Neales <u>et</u> <u>al</u>., 1968).

The studies by Gordon and Larson (1970) indicated that ^{14}C-labelled
carbon was recovered in the expanding bud after being fed to one year
old needles in the spring before bud burst and also when fed to the one
year old needles (then only several months old) the preceeding autumn.
These authors estimated that ten to fifteen percent of photosynthates fed
to needles in the fall are recovered in the terminal buds just before
flush the following spring. Since starch is not stored in the needle over
winter (Clements, 1938; Little, 1961 and 1970; Chabot and Chabot, 1975; and obser-
vations in this work), the photosynthate must be stored in some other form.

In this work, oil bodies were found to gradually increase in size
during the summer in needles of all ages. By mid winter, the oil bodies
were a dominant feature of the winter chlorenchyma and appeared to be
unaffected by the seasonal cytoplasmic changes about them. The oil bodies

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*   This data is from the author of this paper's calculations based on
     data supplied in Clark's 1961 monograph.

did not appear to increase further in size during the winter but were much larger and more numerous in the spring fixation which was the seasonal maxima for oil in needles of all ages that were examined. Harris (1971) noted an increase in cytoplasmic oil bodies in November fixations compared to summer fixations of *Pinus strobus*. But Campbell (1972) did not mention any oil reserves in his studies on summer and early fall needles of *Pinus nigra*. Chabot and Chabot (1975) observed substantial increases in chlorenchyma lipid bodies in the winter fixations of *Abies balsamea*. Winter accumulations of lipid bodies are also found in the resting cambia of conifers (Murmanis, 1970; Itoh, 1971) and in angiospermous cambia (Roberts *et al.*, 1968 and Mia, 1972).

The common if not universal presence of large oil storage bodies in the secondary cambia of both conifers and angiosperms, as well as conifer needles during the winter, and the rapid dissipation of these oil bodies in the spring indicates lipid reserves are significant seasonal storage reservoirs of fixed carbon. The importance of this storage product is undoubtedly increased by the near complete absence of starch in the winter needles.

Labelled tracer studies cited above also indicated that the one year old and older needles mobilize winter storage reserves kept within the needle, plus current photosynthates to supply current season growth of all parts of the tree. These observations may contain a partial answer to the long standing question concerning the competitive advantage of evergreen trees versus deciduous trees. In this work, needles contained starch

reserves and larger oil reserves than seen in winter in the spring fixat-ion conducted on April the twenty-seventh, 1973. However, current needle flush of that season was just begining on June the sixth, 1973, while one year old needles observed on June the sixth had only a few oil bodies, char-acterisic of the summer state. This would indicate that one year and old-er needles had been photosynthetically active to some degree from April the twenty-seventh and that by June the sixth, they had largely expended their oil reserves. Since deciduous aspens growing at the collecting sites were only partially in leaf on June the sixth, Pinus contorta trees would have been photosynthetically active for up to a month before the deciduous trees of the area. Chabot and Chabot (1975) made similar obser-vations on Abies balsamea. These authors detected starch accumulation in early March, two months before new needle growth occurs.

The lysosomal system in higher plants has been described as a system involving cytoplasmic structural entities which maintain an effective compartmentalization of hydrolytic enzymes from the rest of the cellular system (Berjak, 1971). Berjak describes earlier work which have suggest-ed that the developmental sequence of structures that appear to have ly-sosomal properties in differentiating root tips leads to the eventual for-mation of the main or central vacuole as the cells mature. This has de-veloped into the concept that the vacuole is an integral but not the only component of the lysosomal compartment in mature cells.

In the mature conifer chlorenchyma, the main vacuole contains tannin material, a substance characterized by its ability to bind to and dena-

ture proteins. A tannin filled main vacuole would thus not be incompatible with the older but still valid concept of the main vacuole in mature plant cell being a "sink" (Devlin, 1969) for further non-metabolizable byproducts of cellular activity. The presence of tannin may be an answer to the problem of by-products removal from the cell solution in very long lived plant cells by binding and precipitating them. In this context, the observation that the homogeneity of the tannin material breaks down in five year old needles may be relevant. In older needles, the tannin material is characterized by a more heterogeneous appearance due to the presence of various osmiophilic inclusions (figure 67) which may indicate that the tannin may assist in inactivating cellular by-products.

If the tannin material within the tannin vacuole can be viewed as an inert and probable end stage of a cell's lysosomal system, then the persistence of residual ER in the cytoplasm about the nucleus (it was not determined if this was SER or RER in type II cells), dictyosomes and cytoplasmic vacuoles (some of which may contain membranous and possible organelle debris; figures 22. 23 and 25) suggest the active lysosomal compartment is confined to and operates within the cytoplasm. However, confirmation of this suggestion would require enzyme localization studies.

Observations on type II cells in the summer state indicate that the tannin material may be structurally removed from the tonoplast. Figures 22, 23 and 26 indicate that the tannin material contracts as a unit from the tonoplast. The shrinkage of the tannin is believed to be a fixation artifact since the amount of separation between tannin and tonoplast was

fixation dependent. However, in many cases, it was observed that the tonoplast invaginated into the cytoplasm forming an interdigitating complex sectional profile(figure 27). This unusual tonoplast conformation is more pronounced in oblique sections to the tannin vacuole (figure 28). This phenomena is believed to be unrelated to the tannin shrinkage and may represent the type of complex membrane pattern commonly associated with transport activity (Gunning and Pate, 1974). The interdigitation also provides areas where the tonoplast and lumen of the infoldings of the tonoplast are physically separated from the tannin. This type of structure may be the site of most of the enzymatic activity that would be expected by tonoplast and the main cell (tannin) vacuole. Enzyme localization studies would illucidate this suggestion.

In winter, the tannin material contracts to a greater extent than observed in summer. This contraction greatly increases the volume of the protoplast between the tannin material and the inner surface of the cell wall. This contraction may also be an artifact as is believed to be the case in the summer tannin contraction. But studies on frozen fresh winter material with the cryomicroscope indicates the tannin contraction naturally occurs (figure 33) although possibly not as extensively as observed in chemically fixed material.

In winter, the tonoplast was not identified possibly due to very close adhesion to the tannin material which is so osmiophilic that it may mask any other small osmiophilic structure such as a membrane. The maintenance of an intact main cell vacuole (tannin vacuole) in winter was also observ-

ed in conifer needles by Lewis and Tuttle (1923), Parker and Philpott (1961 and 1963), Harris (1971) and Chabot and Chabot (1975). This is in marked contrast to the breakdown of the main cell vacuole in the over wintering cambial tissues of trees (Kadwai and Roberts, 1969; Robards et al., 1969; Mia, 1970 and 1972; Murmanis, 1970 and Itoh, 1971). The breakdown of central vacuole has also been observed in the tillering nodes of winter wheat (Chien and Wu, 1965), bark parenchyma cells (Siminovitch et al., 1968 and Krasvtsev et al., 1971) and wheatgrass hybrid leaves (Khristolyubova and Sanfonova, 1973).

The main difference between the plant cells where there was the observation of central vacuole breadkown into numerous small vacuoles, and the plant cells where the preservation of an intact central vacuole was observed, was the presence or absence of a tannin filled main vacuole. Most authors who studied secondary cambial tissue in winter reported what they believed to be tannin materials in the lumens of the many small winter vacuoles that made up the bulk of the winter protoplast. However, these materials did not have the same structure of homogeneity of the tannin found in the conifer needle main cell vacuole; nor were these materials in the same quantity as the tannin observed in conifer tannin vacuoles. Further more, no tests were mentioned to identify the "tannin material".

The great mass of cytoplasmic vacuoles in the winter chlorenchyma are one of the main features of winter cells. The question of where the vacuoles come from is a question that cannot be answered in this work be-

cause in the August the twentieth, 1972 fixation, they were largely absent

and by November the twenty-sixth, 1972 fixation, they were present. Cy-

toplasmic vacuoles were a common feature of the summer fixations but their

number was greatly less than in winter and they were evenly dispersed with-

in the cytoplasm.

In summer, the cytoplasmic vacuoles were usually separated by ground

cytoplasm and organelles from each other. In winter, however, the cyto-

plasmic vacuoles are found in masses in specific areas within the cell.

The vacuolar region appears to have occupied most of the space formed with-

in the winter cytoplasm by contraction of the tannin material. The vacuol-

ation of the winter cytoplasm appears to be a common feature of over win-

tering cells (see references in the discussion of the tonoplast and tann-

in vacuole above).

Biochemically, the greatly increased synthesis of phospholipids is

one of the main features of stage one of frost hardiness acquisition (Sim-

inovitch et al., 1975). The synthesis of phospholipids and proteins (Sim-

inovitch, 1968) at this time may provide the materials for the greatly in-

creased augmentation of cytoplasmic vacuolar membranes that must occur

sometime in the fall. Similarly, some of the increased lipid bodies in

the spring fixation may represent the lipoidal remains of the winter mem-

brane masses.

Murmanis (1970) and Chien and Wu, (1965) and others who reported vac-

uoles forming in the winter cytoplasm of hardy plants, believed that the

tonoplast of the original main cell vacuole broke down to form the numer-

ous winter vacuoles which in their view, replaced the summer main cell vacuole. In this work, the observation of the tonoplast invaginating into the cytoplasm in summer and the difficulty of resolving it from adjacent cytoplasmic vacuoles in winter may indicate that the cytoplasmic vacuoles observed in this work were also of tonoplast derivation or extension into the cytoplasm. If this is the case, then the only differences in the observations of Murmanis and others, and of the observations in this work is that on the conifer needles, the tannin material retains its structural integrity while the tonoplast proliferates within the cytoplasm as it does in cells without the tannin vacuole.

An alternate suggestion for which there is recent evidence in the literature (Khristolyubova and Sanfonova, 1973) is that the winter proliferation of vacuoles in wheatgrass hybrids originates from the ER. Mollenhauer et al. (1960) observed the responses of plant tissue to mechanical injury; and immediately after the injury, the damaged cytoplasm is characterized by rapid membrane proliferation and vacuolization of the cytoplasm originating from the ER. The endoplasmic reticulum has been observed to proliferate into vacuoles in the root tips of wheat when cooled to near freezing temperatures in wheat (Petrovskaya-Boranova, 1972).

The winter cytoplasmic vacuoles by reason of their extent and seasonal dependance, may play a kay role in the frost tolerance of winter cells. Siminovitch et al. (1975) have biochemically related the extensive augmentation of phospholipids in hardening trees during stage one to the proliferation of cytoplasmic membranes. This membrane proliferation appears to be one of the few features in common between the cytological examinations of different hardy plant species in winter.

Another feature of the winter cytoplasm was the great numbers of os-
miophilic bodies found in the winter cytoplasm. These structures are
found in summer fixations but greatly reduced in size and quantities. The
osmiophilic bodies are characterized in both summer and winter by a close
association with cytoplasmic vacuoles. Usually the osmiophilic bodies
are found within a clearly definable membrane of the cytoplasmic vacuole
(figures 22, 23 and 25). However, in some photomicrographs (in summer
fixations), no bounding membranes could be observed about the osmiophilic
body (lower OB, figure 23). Structurally, the osmiophilic bodies appear
to be closely associated with membrane systems. In summer, 'myelin-like'
structures are occasionally observed (figure 24) while in winter, the os-
miophilic bodies frequently form coatings of varying thickness on the lu-
men side of cytoplasmic vacuoles (figure 49).

The osmiophilic bodies are randomly scattered in the summer cyto-
plasm, but in winter, they are found in very specific locations within
the cell. In winter, the osmiophilic bodies often form a single layer
of osmiophilic bodies between the plasma membrane and organelles deeper
within the cytoplasm (figures 51 and 52). In regions where the chloro-
plasts and the nucleus are not found, they are intermixed with the masses
of cytoplasmic vacuoles found in these areas.

The osmiophilic bodies are characterized in this work by an intense
staining with osmium tetroxide. The osmiophilic bodies in osmium fixed
material are always the most electron dense material within the cytoplasm,
being even more osmiophilic than tannin material. In material fixed only

with aldehydes, they stain weakly with lead or uranium and are difficult to distinquish.

High magnification light microscopy studies of fresh summer material stained with Sudan Black B indicated the presence of numerous very small sudanophilic structues in the cytoplasm (figure 14). This observation may have been a positive indication of a lipid nature for the osmiophilic bodies. However, this type of study was not extensively conducted, and winter light microscopy studies at high magnification with sudan stains were not conducted. In addition, the small size of the structures observed made it difficult to distinquish between positive staining and a high refractility. Harris (1971) and Chabot and Chabot (1975) believed structures resembling osmiophilic bodies were tannin material. Tannin specific stains used in this work gave no trace of any tanniniferous material being present within the cytoplasm (figure 34). In addition, in this work, tannin material always had a fibrillar or granular fine sturcture while osmiophilic bodies did not contain any resolvable fine structure. These observations allow the tentative conclusion to be made that the osmiophilic bodies are not composed of tannin. They may be lipoidal in nature and appear to have some function within the winter tissue. This last conclusion is based on their great increase in numbers during winter and their specific distribution within the winter cell.

A possible function of the osmiophilic bodies that should be investigated is that they may play a role in protecting the winter photosynthetic pigments from photo-oxidation. Chlorophyll is retained in the needles

throughout the winter but the marked winter chlorosis in evergreen conifers, particularly in Pinus species, has been a point of note in the literature. Mohl (1837) suggested organic acids may destroy the green pigments during winter. Haberlandt (1976) suggested high winter light intensity may cause a reduction in chlorophyll content in winter conifers. Holzer (1958) supported Haberlandt's view and reported the observations that pine boughs buried under the snow did not become chlorotic in winter. Carotinoid pigments are yellow and have a role in protection against photo-oxidation (Devlin, 1969). They are also highly unsaturated lipids, and as a result, would stain intensely with osmium tetroxide. The osmiophilic bodies were observed to often lye in tiers near the cell wall between the chloroplast clumps and the plasma membrane. These observations indicated that it may be worthwhile to investigate the unsaturated oil content of seasonally chlorotic needles. A large winter build up of carotinoid-like pigments, possibly as the osmiophilic bodies, may mask the chlorophyll content of the needle enough to account for the seasonal changes.

The last major difference observed in comparisons between winter and summer tissue observed in this work is the change in membrane properties. This was observed on a structural basis and on a more subjective basis in terms of the seasonal responses of pine chlorenchyma to fixation procedures.

In this work, primary and secondary fixation times could be greatly reduced in winter material without affecting the observed ultrastructure.

Similarly, the composition and material concentrations of fixative solu-
tions could be varied in winter to such extremes that they destroyed su-
mmer cellular ultrastructure with minimal or no effect on winter tissue.
Parker and Philpott (1963) commented on the comparative ease in achieving
good winter ultrastructural preservation, particularly when compared with
summer fixations. These fixation properties indicate that the membranes
of the winter protoplast may have lost to a considerable degree their
properties of semi-permeability. The winter protoplast appears remarka-
bly resistant to osmotic shock from fixatives that varied greatly in comp-
osition. Slight changes in fixative compostion in summer at least alter-
ed cell ultrasturcture (and usually disrupted it) and often plasmolized
cells (figure 12). However, in winter, none of the fixative solutions
used in this work plasmolized cells. Plasmolysis would be expected in
highly osmotic fixative containing materials that are normally impermeable
if the cells had not lost some of the properties of membrane semi-permea-
bility.

Structurally, there is also considerable evidence for membrane changes
in winter. Changes in the chloroplast envelope membranes have already
been discussed. Mitochondria envelope and cristae membranes were indist-
inct and amorphous, nuclear envelope membranes contained numerous nuclear
pores and the tonoplast was difficult to distinguish. Only the plasma
membrane remained structurally similar to the summer condition. These
membrane structural changes are probably related to the seasonal processes
in the chlorenchyma since the changes are reversible in summer. Specific
detailed studies would have to be undertaken in each of the seasonally

varying membranes to determine how each observed membrane change may affect frost hardiness. At this stage, one can only conclude that significant seasonal membrane changes occur and that these changes are very organelle or structure specific, i.e., the chloroplast envelope membranes are different at least in winter from thylakoid membranes, nuclear membranes and tonoplast membranes.

III. Discussion of Possible Freezing Mechanisms in the Chlorenchyma

The summer-winter transformation of each major organelle has been discussed above. At this stage, some wholistic concepts can be discussed which may relate what has been observed cytologically in *Pinus* *contorta* with the prevailing literature concepts of frost hardiness.

In the introduction, it was stated that there is a general agreement in the recent literature (Burke et al., 1976) that extremely hardy plants pass through two or more stages of frost hardiness development, with stage one being generalized into a period in which the plant detects the approaching frost season well before severe frost, ceases active division and engages in the active biosynthesis of certain compounds and structures which may have an active role in the frost resistance of the cell; i.e. stage one involves active physiological processes within the cell.

The cytological examination of extremely hardy tissue during the winter months would reveal the materials synthesized during stage one of frost hardiness development. Winter fixations of *Pinus* *contorta* chlorenchyma indicated the following materials that were different from, or more numerous than type II summer cells: lipid bodies, osmiophilic bodies, the membranes of cytoplasmic vacuoles, nuclear pores, chloroplast stroma and structural and physiological changes in membranes. The lipid bodies as previously discussed are not believed to be involved with frost hardiness mechanisms. However, the remaining material presumably was actively synthesized in the fall and was present in what appeared to be uniform quantities throughout

the winter and subsequently became absent or greatly reduced in quantity the following summer. It thus seems reasonable to assume that these materials had some role in the frost hardiness mechanism of the cell. The increased numbers of nuclear pores and changes in the morphology of chloroplast and mitochondrial membranes may reflect the specific response of each type of organelle to the problem of releasing free water during freezing. No explanation for the apparent increase in chloroplast stroma or the increase and specific distribution of osmiophilic bodies during winter is possible based on the observations in this work. Suggested functions during winter for the osmiophilic bodies has been discussed previously.

Some insight into what roles the observed cytoplasmic changes may play in achieving frost hardiness may be obtained by considering what happens in stage two of frost hardiness development. In this stage, it is believed that the cytoplasm physically accomodates itself to the increasing severity of frost or more specifically ice in the environ of the cell and the corresponding loss of liquid water from the cell. The essential step involved with phase two of hardiness aquisition is that over wintering tissue must be exposed to specifc moderate frosts that are species or varietal specific before it can tolerate very heavy frosts up to its ultimate hardiness level (Sakai, 1966 and 1973). What is generally believed to be happening during phase two is that the cytoplasm is dehydrating and releasing water to extracellular ice nucleating sites (Weiser, 1970). This frost plasmolysis phenomenon is in equilibrium with whatever the tissue temperature is at a given instant, and at moderate frost intensities, the bulk cell water will be released to ice nucleating sites. But if this water is not allowed

enough time to migrate out of the cell gradually to freeze externally, then intracellular freezing will occur (Weiser, 1970). Intracellular freezing in hardy mature plant tissue is considered fatal by most authors (Tumanov, 1967 and Weiser, 1970). Frost plasmolysis of frozen plant cells has been well documented at the light microscopic level. Improtant early studies on the subject are by Iljin (1934),   Kessler and Rhuland (1938), and more recently, Samygin (1966).

A key step for the frost hardy cell would thus be to get the freezable free water out of the cytoplasm to external ice nucleation sites. This could be achieved by the cell loosing its membrane semi-permeability. Without the loss of semi-permeability, there would be an osmotic gradient for water opposite to the direction in which it must flow to freeze externally. The observation in this work of the resistance of the winter cytoplasm to extreme changes in fixative osmotic concentration whether electrolytic (seawater) or non-electrolytic (sucrose) supports this concept. The fact that the winter chlorenchyma cells were also never plasmolyzed also indicates a loss of semi-permeability since the plasmolysis that may occur during freezing, "frost plasmolysis", will involve the collapse of the protoplast due to loss of liquid water and may have little to do with the semi-permeability of membranes as in osmotically induced plasmolysis. If this concept is correct, it will explain studies such as those conducted by Scarth and Simiovitch (cited by Siminovitch et al., 1968) which indicated that nearly twice the concentration of plasmolyzing fluid was required to achieve the same degree of plasmolysis in hardy cells as in non-hardy cells. If the protoplast had partially lost the property of semi-permeability, then in-

creased osmotic solutions would be necessary to achieve the same degree of osmotic plasmolysis.

The fact that at least some hardy tissues become more permeable in winter has been known for some time (Scarth and Levitt, 1937) and more recently, supported by McKenzie et al. (1974). Studies have also indicated increase frost resistance in non-hardy tissue treated with compounds that increase membrane permeability (Kuiper, 1967).

In this discussion, the loss of membrane semi-permeability has been discussed with regard to the protoplast as a whole. In reality, the various membrane systems within the protoplast may not uniformly gain the same degree of permeability. In this work, there is evidence that the chloroplasts at least retain some degree of osmotic responsiveness to differing fixative solutions (particularly the seawater electrolyte fixative solution, figure 60). The retention of some degree of turgidity by specific organelles could affect the distribution of organelles in the winter cytoplasm by the orangelles acting as structural units in the plasmolyzing protoplast during freezing. If plasmolysis is not uniform throughout the cell, then these organelles could be pushed or squeezed into specific locations by the collapsing cytoplasm.

When considering an hypothesis to explain what may occur when the winter pine chlorenchymal cell freezes, the assymetry of cytoplasmic structures observed in this work should be considered. It was noted that the clumped chloroplasts, nucleus and most summer-like ground cytoplasm was

found in specific parts of the cell that were defined by the presence of trabiculae invaginating from the cell walls. In the regions where the long trabiculae invaginate from cell wall face C, it was observed that the plasmodesmata also occured. The plasmodesmata represent sites of plasma membrane bondage to the cell wall. Thus if the cytoplasm plasmolyzed and retreated from the cell wall, plasmolysis would have to occur with the cytoplasm still attached to the plasmodesmatal regions of the cell wall.

In winter, the collapsed portions of the cytoplasm in the pine chlorenchyma would be the areas of extensive cytoplasmic vacuolization while the chloroplast clumps tend to occur in the areas near the plasmodesmata. This hypothesis also would offer a mechanical explanation of how the summer organelle distribution is disrupted. In this concept, the plasmolysis of the cytoplasm occurs in the region of extensive vacuolization. The vacuolization may be a protective device to allow for the expansion and collapse of the cytoplasm via highly permeable plasma and vacuolar membranes. The freeze-thaw cycles that must occur on warm days and sub-freezing night temperatures may squeeze the chloroplasts and the nucleus into the parts of the cell where the cytoplasm cannot collapse due to the attachment to plasmodesmata.

In this mechanical explanation to account for the winter chloroplast and nuclear distribution, plasmodesmata alone cannot be the only sites where complete plasmolysis of the cytoplasm does not occur because small clumps of chloroplasts and occasionally the nucleus are also found in the

cytoplasm near where the small trabiculae invaginate. The trabiculae themselves may provide increased areas of attachment between the plasma membrane and the cell wall, thus making plasmolysis more difficult in the cytoplasm adjacent to trabicular areas.

One feature of plasmolysis caused by freezing that has long been observed in cases where ice forms outside the cell wall in primary and thin walled tissue is the fact that the cell tends to partially collapse as cell turgar pressure is lost (see works cited in Levitt, 1972). If this is the case, the most likely site for extracellular ice formation in the pine mesophyll is in the transverse chlorenchyma air spaces between the transeverse files of mesophyll cells. In this case, ice would form opposite cell face A in figure 2. The central parts of this cell face would also be the weakest cell facet structurally because the trabiculae would offer a bracing effect to the cell walls about the perimeter of the cell (in the transverse plane of the needle). The support given to the specific areas of the cell wall by the trabiculae may thus offer protection from cell wall collapse in the trabicular areas. Chloroplasts and other organelles would accumulate near the trabicular areas by the squeezing effect of wall collapse in other parts of the cell. In this concept, the trabicular walls are assumed to function as structural supports for the chlorenchymal cell wall. The structural arrangement of the mesophyll can be described as radially extending (from endodermis to hypodermis) single sheets of cells in the transverse plane of the needle. This cell sheet is particularly thin due to the minor axis of the chlorenchyma cells being in the longitudinal plane of the needle. Thus the chlorenchyma cells may require additional strength and bracing to counteract

forces normal to the transverse plane of the needle, since this would be
the weakest plane of the chlorenchyma cell sheet. The trabiculae (arrang-
ed about all cell walls not lying in the transverse plane) are ideally po-
sitioned to give maximal bracing support to the cell sheet in this direct-
ion. They are also positioned to prevent the transverse faces of the chlor-
enchymal cells (face A) from ballooning out under excess turgor pressure
and thus occluding the transverse air spaces within the needle.

The above discussion is based on the premise that the winter cytoplasm-
ic arrangement provides clues to the distortion and physical rearrangements
which may occur in the cytoplasm as a result of the freezing process. The
retention of plasma membrane-cell wall connections and the possible dist-
ortion of specific parts of the cell wall due to extracellular ice format-
ion and/or a loss of turgor pressure are proposed mechanisms which may ac-
count for the observed arrangement of the winter _Pinus_ cytoplasm. This is
only a proposal suggested as a basis for further investigation. It should
be noted that the Russian authors (Genkel and Kurkova, 1971) believe that
plasmodesmatal strands breaks or separate from primary pit fields during
ice induced plasmolysis. These observations if correct would invalidate
the concept presented above,which is based on the plasma membrane remain-
ing attached to the cell wall in primary pit field areas. Levitt (1972)
is skeptical of the Russian concept of plasma membrane attachment to pri-
mary pit fields breaking during winter. He cites several early microscopy
studies which clearly showed cytoplasmic strands from ice induced,plasmol-
yzed cells remaining attached to the cell wall.

Bold (1957) suggested that the trabiculae serve in the leaves of _Pinus_ to increase the gas exchange surface of the chlorenchyma cells. This concept is based on the frequent observation of chloroplasts lining the trabicular walls deep within the cell interior. Haris (1971) extended this concept by observing the splitting of the middle lamella at the ends of trabiculae forms an extracellular air space or tube extending through the chlorenchymal cell linking the two cell faces labelled A in figure 2. This air space was also observed in this work and the air spaces are thus illustrated at the tips of the trabiculae in figure 3. The observations in this work also support the above concepts but it is suggested that the structural role of the trabiculae may be the wall structures' primary function.

Trabiculae are not found in the chlorenchyma of other conifer needles (see Esau, 1965, for references) even though at least some needle bearing conifers have a mesophyllic arrangement similar to that observed in _Pinus_ _contorta_ (Gambles _et_ _al_., 1974). A possible explanation for this is the shape of pallisade chlorenchyma cells in other conifers is usually a cylindrical shape which would be a stronger basic structure to lateral forces to the cylinder's major axis than to the thin rectangular box or oval shape of the pine mesophyll chlorenchyma.

On the basis of the above discussion, it would be expected that due to frost plasmolysis, the most likely place for the cell wall to partially collapse in _Tsuga_ _mertensiana_ would be the cell wall area with the least mechanical strength and the wall area bounded by extracellular air spaces where ice crystal growth can occur. In the cylindrical cells of _T_. _mert-_

ensiana, the air spaces border the cylindrical walls of the cell which could be expected to be the weakest part of the cell wall. The dome shaped ends of the cell firmly attached to adjacent cells would be the strongest part of the cell. It is interesting to note that chloroplast clumps and nuclei are found at the ends of the T. mertensiana cell in winter and the cytoplasm along the cylindrical walls is predominently filled by cytoplasmic vacuoles (figure 18).

The concept of ice plasmolysis of the cytoplasm, particularly in the areas of the central parts of the cell wall face A with the resultant squeezing of some organelles into the cytoplasm near the trabicular regions becomes a more logical suggestion if the observations of Parker (1960), which indicate the gellation of the tannin vacuole during winter are correct.

The concept of protoplast gellation during winter in frost hardy cells has long been a matter of debate. Scarth and Levitt (1937) believed that the cytoplasm remained fluid while other authors (Lewis et al., 1920 and Kessler et al., 1938) maintained that the protoplast became more viscous or gellated. An explanation of at least part of this controversy may be the failure of some of the early authors to distinguish between what is occuring in the main vacuole verus the cytopalsm, and also the use of plasmolyzing fluids to measure cytoplasmic viscosity or gelling. The later precedure would give incorrect results if protoplast permeability was increased to a wide range of substances and the differential permeability of the protoplast membranes decreasd as is indicated by the observations in this work.

It is difficult to conceive of how all parts of the cytoplasm as ob-
served in the winter pine chlorenchyma could exist in a gell state. For
example, there is no evidence for any electron density within the cytoplasm-
ic vacuoles other than the osmiophilic bodies that often coat the inner
surface of the vacuole membrane. From this observation, the cytoplasmic
vacuoles may only contain material that exhibits no staining properties or
electron density when prepared by ultrastructural techniques. Such mater-
ials could only be cell water and low molecular weight organic compounds
such as amino acids, sugars etc. Such materials would not be able to gel-
late.

The tannin material of the tannin vacuole however could possibly gel-
late. It meets many of the characteristics required of a gell outlined by
Levitt (1956) and Tumanov (1967). Some of these characteristics of tannins
are a three dimensional macromolecular polymeric configuration of very
large and indeterminable molecular weight compounds and containing hydro-
philic moieties to interact with the gell solvent (water) (Swain, 1965).
The gell polyer must also be very evenly and uniformly dispersed within
the solvent in order to gell (Levitt, 1956). Ultrastructural observations
on the tannins is _Pinus_ in this work, and elsewhere (e.g., Ledbetter and
Porter, 1970 and Haris, 1971) consistently indicate that the tannin is ev-
enly dispersed and remarkably homogeneous within the tannin vacuole.

The above observations in themselves would not be sufficient arguments
for the gellation of the tannin vacuole were it not for the duplication in
this work of the experimental observation on a cryotome stage of Parker

(1960) that the tannin vacuole of <u>Pinus</u> needles in winter can be physical-
ly dislodged from the protoplast as an intact unit. This observation in-
dicates that at least at sub-freezing temperatures. the tannin vacuole is
in a form that can maintain its structure independent of external support.
If the tannin is in a gelled state during winter, it is probably not in a
gelled state during summer. Evidence for this is the observed lysis of
summer cells when allowed to thaw on a cryomicroscope stage and also the
observation of tannin material becoming dispersed throughout the cytoplasm
in summer chlorenchyma that had been fixed after freezing (figure 72).
Parker (1960) also did not believe the tannin vacuole gelled in summer.

There are also theoretical arguments in favor of the gelling concept
for at least parts of winter cells. For example, the cell must maintain
some form of structural integrity after the loss of freezable water (Tuma-
nov, 1967). Experiments with artificial organic gells indicate that at
slow freezing rates, they tend to release free water to external ice nucle-
ation sites rather than allowing ice nucleation to occur within the gell (Per-
sidsky and Luyet, 1975). Gelling of tannin would reduce the risk of it en-
tering the cytoplasm during the physical stresses of winter.

If the tannin vacuole does gell in winter, then the concept of ice
plasmolysis of the cytoplasm which leads to the squeezing of the chloro-
plast and the nucleus into specific areas of the cell where the wall may
not be able to collapse is considerably more attractive. With a semi-sol-
id tannin deposit on the inside of the cell and a collapsing cell wall on
the exterior of the cytoplasm, there would now be two solid entities bet-
ween which the cytoplasm would be crushed during ice plasmolysis.

The above discussion of what may occur to cause some of the charact-
eristic features of the winter cytoplasm can only be considered a prelimin-
ary theory. Considerably more extensive study most be conducted before
these concepts could be verified.

The above discussion is based on the concept that the winter cytoplas-
mic arrangement in Pinus and T. mertensiana is largely induced by physical
deformations within the cytoplasm. There is the possibility that some or
all of the organelle arrangements that were observed in winter may be due
to physiologically based movements of organelles for protective or other
reasons. For example Haberlant (1876) has suggested that chloroplast clum-
ping may be a physiologically induced mechanism for chloroplasts to gain
added protection from the photo-oxidizing effects of light during the long
dormant state of winter. Presumably, within the chloroplast clumps, the
masking effects of the organelles all being densely clustered in one area
could be an advantage. The principle of chloroplast movements in rela-
tion to external stimuli (usually varying light intensities) is well known,
(e.g., Inoue and Shibata, 1973). Such physiologically based possibilities
have not been discussed in this work but should be seriously considered
as alternatives to the concepts presented in this work. Studies of the
chlorenchyma during the fall while the cells are becoming frost hardy but
are still physiologically active should illucidate whether organelle move-
ments are based on physiological rather than physical, ice related factors.
Similarly, the study of frost hardy cells immediately before and after the
first severe seasonal frost may indicate whether physical forces related
to the frost have altered the organelle arrangement within cells.

Further study of the cytology of frost resistant processes in very hardy plants should be conducted. Such studies would illucidate the cytological basis of the first phase of frost hardening during the late summer and early fall (a period of the seasonal cycle not covered in this work) and could, with presently available techniques, clarify the physical processes occuring wihtin the cell as the frost hardy tissue is frozen. A cryomicroscope with mechanisms to precisely control stage temperature such as the one developed by Diller et al. (1971) would allow the study of ice formation and melting within specific temperature ranges. The use of the freeze substitution fixation and embedding techniques such as those used by Rapatz et al. (1963) would allow direct electron microscopic observation of the cytoplasm in the state of ice plasmolysis. The production of freeze-etched platinum-carbon replicas of material frozen to pre-arranged temperatures at controlled rates before submersion into liquid nitrogen may offer the greatest potential of all present techniques in the study of frozen tissue since the presence of ice crytals themselves can be seen at the resolution level of the electron microscope (Nei,1976). In this work, the freeze-etch technique had been attempted, but the major difficulty encountered was the failure to successfully remove replicas from tissue pieces.

IV.   Discussion of Experiments in Controlled Environment Chambers

The experiments on pine seedlings in controlled environments were de-
signed to study cytologically the frost hardening process between the non-
hardy growth state and stage two. It has been noted that stage one is be-
lieved (Weiser, 1970 and Tumanov, 1967) to be a frost season detection and
synthesis of cryoprotective materials stage. Thus it was hoped that a fix-
ation of needles from seedlings that had been exposed to two weeks of short
photo periods and progressively lower temperatures may reveal the following:
any component or components of the winter cytological state due to prior
specific chemical sythesis and preparation by chlorenchyma cells; and any
component or components due to the mechanical deformation of the cytoplasm
by deep freezing (stage 2 of frost hardiness acquisition).

Unfortunately, the fixation of control trees and the fixation of trees
fixed at various times during the artificial frost acclimatization period
in stage one were not successful. Attempts to repeat the experiment were
prevented by repeated failures of the growth chambers until the supply of
seedling trees ran out.

The fixation conducted after the acclimatized trees were placed in the
freezer at $-18^{o}C$ for eight hours was successful. However, the observations
recorded on this material are not identical with those described for the
winter condition in the field.

The separation of chloroplasts from the cell wall, their adherence in

small clusters, the formation of cytoplasmic vacuoles and the contraction
of the tannin vacuole imply that some of the features of the winter ultra-
structure were obtained. The fact that the trees were severely damaged
after freezer exposure indicates that artificial hardening was at most only
a partial success. However, that the control trees were killed immidiate-
ly during or after freezer exposure indicates that some degree of artifi-
cially induced hardening was achieved.

Two very interesting observations were made on trees artificially hard-
ened and exposed to the freezer temperatures. The first is that the needles
failed to yellow until after the frost exposure, and the degree of yellowing
was very slight. The second is that very few osmiophilic bodies were ob-
served in the needles. This may be   indirect evidence that the chloro-
plast clumping and/or the osmiophilic bodies may be responsible for the win-
ter chlorosis of Pinus contorta ssp. latifolia needles. This type of cont-
rolled environment study would be a most interesting area for further work.

Pinus contorta can be described as a moderate xerophyte (Mirov, 1967).
Tumanov (1967),and Alden and Hermann (1971) cite literature which discusses
the possible correlation between drought resistance and forst hardiness.
Li and Weiser (1970) succeeded in slightly increasing the frost hardiness
of stem tissue pieces by partial dehydration before frost exposure. A
drought exposure experiment was conducted on trees with the object of:

    a.   Determining if drought exposed trees were more frost resistant.

    b.   Determining if the ultrastructure of drought exposed needles
           had any resemblance to winter frost hardy needles.

Neither part of this experiment indicated an obvious cytological cor-relation between drought conditions and frost hardiness. The ultrastruct-ure of drought exposed needles did indicate some separation of chloroplasts from the cell wall. However, this was occasionally noted in summer fixat-ions and may be fixation artifact.

SUMMARY

This work has identified two seasonally dependent, cytoplasmic states in the mesophyll chlorenchyma of <u>Pinus</u> <u>contorta</u> spp. <u>latifolia</u>. In one state, the cytoplasmic constituents of most mature chlorenchyma cells (in needles varying in age from just after the completion of needle elongation to needles five years of age) appear very similar to the structural and positional organization of cell organelles and constituents described in the literature for the assimilatory tissue of other higher plants. The chlorenchymal cells found in the state described above were all observed during the growing season (June through August). This state is referred to here as the summer state.

Fixations on chlorenchymal cells collected during the winter months, (December through March), revealed a consistent but unusual cytoplasmic state, (termed the winter state), which was remarkably different from the structural and positional appearance of almost all cytoplasmic structures and organelles observed in the summer state. The winter cytoplasm can be described and compared to the summer state on the basis of specific individual organelles or structures.

Chloroplasts:

In summer, the chloroplasts were discoidal in shape, ly-

ing near the cell wall with their long axes (in sectional profile) parallel to the cell wall. The distribution of chloroplasts was uniform about the cell wall and trabiculae.

In winter, the chloroplasts were aggregated into either large clumps of at least ten , or small clumps of less than ten. Generally, the large clumps were in the parts of the cell where the long trabiculae are found and the smaller clumps, in the areas near the small trabiculae. No chloroplasts were found in the cytoplasm adjacent to the cell wall areas without trabiculae.

The winter chloroplasts were approximately circular in sectional profile and slightly larger in volume than summer chloroplasts. The increase in volume appears to be due to a transformation of the chloroplast's shape from discoidal in summer to the spherical shape. The added volume is due to an increase in stroma. The winter thylakoid system of the chloroplast usually retained the discoidal appearance. Winter chloroplasts were also characterized by their near complete absence of starch. The envelope membranes of winter chloroplasts were not as distinct in winter as in summer, they appeared amorphous at high magnification. Thylakoid membranes appeared to be sharp and distinct in both summer and winter fixations.

In winter, the outer membranes of the chloroplast envelopes of adjacent chloroplasts within clumps may fuse to form regions of close membrane association as the result of fusion between neighboring chloroplasts. Many winter chloroplasts contained two

or more thylakoid systems within one membrane envelope.

Mitochondria:

In summer, Mitochondria were polymorphic in cross section and were usually distributed uniformly between chloroplasts or oil bodies.

In winter, mitochondria were considerably smaller in sectional profile, and usually spherical in shape. They are usually loosely associated with chloroplast clumps and the remainder are randomly scattered in the cytoplasm. No mitochondrial membrane fusion was observed between mitochondria or between mitochondria and other organelles. The cristae were reduced in number and the mitochondrial membranes were not as distinct as observed in the organelles in summer.

RER, SER and Dictyosomes:

These structures were rare in both winter and summer cytoplasm. RER and SER were usually found near the nucleus in both summer and winter fixations.

Nucleus:

In summer, the nucleus is spherical and is usually found in a central location within the cell. The tonoplast usually nearly encircles the nucleus except for broad cytoplasmic strands connecting the perinuclear cytoplasm to the peripheral cytoplasm. The nuclear envelope is distinct but with few nuclear pores. The nucleoplasm contains very dense regions of heterochromatin interspersed with relatively

clear regions of nucleoplasm containing some granular material and fine fibrillar material.

In winter, the nucleus is found in the peripheral cytoplasm, usually in the ends of the cell where the long trabiculae are found. It is usually ovoid in shape with the long axis parallel to the adjacent cell wall. The nuclear envelope is characterized by a very high density of nuclear pores. The nucleoplasm appears finely granular with the dense heterochromatin areas being more dispersed in winter. The nucleolus appears similar in the winter and summer nucleus.

The tannin, main vacuole and tonoplast:

The bulk of the cell volume in both summer and winter is occupied by the main cell vacuole. However, in the pine chlorenchyma, this vacuole is filled with a homogeneous, highly osmiophilic matrix which was identified through specific staining as being tannin material. The main vacuole was thus referred to as the tannin vacuole.

In summer, the tonoplast was very prominent due to a slight shrinkage of the tannin matrix during most fixations. The summer tonoplast was particularly sensitive to changes in fixative osmolarities. The volume of the tannin vacuole was greater and the cytoplasm was confined to: a thin peripheral sheet about the inner surface of the cell wall, in slender strands through the main vacuole to the nucleus, and a thin region of perinuclear cytoplasm.

In winter, the tonoplast was obscured by tannin material and changes in the osmolarity of fixative failed to affect the tonoplast or the tannin material. The tannin vacuole was reduced in volume and had retracted to the central area of the cell. The vacuole was more spherical (not following the contours of the inner surface of the wall as closely) and no cytoplasmic strands were observed transversing it. The contraction of the tannin vacuole greatly increased the volume of the peripheral cytoplasm about the inner surface of the cell wall. This increase in cytoplasmic volume was greatest near the ends of the cell where the long trabiculae are found. The shape of the tannin vacuole in winter was confirmed by observations on cryosections of chemically untreated tissue.

Cytoplasmic vacuoles and osmiophilic bodies:

In summer, small intensely osmiophilic bodies were observed within the cytoplasm. They were not found in large numbers and were randomly distributed.

In winter, the osmiophilic bodies were present in great number and were a very prominent feature. They were localized in two general areas within the cell: near the plasma membrane about the entire cell as a single uniform layer, and randomly distributed deep in the cytoplasm about the cytoplasmic vacuoles, particularly in areas not occupied by the chloroplast clumps or the nucleus.

In summer, small cytoplasmic vacuoles are found scattered

throughout the cytoplasm. They usually contain osmiophilic bodies.

In winter, the osmiophilic bodies remained associated with masses of cytoplasmic vacuoles, mainly in areas of cytoplasm not occupied by the chloroplast clumps or the nucleus. The dense masses of vacuoles can be described as being "foamy" or "spong-like" in appearance.

Oil bodies:

In summer, there are some oil bodies observed within the cytoplasm, usually near the nucleus. In winter, these oil bodies are greatly increased in size and number and are a prominent feature of the winter cytoplasm.

Plasma membrane:

The plasma membrane appeared as a clearly defined membrane closely appressed to the cell wall in most summer and all winter fixations. In some summer fixations, the plasma membrane was occasionally slightly separated from the cell wall due to some degree of cell plasmolysis.

Experiments with differing fixative and preparative procedures resulted in improved methods of preparing pine needle chlorenchyma for electron microscopy. These experiments also revealed that the winter cytoplasm is remarkably insensitive to radical changes in the osmotic and ionic nature of fixative solutions when compared to summer tissue. Chemical preparative

procedures for electron microscopy could be substantially reduced in durat-
ion for winter tissue without any detectable deterioration of ultrastruct-
ural preservation. These differences in response to chemical treatments
are interpreted as being indicative of a fundamental change in the permeä-
bility of cytoplasmic membrane systems in winter.

It is concluded that the dramatic transformation in chlorenchyma ult-
rastructure between the summer and winter states represents the cytologi-
cal response to the physiological and physical changes within the pine
needle mesophyll. The physiologically induced ultrastructural changes in-
clude the changes in cytoplasmic membrane permeability and structure in
winter, thus allowing the rapid penetration of fixative solutions and pre-
venting any extensive osmotic response by the protoplast or its organelles
to significant changes in fixative concentration or composition. The in-
creases observed in cytoplasmic vacuolation, osmiophilic bodies, oil bo-
dies, chloroplast stroma and nuclear envelope pores must also be the result
of physiological and biochemical processes which must have occurred while the
chlorenchyma was still in an active state some time in early fall. Simi-
larly, the reduction in mitochondrial volume and the assumption of simpli-
fied spherical shapes, and the hydrolysis of chloroplast starch grains
must also have occurred when the tissue was still active metabolically. It
is concluded in this work that the above cytoplasmic changes between summer
and winter states may be the cytological response of the stage one phase
of frost hardiness preparation.

Some of the structural differences between summer and winter chlorenchyma ultrastructure may be related to physical rather than physiological or biochemical changes in the chlorenchyma. The physical changes are believed to be due to the distortions induced within the protoplast due to ice plasmolysis. These physical distortions may represent the cytological response of stage two of frost hardiness development, in which the protoplast is believed to physically adapt and accomodate ice plasmolysis and the presence of ice crystals. Cytological manifestations of ice plasmolysis would lead to the aggregation of chloroplasts in certain specific cytoplasmic areas and finally the fusion of these organelles. The translocation and change in shape of the winter nucleus and the distribution of cytoplasmic vacuoles are also believed to be the result of ice induced plasmolytic forces.

An hypothesis based on physical forces due to the freezing of the winter chlorenchyma is presented to explain some of the winter ultrastructural changes observed. This hypothesis is based on the concept of differential plasmolysis in which the winter cell plasmolyzes in regions of extensive cytoplasmic vacuolization, but does not plasmolyze as extensively in cell regions where the chloroplast clumps and the winter nucleus are found. A possible explanation for this differential plasmolysis is based on the concept that the plasma membrane may adhere to the cell wall more extensively where the primary pit fields are located , while the protoplast collapses readily in the presence of ice dehydration in the regions of extensive vacuolization. Another important aspect of this hypothesis is that the tannin vacuole enters a gel-like state with subsequent structural integrity

at low temperatures and/or in a dehydrated state due to the removal of cell free water by ice nucleation sites external to the protoplast. Nuclear and chloroplast migrations from summer locations would occur by the squeezing of these organelles from summer locations as the cytoplasm collapses against the tannin material.

The fundamental transformation of the summer pine chlorenchyma cell ultrastructure to the winter state is completely reversible. The summer state of needles after winter is very similar to the summer before exposure to fall and winter. What may be an intermediate or spring transitional state has been described in this work for chlorenchyma fixed in April.

Pine chlorenchyma from needles that had been kept in a frozen state from winter collection were observed in the frozen state without any chemical additives. These observations indicated that the tannin vacuole was in the same position as observed in chemical fixations of winter cells. The position of oil bodies and the nucleus were also similar to that observed in chemically fixed material. No other cytoplasmic details could be observed in frozen sections. It is concluded that the observations on frozen material indicates that the appearance of chemically fixed winter tissue may be very close to the true physical state of the winter frozen cell.

Studies in this work indicated that a minority of chlorenchymal cells in needles of all ages were senescent or dead. The numbers of cells in these two conditions were proportional to needle age, but were independent of season.

The chlorenchyma of Pinus albicaulis and Tsuga mertensiana were also
examined at the light microscopy level in summer and winter. These obser-
vations indicated a seasonal cycle between summer and winter cytoplasmic
states which is very similar to that studied in P. contorta.

The structure of the mesophyll chlorenchymal cells were studied in
detail by serial sectioning and scanning electron microscopy. The shape
of the mesophyll cell was observed to be related to the distribution of
certain cellular components in the winter state.

Experiments were conducted to artificially induce frost hardiness in
laboratory grown Pinus contorta seedlings, but these experiments were only
partially successful and no definitive conclusions can be based on them.

APPENDIX I

Specific Stain Solution

1. **Modified Johansen (1940) Stain and Fixation for Tannins**

A dark brown staining of vacuoles will occur with longitudinally cut needles fixed for 12 hours in the following solution:

> Ferrous sulphate   0.5 gm
> 1.5% freshly made formalin, made up in
> Sodium cacodylate buffer

2. **Reeve Stain for Polyphenols (1951)**

To fresh sections, the following solutions are added in succession.

> 10% sodium nitrite
> · 20% urea stabilizer
> 10% acetic acid
>
> After 3 to 5 minutes, add 2 N sodium hydroxide.

Slow in developing light pink areas were interpreted to indicate the presence of tannins. The bright cherry-red staining of tannins described by Reeve was never observed. Only a light pink coloration of the tannin vacuoles was observed.

3. **Sudan Black B Solution**

Stain was made up in absolute alcohol and a clearing solution of 90% alcohol - 10% distilled water was used. Staining was done for five minutes followed by 1 minute in clearing solution, followed by two washes in distilled water. Dark spherical desposits in the protoplasts were interpreted as positive tests for lipids.

APPENDIX II

Cryosectioning and Light Microscopy of Frozen Tissue

In order to view tissue in the frozen state and unthawed state, (field collection was at below zero temperatures), a Richert cryomicrotome and Nikon biocular microscope with camera attachment were modified for this purpose. The procedure and apparatus used is described as follows with reference to figures 78, 79 and 80.

A. Cryosectioning

Needles stored in a commercial freezer at $-18^{o}C$, were subdivided transversely within the freezer into approximately 0.5 cm long segments. They were then placed in a sealed vial which was immersed in a brine-ice bath (A, figure 78), and transferred in this bath to the apparatus illustrated in figure 78. The apparatus consists of a standard Richert CO_2 expansion cryotome, ((B), CO_2 bottle; (C), cutting arm; (D), cooling stage). A large razor blade with a well made of plasticine attached to its upper surface was used as the sectioning knife. Liquid nitrogen was transferred from the Dewar liquid nitrogen reservoir (K) to the plasticine well by an aluminum foil ladle (E). When vigorous bioling of the liquid nitrogen in the well stopped, the knife was ready for sectioning. Several drops of gum arabica support media was cooled on the cryotome stage (D) until ice began to form. Tissue was then removed from the brine-ice bath with insulated forceps

(G), the tips of which had been cooled in liquid nitrogen. The specimen was dipped into liquid nitrogen and thrust into the support media. The extreme low temperature of the specimen froze the support media immediately. The specimen and support media were maintained in the frozen state by expanding CO_2 gas.

Sections could now be cut since both specimen and knife were at very low temperatures. Approximately 20 μm sections were obtained. Plastic coverslips (H) were cut into 6 mm squares (arrows). Some of the coverslip pieces were then coated about the edges with a thin band of silicone vacuum grease. A cold sink was used to maintain the coverslips at low temperature and it consisted of a 650 gm. block of iron (J) which had previously been immersed in liquid nitrogen until boiling stopped. The manipulation, storage, and transport of coverslips and sections were conducted on the surface of this sink which could be used for up to twenty minutes before re-cooling became necessary. Sections were transferred from the knife to the greased coverslip and a non-greased coverslip placed on top. This formed a sealed wafer consisting of the two coverslips, the sections in between, and the edges sealed with vacuum grease.

B.   Cryomicroscopy

The apparatus used to observe the frozen sections is illustrat-
ed in figure 79. A Nikon binocular with a camera attachment (P) was
used. The stage controls were removed and replaced with a handmade
cooling apparatus (M). This apparatus will be described with refer-
ence to the cross-sectional diagram shown in figure 80. The stage
was covered with aluminum foil (not shown) and then coated with a
layer of plasticine (stippled areas). A heavy gauge 4 X 10 cm strip
of copper with a 2 mm aperture drilled near one end was placed in the
plasticine with the aperture over the optical axis of the microscope.
The other end of the copper sheet was bent upwards into a liquid ni-
trogen reservoir and coated with vacuum grease to prevent leaking.
The heavy guage copper sheet acted as the conducting medium to lower
the stage temperature by conducting heat from the stage to the liquid
nitrogen reservoir. A 1 cm area on the upper surface of the copper
sheet above the aperture was not covered with plasticine. The appar-
atus was deemed ready for use when a drop of 50% ethylene glycol sol-
ution frozen quickly when placed on the exposed copper above the aper-
ture. Only the 20X and the 40X objectives were used and the conden-
ser lense was turned down to prevent it from coming too close to the
cooling system.

Freon gas (N) was cooled to liquid in a liquid nitrogen bath (O).

The exposed copper on the stage was brushed with liquid freon and the specimen wafer quickly dipped into the freon just before observation in order to quench the material and remove water vapour frost from the system. The sepcimen wafer was manipulated on the stage with cooled insulated forceps (G).

PLATES AND EXPLANTIONS

LEGEND

C	Cuticle
CC	Chloroplast clump
CE	Chloroplast envelope
Ch	Chloroplast
Cr	Cristae
CV	Cytoplasmic Vacuole
Cy	Cytoplasm
D	Dictyosome
E	Epidermis
ER	Endoplasmic Reticulum (used when distinction between RER and SER cannot be determined).
En	Endodermis
ES	Extracellular Space
FC	Fiber Cells
FH	Fiberous Hypodermal Cells
G	Grannum
Gd	Guard Cells
GC	Ground Cytoplasm
HC	Heterochromatin
M	Mesophyll
Mt	Mitochondria
MV	Main Vacuole
N	Nucleus

NE	Nuclear Envelope
Nu	Nucleolus
O	Oil body
OB	Osmiophilic Body
P	Polysomes
Pd	Plasmodesmata
Pg	Plastoglobuli
PM	Plasma Membrane
RD	Resin Duct
RER	Rough Endoplasmic Reticulum
S	Stroma
Sc	Starch Center
SER	Smooth Endoplasmic Reticulum
St	Starch Grain
T	Trabiculum
TM	Tannin Material
To	Tonoplast
TP	Transfusion Parenchyma
TT	Transfusion Tracheids
TV	Tannin Vacuole
VB	Vascular Bundle
W	Cell Wall
WL	"Water Layer" of the Hypodermis

PLATE I

Needle Cells and Tissues

Figure 1   One half of the transverse area of fresh mature needle
material from an approximately 20μm cyrosection section is
shown by dark field illumination. Cells and tissues from
outer surface to needle interior are: thick cuticle (C, ar-
row), the outer dermal layers; single tier of epidermal
cells (E) without visible cell lumina; a single tier of
thin-walled hypodermal cells (the "water layer", WL) and a
one or two cell layer of fibrous hypodermal cells (FH). The
mesophyll (M) consists of crenulated walled chlorenchyma,
e.g. cell labelled (A). Note medially (to the left of the
plate), there are two tiers of rectangular chlorenchyma cells
and laterally (to the right) along the needle edges, there
are more than two cell layers. Cells in this area are us-
ually ovoid in transection. In the lateral mesophyll, a
resin duct (RD), with duct lumen bordered by a thin walled
secretory epithelium (SE) and a fiber cell layer (FC) is
found. Note the boxed area in the upper medial mesophyll
represents the area where most sections studied in this work
were taken. The endodermis (En) of one cell layer encloses
transfusion tissue of two cell types: transfusion tracheids
(TT) and transfusion parenchymal cells (TP). One of the two
vascular bundles (VB) is shown.

A 100 μm scale is shown: 175x.

1
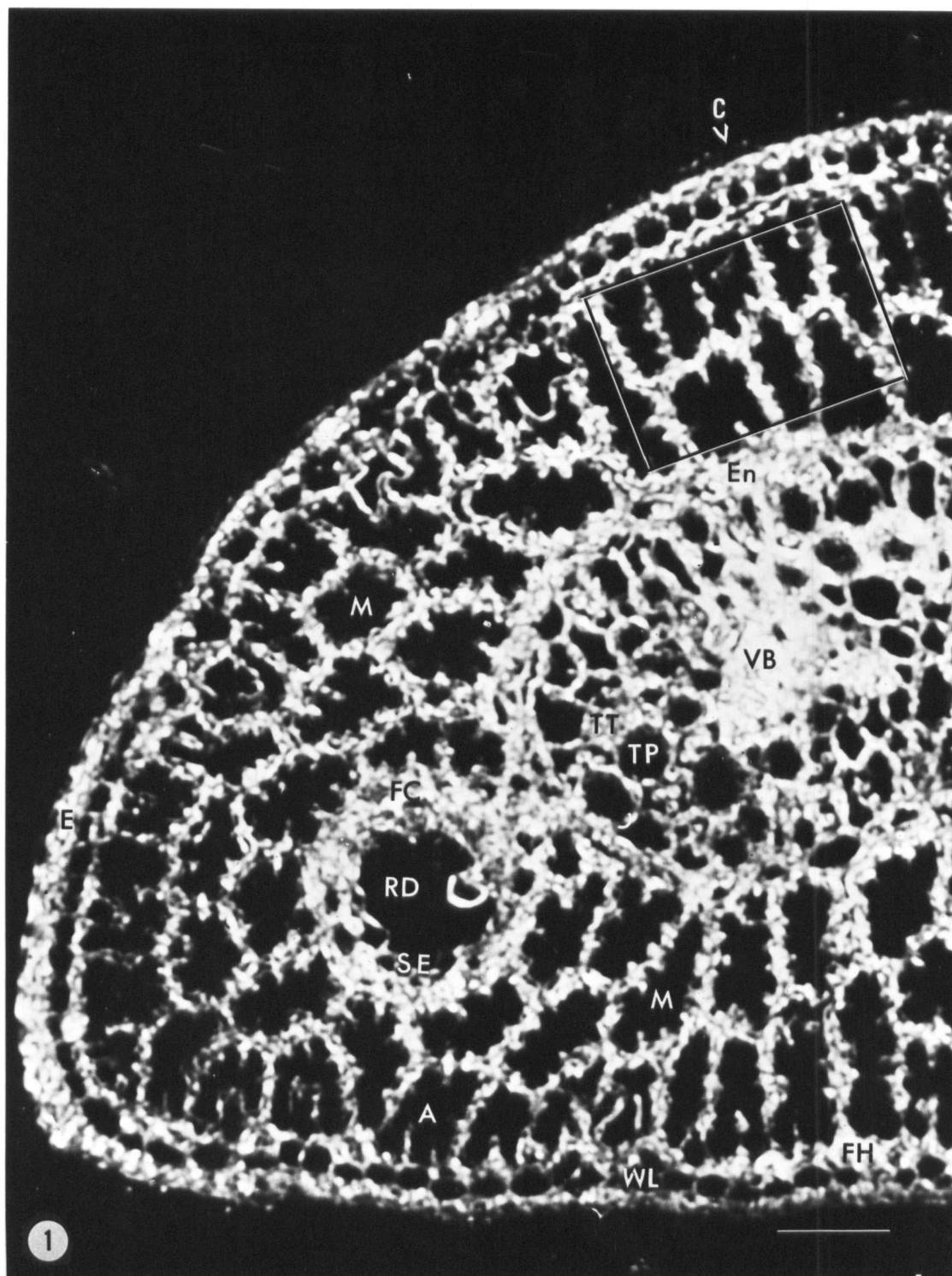
PLATE II

The Shape and Sectional Interpretations of

a Generalized Pine Mesophyll Cell

Figures 2 to 6 show the three dimensional shape of a generalized medial chlorenchymal cell and the interpretation of various sectioning planes through it.

Figure 2   Diagram of the surface of a generalized chlorenchymal cell. Three cell faces are shown with respect to the needle:transverse face (A), radial face (B) and one of the ends of the cell (tangential face, C). Dark surface grooves are trabicular wall invaginations.

Figures 3 to 6 all have the same key to the cellular components shown. Nucleus, (circle and N), light stippling (cytoplasm), dark stippling (tannin material of the tannin vacuole), dark lines (cell walls and the trabicular invaginations). All section profiles are of sections which could be cut from figure 2.

Figure 3   The generalized appearance of a transverse needle section, in a plane parallel to face A figure 2.

Figure 4   A tangential needle section near the end of the cell (face C). Note that the end wall trabiculae cause the section to appear as three, cell-wall-bounded compartments.

Figure 5   A tangential needle section cutting the mid-region of the cell below the region of trabicular penetration.

Figure 6   A radial needle section of the narrow side of the cell in a plane parallel to face B and close enough to cut the trabiculum. Note the trabiculum which invaginates in from face B divides this section into two compartments.

2

3

4

5
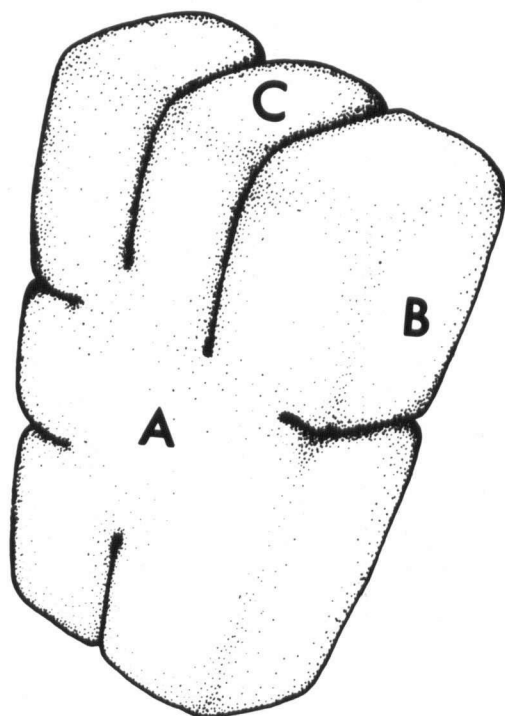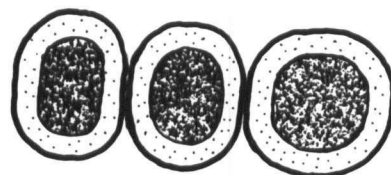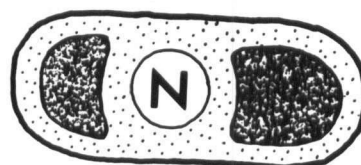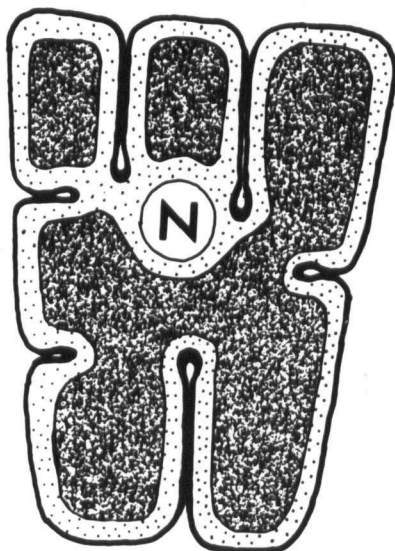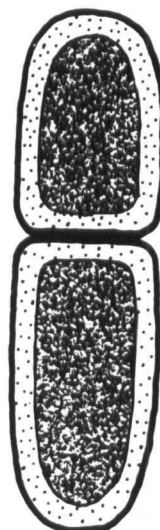
6

PLATE III

Scanning Electron Microscopy of Mesophyll Chlorenchyma

Figure 7   A transverse view of the lateral mesophyll. (FH) = fibrous hypodermal cells. Note the chlorenchyma cells are ovoid in outline, trabiculae of uniform length invaginate about each cell. Cell wall debris from a cell in the sheet of cells that was above the cells shown can be seen still attached (solid arrow). Open arrow shows where the cell wall has broken off the cell surface.

      Approx. 600x.

Figure 8   Two cells (A' and A) are viewed obliquely in the transverse plane of the needle. These cells are more angular in outline being from the more medial mesophyll areas. The cell faces labelled A and A' correspond to face A in figure 2. Similarly, face C corresponds to face C in figure 2. Note the long trabiculum (arrow) and the hollow to the left where the cytoplasm which has been dislodged. Arrowhead indicates cytoplasmic areas where the cell wall has broken off.

      Approx. 850x.

Figure 9   Higher magnification of two adjacent cells. The arrowhead shows the invagination of a short trabiculae. Note how the trabicular folds completely back on itself leaving what appears to be a pit into cell interior. Arrows point to trabiculae whose walls have not folded back on themselves completely but have formed "U" shaped cleavages in the cell walls. Note near the top arrow, a patch of cell wall has broken off exposing the cytoplasm.

      Approx. 1,000x.
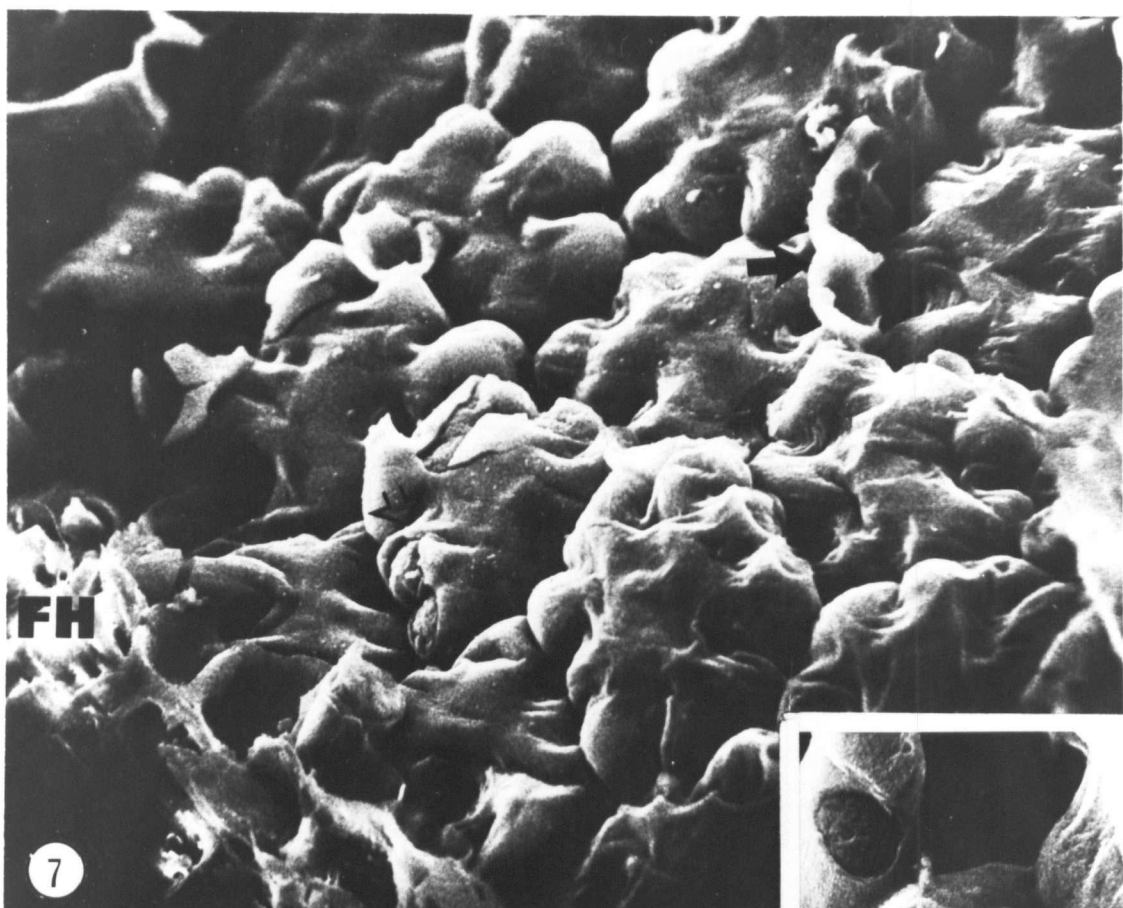
PLATE IV

Light Microscopy - Summer Chlorenchyma

Figure 10   The section was prepared and observed by the procedure in appendix II. An entire chlorenchymal cell is visible, a "U-shaped" extracellular space extends into the cell. The guard cells (Gd) of a stomata can be seen. A nucleus (N) and chloroplasts tightly appressed to the cell wall (arrow) can be seen.

A 10 μm scale is shown: 1,400x.

Figure 11   Formalin fixed material. The cell walls of two cells (W), a trabiculum (T) and nucleus (N) are visible. Individual chloroplasts (arrowheads) uniformly line the wall and the trabiculum.

A 10 μm scale is shown: 1,700x.

Figure 12   Tissue stained in the formalin fixative and tannin specific stain solution given in appendix I. Two types of trabiculae are shown: the long end wall trabiculum (T) and the shorter lateral wall trabiculae (T'). The cytoplasm can be seen as a thin clear parietal band (between arrowheads). Note the heavily stained tannin vacuole (TV) and a nucleus (N) at the tip of the long trabiculum.

A 25 μm scale is shown: 850x.

Figure 13   Two large chlorenchymal and several endodermal cells (En) are shown in unfixed material stained with Sudan Black B. Small sudanophilic bodies are found in the cytoplasm (open arrows) and larger bodies are found in the endodermis (En, arrow).

A 25 μm scale is shown: 750x.

Figure 14   High magnification of the type of material in figure 13. Note the sudanophilic bodies (O) are close to the cell wall. Some dark staining material is near the resolution of the microscope (White arrows).

A 10 μm scale is shown: 2,000x.
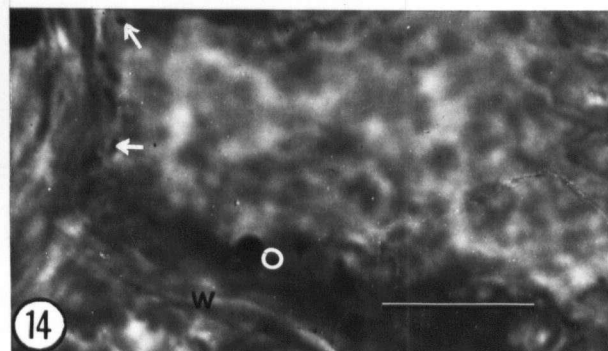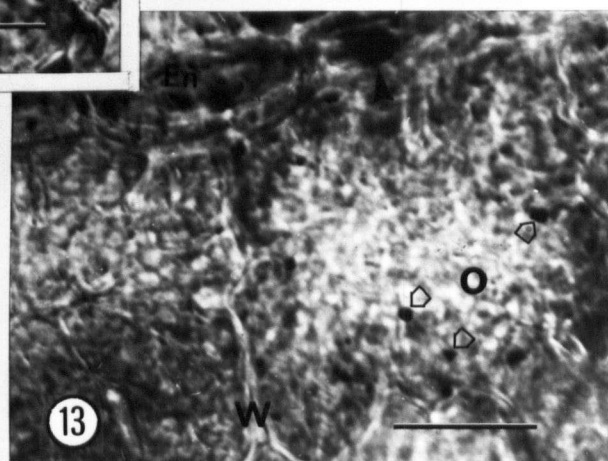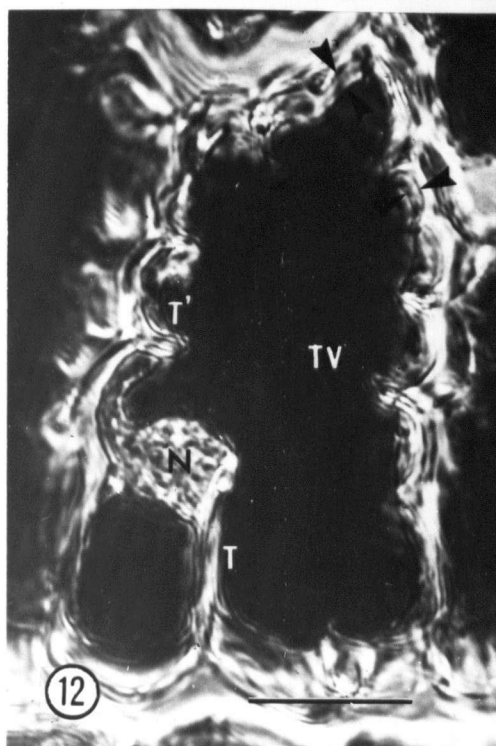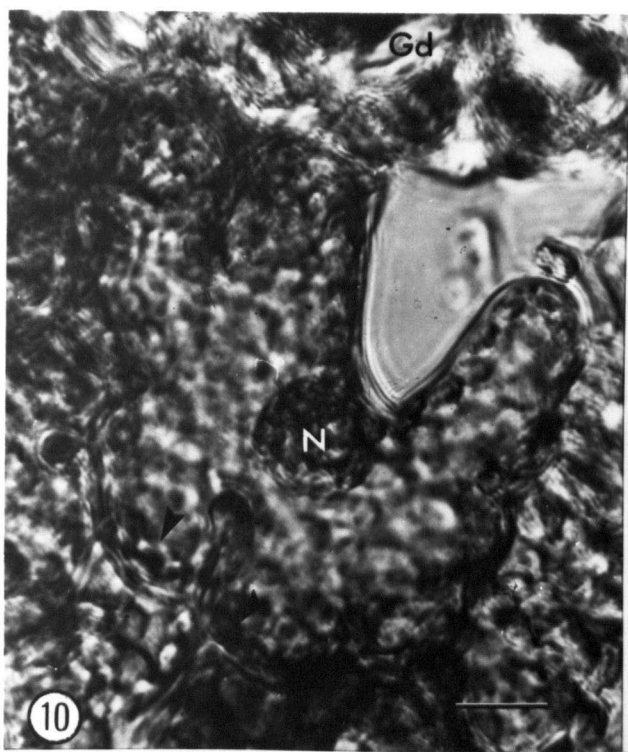
PLATE V

The Summer - Winter Light Microscopy

Cytology of Tusga mertensiana

Summer fixations.

Figure 15   Transverse section of mountain hemlock needle. Note tan-
                  nin (TV), circular nuclei (N) and chloroplasts lining the cell
                  wall (arrowheads). Part of the endodermis (En) is also visi-
                  ble.

                      700x.

Figure 16   A portion of a mesophyll cell. Individual chloroplasts
                  (Ch) are clearly seen with ovate profiles, a cytoplasmic va-
                  coule is shown (CV). Starch grains (St) are present in the
                  chloroplasts and what may be osmiophilic bodies (arrowheads)
                  are seen along the cell wall. TV = tannin vacuole.

                      1,600x.

Winter fixations.

Figure 17   Chloroplasts clumps (CC) are visible in all cells shown.
                  Note the position of the ovate nucleus (N) in the cytoplasm
                  with the two prominent nucleoli (arrows). Cytoplasmic vac-
                  uoles (CV) and osmiophilic bodies can be observed (arrowheads).
                  Open arrow points to a primary pit field between two cells.
                  TV = tannin vacuole.

                      1,600x.

Figure 18   Chloroplast clumps (CC) in two adjacent cells, note the
                  vacuolated cytoplasm between the clumps, the tannin vacuole
                  (TV) and the cell wall. The vacuoles along the cell wall, in
                  particular, contain numerous osmiophilic bodies (arrowheads).

                      1,500x.
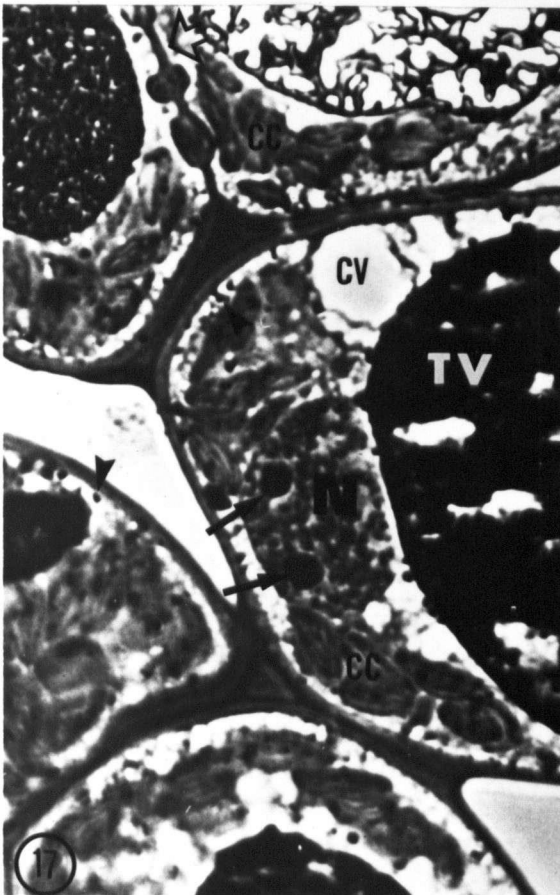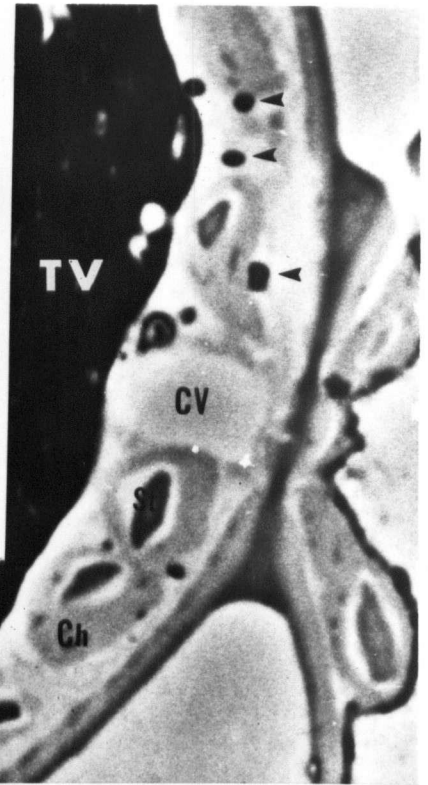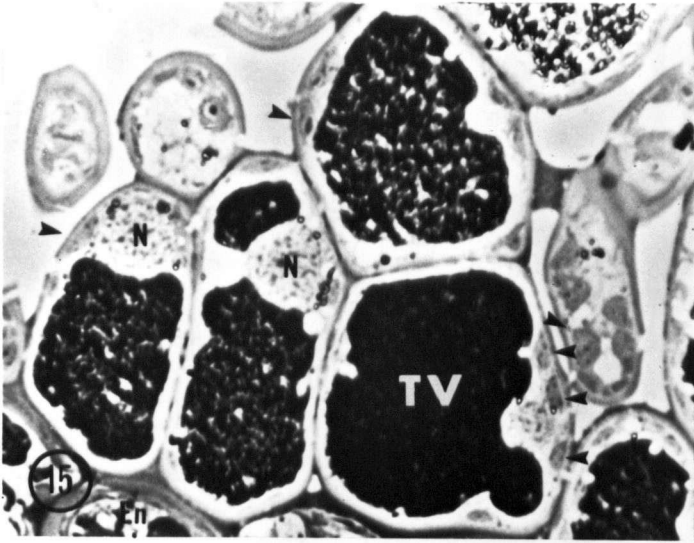
PLATE VI

Electron Microscopy of Summer Chlorenchyma.

Figures 19 to 32 are electron photomicrographs of summer tissue fixed
at dawn and fixed in the standard fixative solution.

Figure 19   Parts of four cells sectioned in the region illustrated
in figure 5. This is a three month old needle (August fixat-
ion). The cells are dominated by the homogeneous osmiophilic
tannin vacuoles (TV). In the parietal cytoplasm is found:
chloroplasts (Ch) with starch grains (St), polymorphic mito-
chondria (Mt), homogeneous and largely non-vacuolated ground
cytoplasm, a few cytoplasmic vacuoles (CV) associated with the
osmiophilic bodies (OB), and small (usually 2 microns or less)
oil bodies (O).

              A 5 μm scale is shown: 5,000x.

Figure 20   A 2 mm needle. Tannin has only just begun to be formed
and none is visible in the main vacuole (MV). The chloroplast
has fully developed with small grana (G, white arrow). Plasto-
globuli (dark arrows) are very small and few in number. The
cell wall (W) and part of the extracellular space (ES) is shown.

              A 1 μm scale is shown: 17,000x.

Figure 21   A chloroplast and mitochondria (Mt) from a two year old
needle. The membranes shown are distinct. The plasma mem-
brane (PM), double membraned chloroplast envelope (CE, arr-
ow) and mitochondrial envelope membranes (open arrowhead) can
be seen. Grana contain few thylakoids but partition regions
are extensive (open arrow). Plastoglobuli (Pg) are large, num-
erous, and usually associated with the thylakoid membranes. The
ground cytoplasm (GC) is relatively empty of identifiable stru-
ctures. except for fine granular and fibrilar material. A
starch grain (St) is shown within the chloroplast.

              A 1 μm scale is shown: 29,000x.
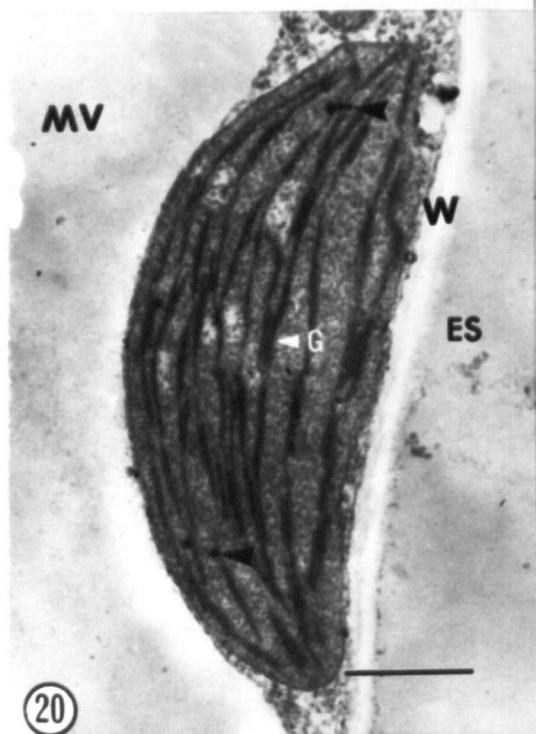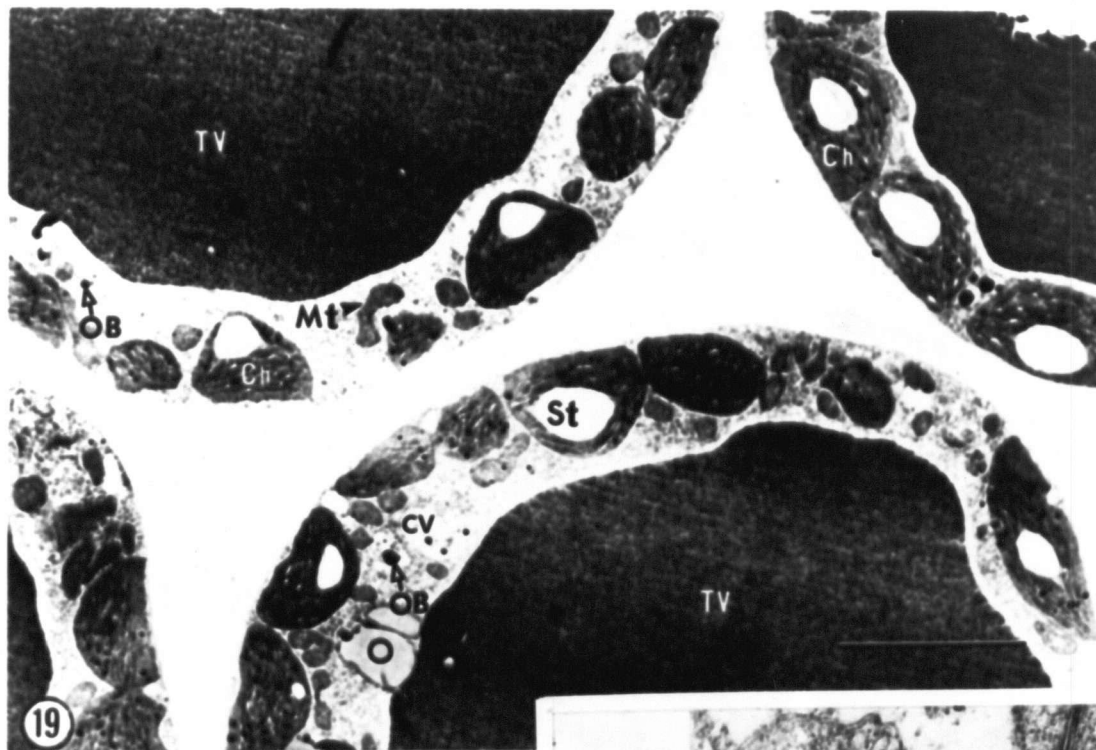
PLATE VII

Electron Microscopy of Summer Chlorenchyma: Continued

Figure 22 The organelle rich cytoplasm near the nuelcus (N) is shown in this section from a two month old needle. A chloroplast (Ch) containing a starch grain (St) and with some of its thylakoids slightly swollen is shown along the cell wall (W), the plasma membrane (PM), several cytoplasmic vacuoles (CV) containing osmiophilic bodies (OB) and membranous and fibrillar material, rough endoplasmic (RER), a dictyosomes (P, arrowheads) are shown.

  A 0.5 μm scale is shown: 38,000x.

Figure 23 Two osmiophilic bodies (OB) are shown in three month old needle tissue. The upper is enclosed within a cytoplasmic vacuole (CV) which also contains some membranous material (solid arrowhead). The lower osmiophilic body appears not to be membrane bound. Note the tonoplast (To, open arrows) appears separated from the tannin material of the tannin vacuole (TV). Fibrillar material appears to stretch between the tannin and the tonoplast (open arrows). W = cell wall.

  A 0.5 μm scale is shown: 40,000x.

Figure 24 An osmiophilic body (OB) from fourteen month old needle tissue is shown associated with several concentric membranes (arrowheads). Also shown are the tannin vacuole (TV), and the cell wall (W).

  A 0.5 μm scale is shown: 33,000x.

Figure 25 A small area of cytoplasm is shown from three month old needle tissue. Cytoplasmic vacuoles (CV) are shown containing osmiophilic material (solid arrohead). Two dictyosomes (D) are shown surrounded by numerous small vesicles (open arrowhead). TV = tannin vacuole.

  A 0.5 μm scale is shown: 35,000x.
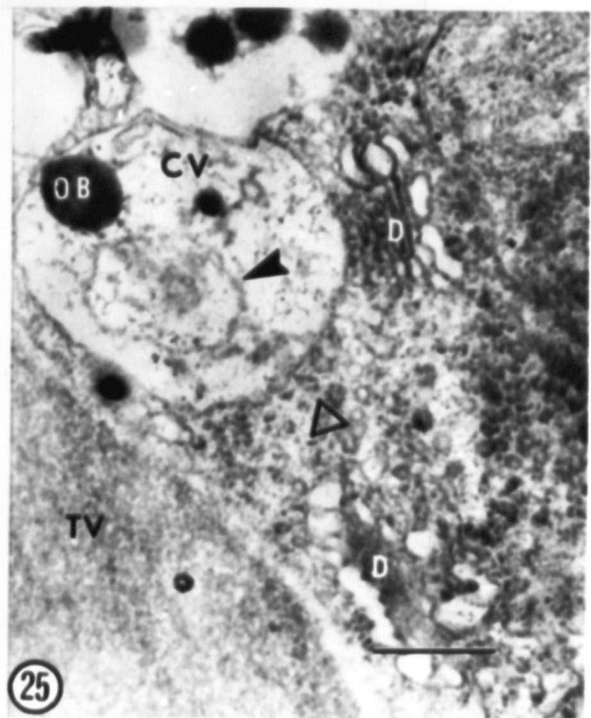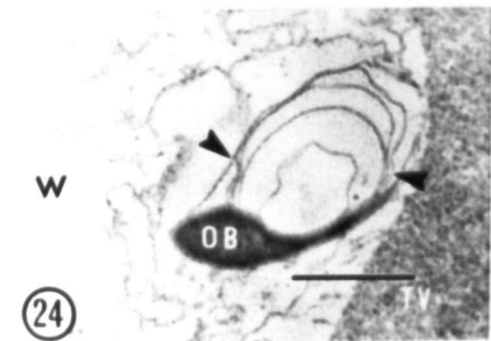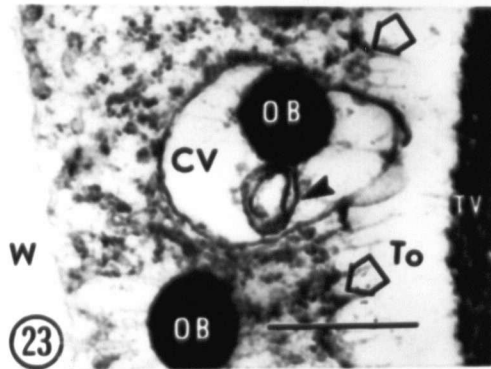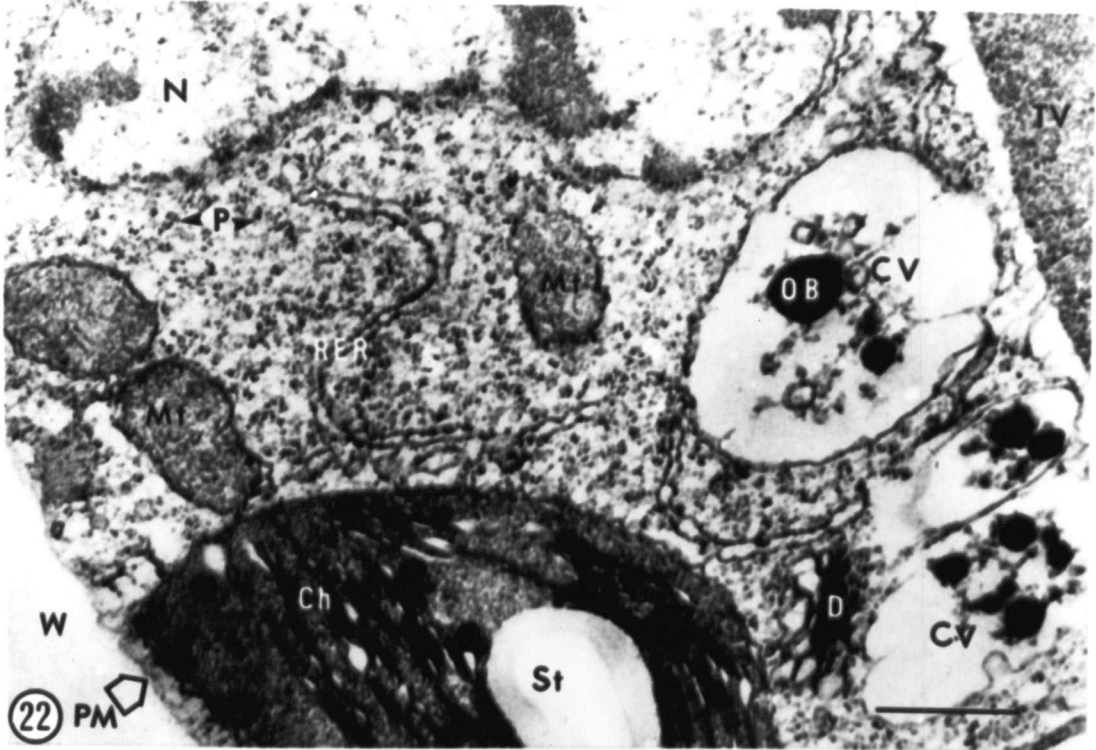
PLATE VIII

Electron Microscopy of Summer Chlorenchyma: continued

Figure 26   A small area of cytoplasm is shown from two month old needle tissue. Several dictyosomes (D) are visible and surrounded by vesicles. From what may be part of a dictyosome (in the upper center), a series of cytoplasmic vacuoles (solid arrows) can be seen. The largest is closest to and appears to have fused with the tonoplast (open arrowhead). Note the fibrils (arrows) which appear to connect the tannin material with the tonoplast (To). TV = tannin vacuole.

A 0.5 μm scale is shown: 28,000x.

Figure 27   An area of cytoplasm from a young needle (one month old) is shown. A dictyosome (D) with a series of cytoplasmic vesicles between it and the cell wall (W) is shown. An unusual section of a mitochondrion (Mt) and part of the tannin vacuole (TV) is also shown.

A 0.5 μm scale is shown: 35,000x.

Figure 28   This section is parallel to, and just under the cell wall in a young (two month old) needle showing a multilobed mitochondria (Mt). Note that the matrix is filled with extensive tubular cristae (Cr, arrowheads). Part of the surface of a chloroplast (Ch) is also shown.

A 0.5 μm scale is shown: 34,000x.

Figure 29   An oil body (O) is shown with the mitochondria (Mt) nearby in one year old needle tissue. Two osmiophilic bodies (OB) are also shown along with part of a chloroplast (Ch) and the cell wall (W).

A 0.5 μm scale is shown: 38,000x.
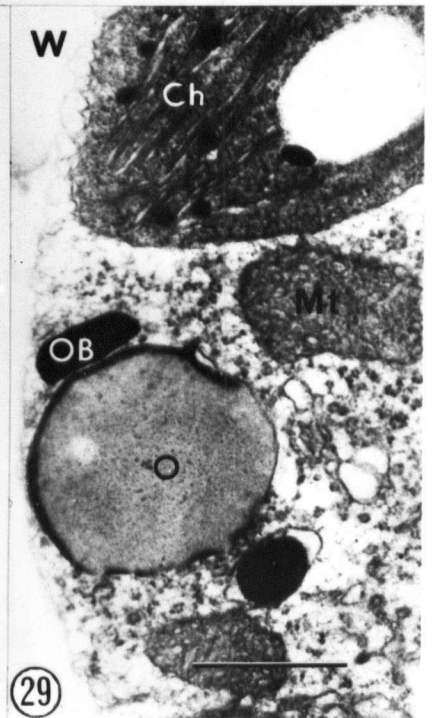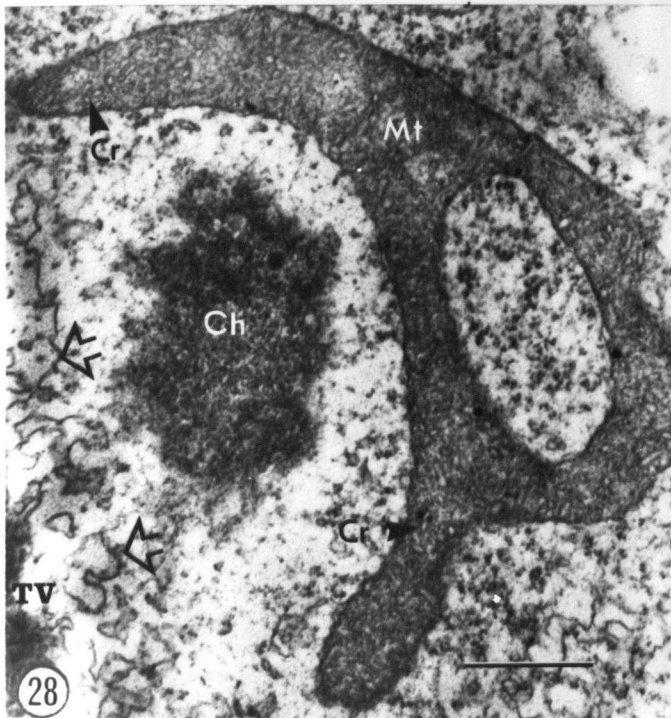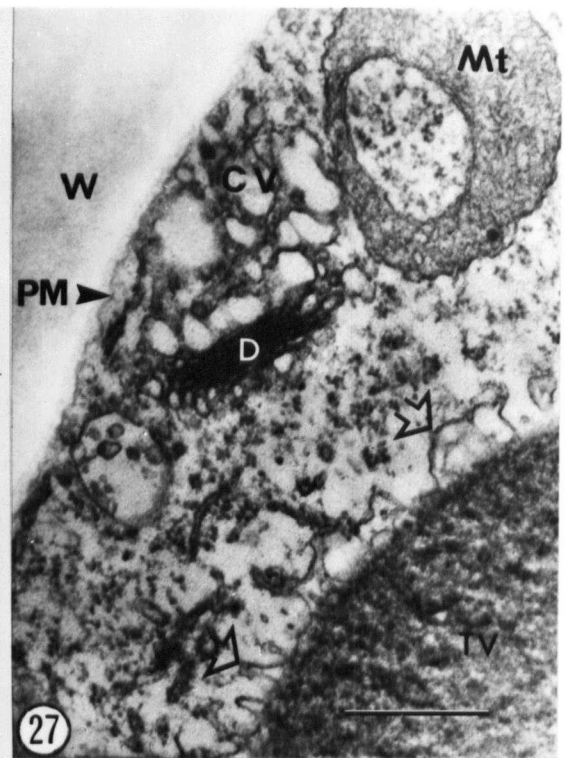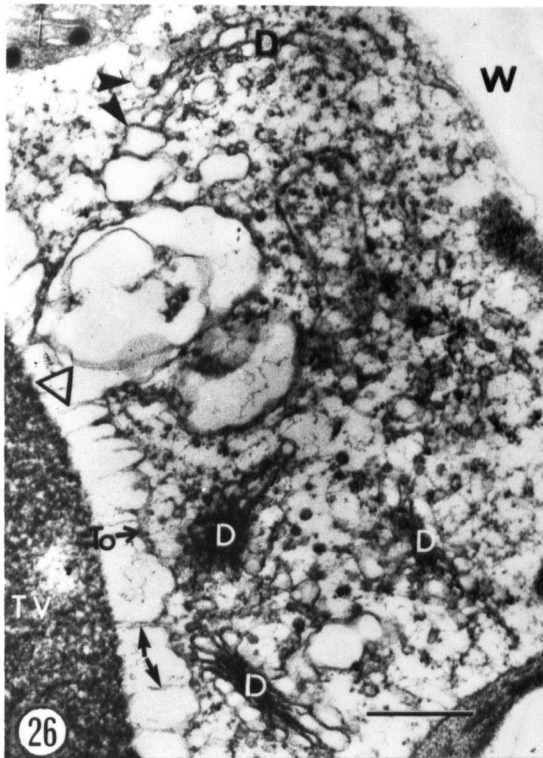
PLATE IX

Electron Microscopy of Summer Chlorenchyma: Continued

Figure 30   A nucleolus is shown from a two month old needle contain-
ing largely pars granulosum. It is separated from the adja-
cent heterochromatin by a ring of clear nucleoplasm containing
only fine fibrillar material.

A 0.5 μm scale is shown: 30,000x.

Figure 31   A region between two cells is shown with the nucleus (N)
of one cell near the cell wall of a three month old needle.
This is a 1 μm plastic section stained with osmium, toluidine
blue and Saffranin and viewed with phase contrast optics.
Three nucleoli (Nu, arrowheads) are visible, each with a clear
ring of nucleoplasm about them. The dense and irregular heter-
ochromatin is visible (Hc). Starch grains (St) are seen in
chloroplasts (Ch) spaced evenly along the cell wall. The tan-
nin vacuoles (TV), extracellular space (ES), and a trabiculum
(T) are also shown.

A 5 μm scale is shown: 3,100x.

Figure 32   Most of a section of a nucleus from a three month old nee-
dle is shown. A clearly defined nuclear envelope (NE) consist-
ing of two distinct membranes in which few nuclear pore pro-
files can be seen (arrows). The nucleoplasm contains two nu-
cleoli (Nu) and irregular dense aggregates of granular hetero-
chromatin (Hc, arrowheads). In the cytoplasm near the nucleus
rough endoplasmic reticulum (RER), an oil body (O), chloroplasts
(Ch), and the tannin vacuole (TV) are shown.

A 1 μm scale is shown: 12,000x.
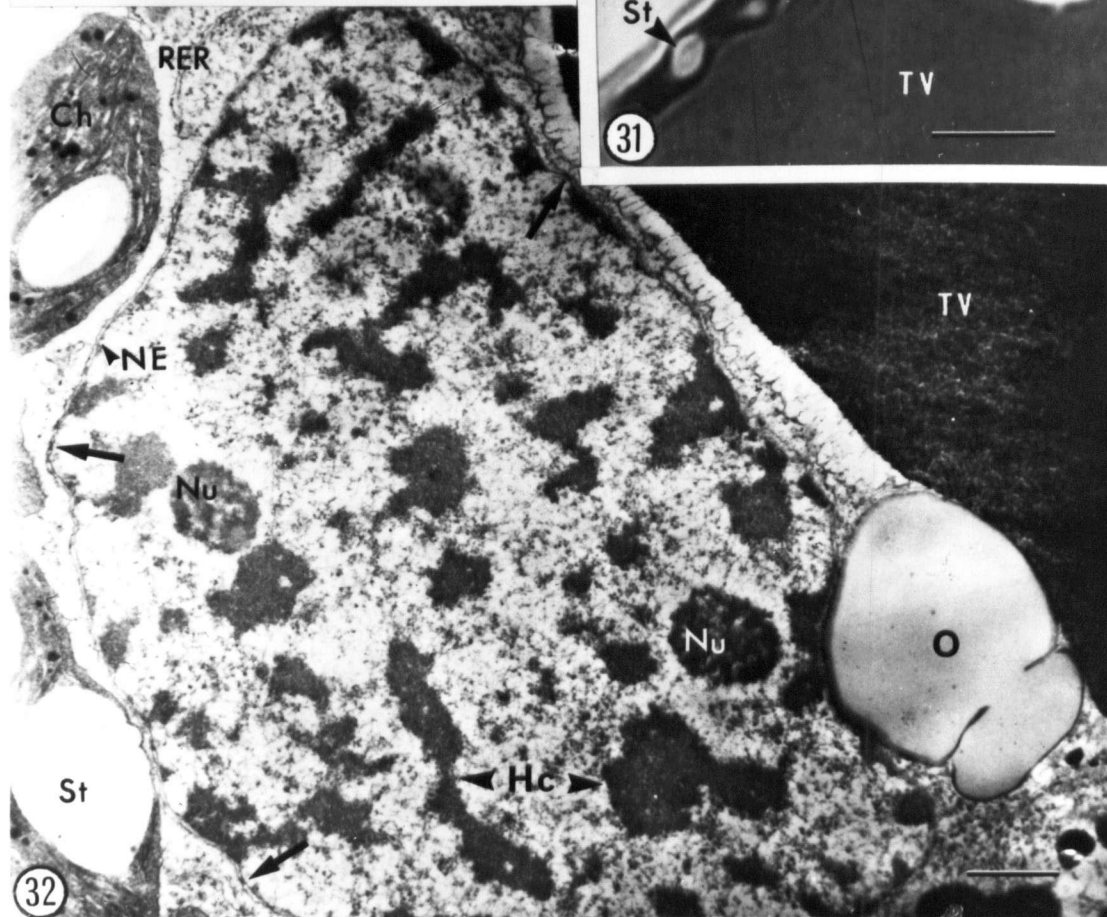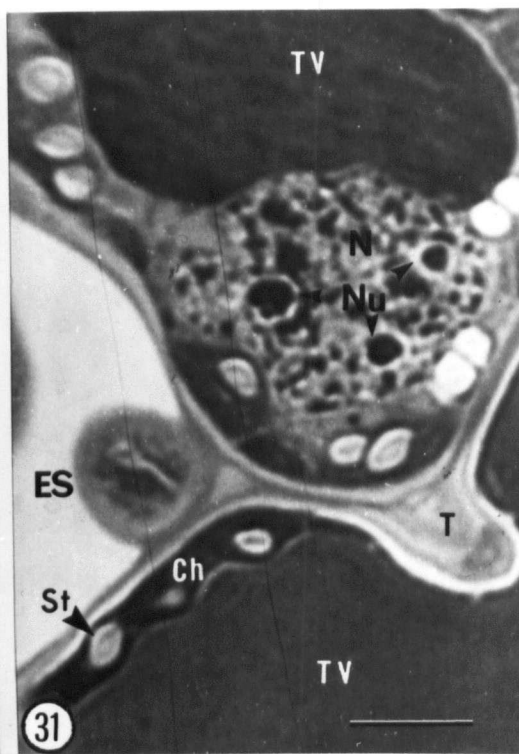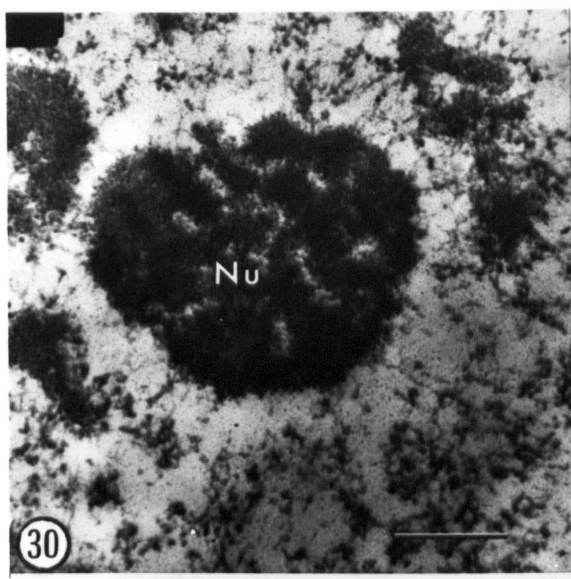
PLATE X

Light Microscopy of Winter Tissue

Figure 33
An unstained, non-fixed, frozen section of several cells is shown prepared by the procedure outlined in appendix II. Note the wide band (between arrowheads) of granular cytoplasm along the cell wall. In most areas the cytoplasm is as wide as the distance the trabiculae (T) penetrate into the cell. The only other recognizable features are the tannin vacuoles (TV) and a nucleus (N) in one cell. Nothing resembling chloroplasts can be seen. Material from eight month old needle.

A 25 μm scale is shown: 800x. Section about 20 μm thick.

Figure 34
A cryosection of cells stained and fixed in the specific formalin fixative for tannin given in appendix I. Note that there is an uniform and dramatic positive tannin staining for the tannin vacuoles (TV), but none in the cytoplasm, endodermis (En) or transfusion tracheid (TT). The several nuclei (N) visible are located along the cell wall. No substructure can be seen within the cytoplasm except for numerous large, highly refractile spheres (arrowheads). These stain positively with Sudan Black B.

A 50 μm scale is shown: 380x.

Figure 35
A 1 μm plastic section of two cells with osmium, toludine blue and Safranin staining is shown. The irregular nature of the tannin vacuole (TV) is an artifact due to tearing as a result of sectioning. Note the extensive regions of vacuolated cytoplasm (CV). Some osmiophilic bodies (arrowheads) are large enough to be seen amongst the cytoplasmic vacuoles. Note the large number of chloroplasts which are found in the chloroplast clumps (CC). Note that the dark thylakoid system of each chloroplast is contrasted against a surrounding lightly stained region of stroma. FH = part of a fibrous hypodermal cell.

A 10 μm scale is shown: 1,700x.

Figure 36
One cell and parts of several others are shown in this figure of an unfixed and approximately 20 μm cryosectioned material stained with Sudan Black B. Note the several very large oil droplets (O) found within the cell. T = long trabiculae of the cell.

A 10 μm scale is shown: 1,400x.
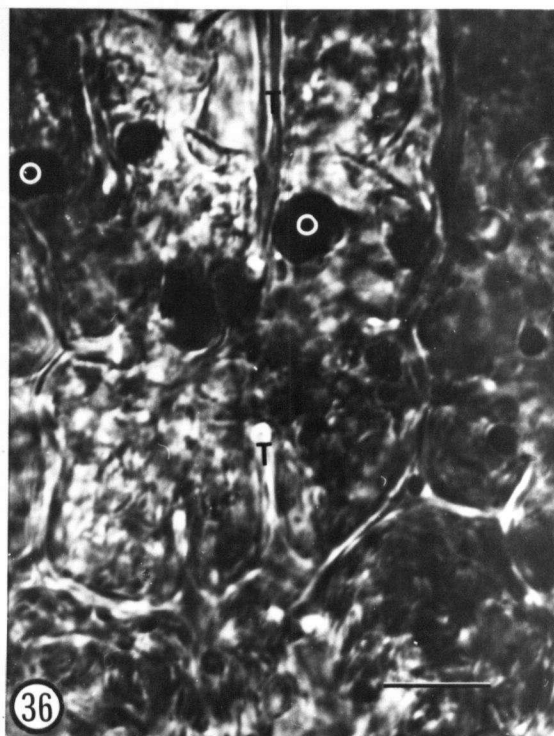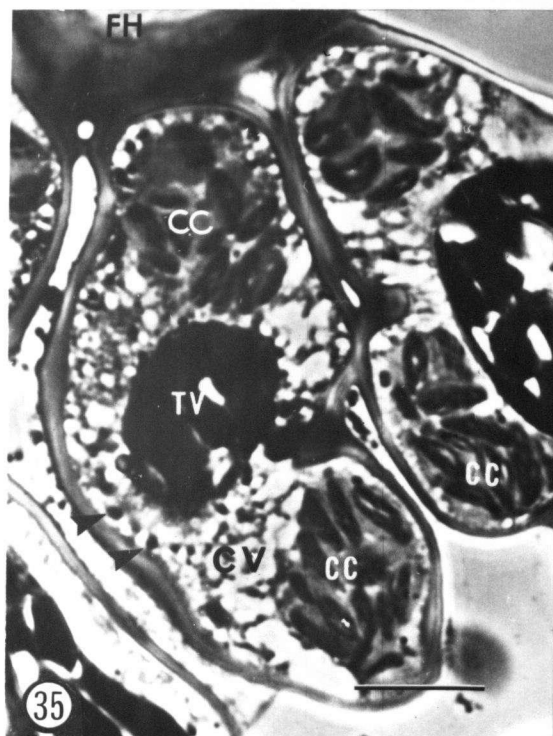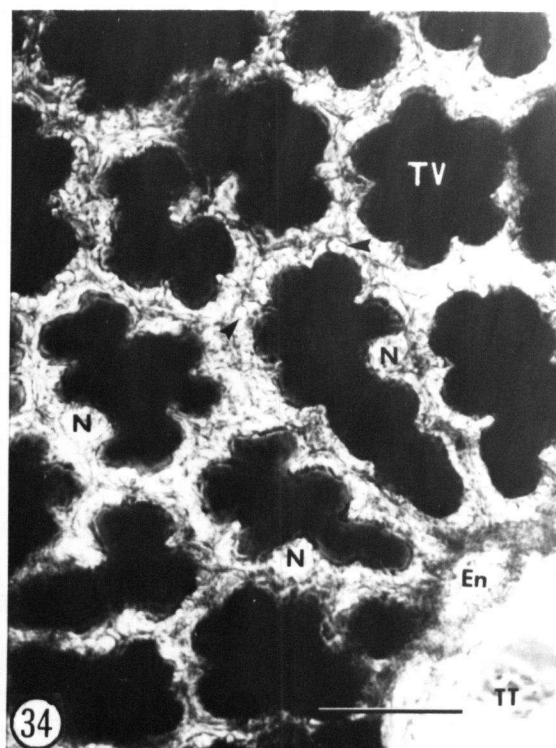
PLATE XI

Electron Microscopy of Winter Chlorenchyma

Figure 37   A needle tangential section showing several cells in the
plane of figure 5. Note how the tannin vacuoles (TV) are
rounded and do not follow the contours of the cell wall (W)
with the resultant irregular sectional width of the cytoplasm.
Small chloroplast clumps (CC) are found along the walls cor-
responding to face B (figure 2) while few organelles are found
along the walls corresponding to face A (figure 2), asterisk.
Circular mitochondria are found about the chloroplast clumps
(Mt, arrowheads). The open arrow points to blebs of tannin
along the edge of the tannin vacuole. OB = osmiophilic bodies,
ES = extracellular space, n = nucleus, seen in the lower left
hand corner. Needle, twenty months old.

A 5 μm scale is shown: 4,400x.
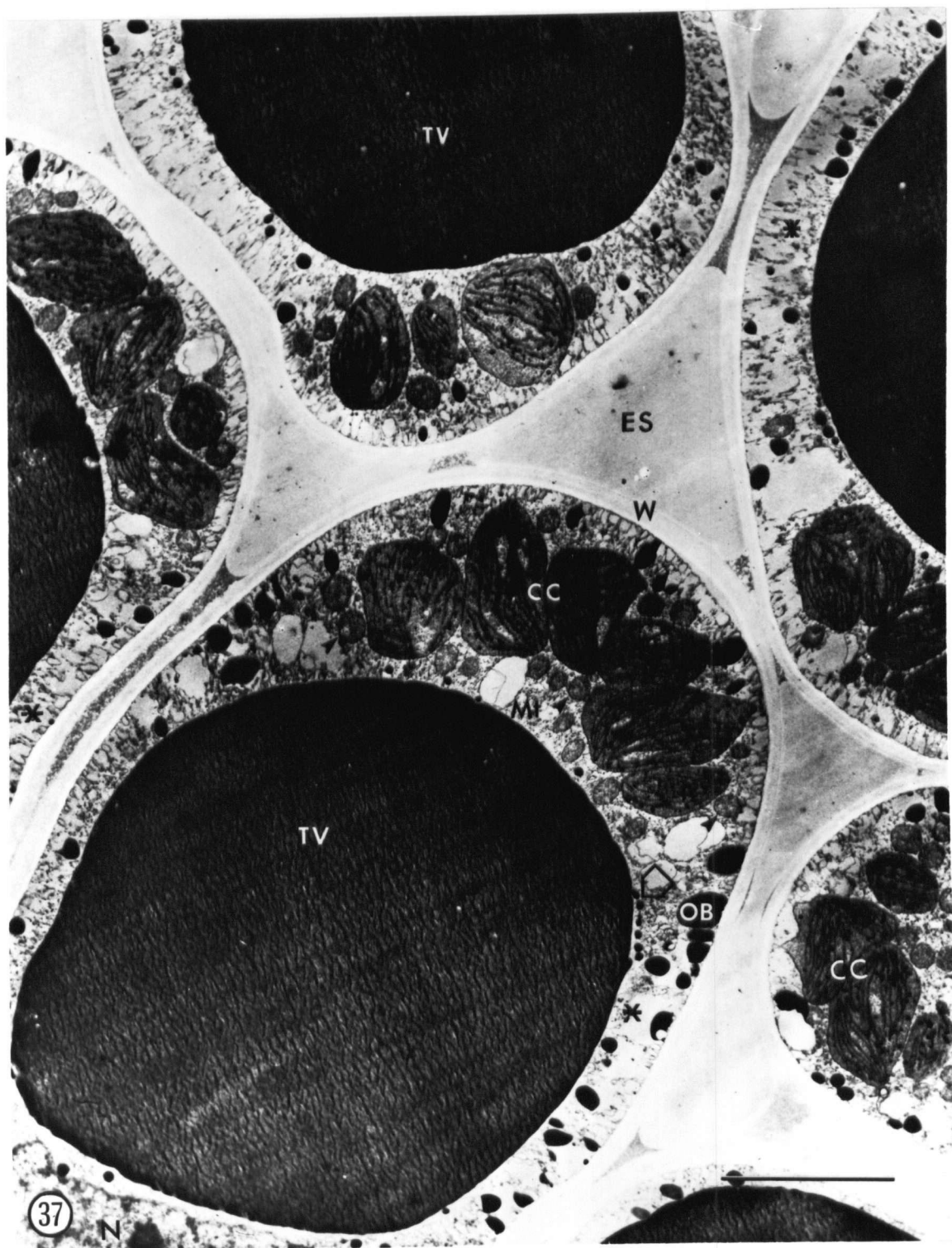
PLATE XII

Electron Microscopy of Winter Chlorenchyma: Continued

Figure 38   Cell section in the plane of figure 6 showing nearly an
entire cell from a nine month old needle. Note that the lar-
gest chloroplast clumps are in the ends of the cell (left and
right parts of the figure). Cytoplasmic vacuoles tend to be
orientated normal to the tannin vacuole (TV) surface (double
ended arrows). The cytoplasmic vacuoles are also associated
with the osmiophilic bodies. An oblique section of a short
trabiculum (T) is also shown.

A 5 μm scale is shown: 4,400x.

Figure 39   Cell section in a plane similar to figure 3 showing part
of a eight month old needle. The chloroplast clumps (CC) are
located in the ends of the cell (to the left of the figure)
and along the wall corresponding to face B, figure 2 (clumps
in the center and to the right, near the short trabiculae (T)).
A large oil body is seen (O). Note how close the tannin va-
cuole (TV) comes to the ends of the trabiculae. The edge of
the tannin vacuole has fragmented into some smaller pieces of
tannin material (arrowhead). OB = osmiophilic bodies. ES =
extracellular space filled with what appears to be cellular
debris.

A 5 μm scale is shown: 46,000x.
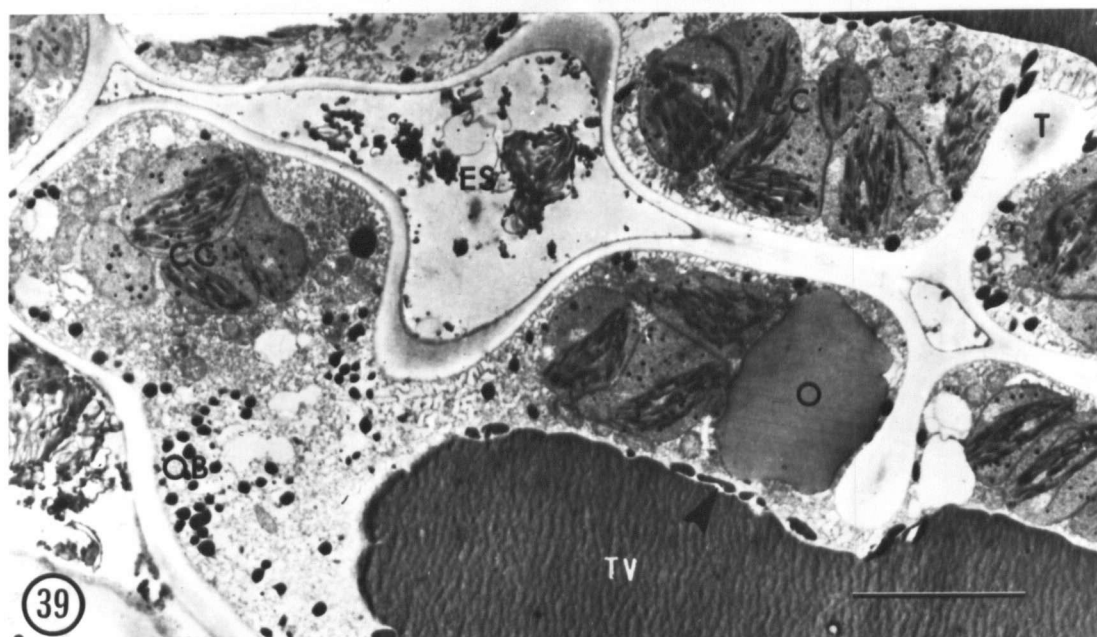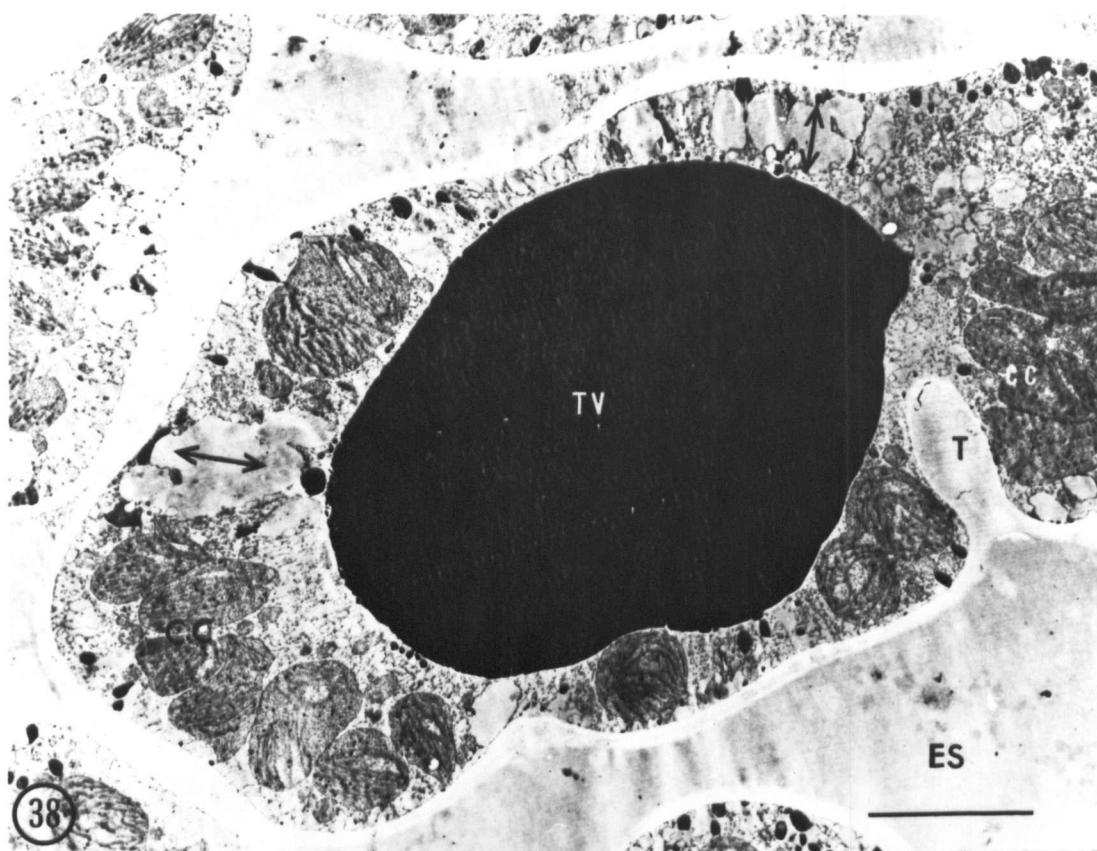
PLATE XIII

Electron Microscopy of Winter Chlorenchyma: Continued

Figure 40   A tangential section of an eight month old needle (appro-
ximately the plane of figure 4). Note the large chloroplast
clumps (CC) consistently found near the end cell walls of these
cells. This section has just cut into the surface of a tannin
vacuole (TV). Note the irregular surface and what appears to
be tannin blebs (solid arrowheads) in the immediately adjacent
cytoplasm. Plasmodesmata (Pd) are seen between adjacent chlo-
renchymal cells. Note that the plasma membrane is consistent-
ly appressed to the cell wall (open arrowhead).

A 5 μm scale is shown: 5,000x.

Figure 41   One of the regions of extensively vacuolated cytoplasm des-
cribed as "foamy" or "spongy" is shown by the asterisk, separat-
ing two chloroplast clumps (CC) in an nine month old needle.
Note the very small amounts of ground cytoplasm (arrowheads)
found in the regions of the most extensive vacuolation.

A 2.5 μm scale is shown: 75,000x.

Figure 42   Part of a chloroplast clump of an eight month old needle
is shown. Note how the chloroplasts are interlocking with each
other with no open spaces between adjacent plastids. The junct-
ional regions between the chloroplasts appear to consist of se-
veral parallel membranes (solid arrowheads). Between the chlo-
roplasts and the motochondria, there is a uniform gap of cyto-
plasm (open arrowheads). Most of the irregular shaped portions
of the chloroplasts consist of stromal regions (S) containing
no thylakoids. Note two thylakoid systems (each labelled with
an asterisk) lie at right angles to each other within one chlo-
roplast envelope.

A 1 μm scale is shown: 13,000x.
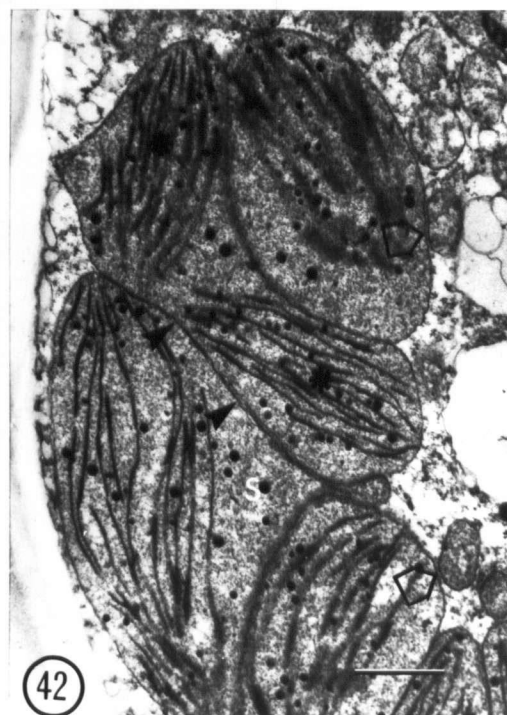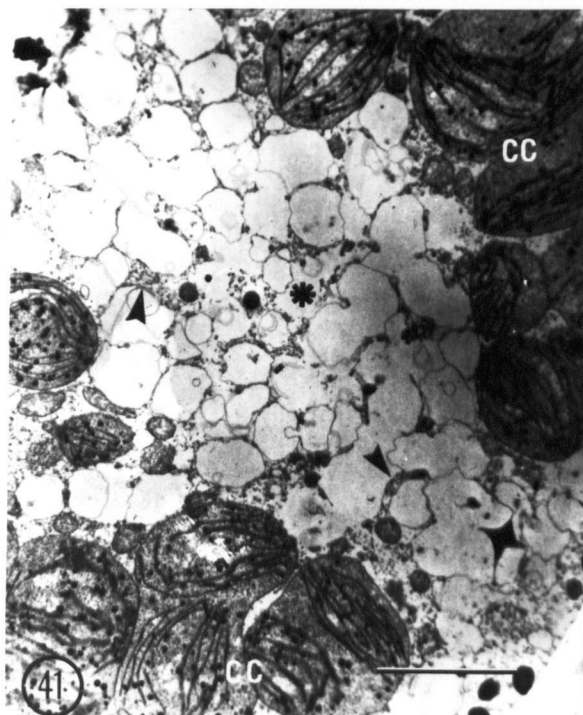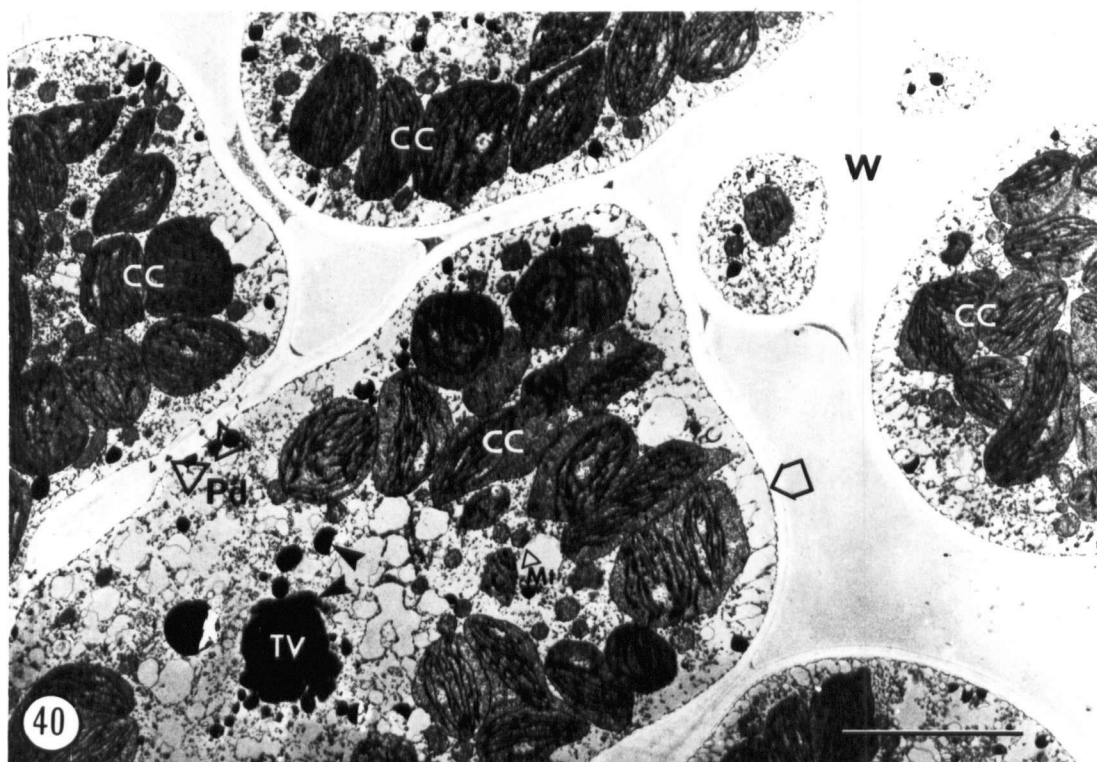
PLATE XIV

Electron Microscopy of Winter Chlorenchyma: Continued

Figure 43   Two chloroplasts from an eight month old needle are shown. The oblique section of the chloroplast envelope (CE, solid arrowhead) appears as a uniform granular area. Cross sections of the envelope at low magnification appear as single lines (open arrows). Note the two internal thylakoid systems (asterisks) lie within one chloroplast envelope, separated by a large area of stroma (S).

    A 2 μm scale is shown: 15,000x.

Figure 44   A clump of chloroplasts from a fifty-five month old needle is shown. The chloroplasts have little exclusively stromal regions, long granal areas of no more than two or three thylakoids and numerous larger plastoglobuli (Pg, solid arrowheads) are shown. The chloroplasts occasionally have contrictions or narrow isthmuses between them (open arrow). Also shown is the ground cytoplasm (GC) containing little except scattered fibillar material and a very large oil body (O). W = cell wall.

    A 1 μm scale is shown: 10,000x.

Figure 45   Part of the areas of contact between three chloroplasts in an eight month old needle. The chloroplasts envelope (CE, arrowhead) is very difficult to resolve into its two membrane components, and appears as a series of vesicles approximately 25 nm to 40 nm in diameter. In the regions of chloroplast contact, parts of three membranes can sometimes be seen (white arrows).

    A 2 μm scale is shown: 27,000x.

Figure 46   An optical magnification of the boxed region of figure 45. The tripartite nature of the membranes in the region between adjacent chloroplasts is visible (between arrowheads). Two thin outer membranes and a thicker central membrane can be seen.

    A 0.5 μm scale is shown: 54,000x.

Figure 47   A chloroplast and mitochondrion (Mt) of an eight month old needle are shown. Note the indistinct chloroplast envelope (CE, arrowhead). In contrast, the thylakoid membranes in the unusually large grannal area (G) are very distinct. Plastoglobuli (Pg, arrow) are evenly distributed throughout the plastid, even in the large stromal (S) areas. The clear region (sc) may be the starch formation center. Mt = mitochondria.
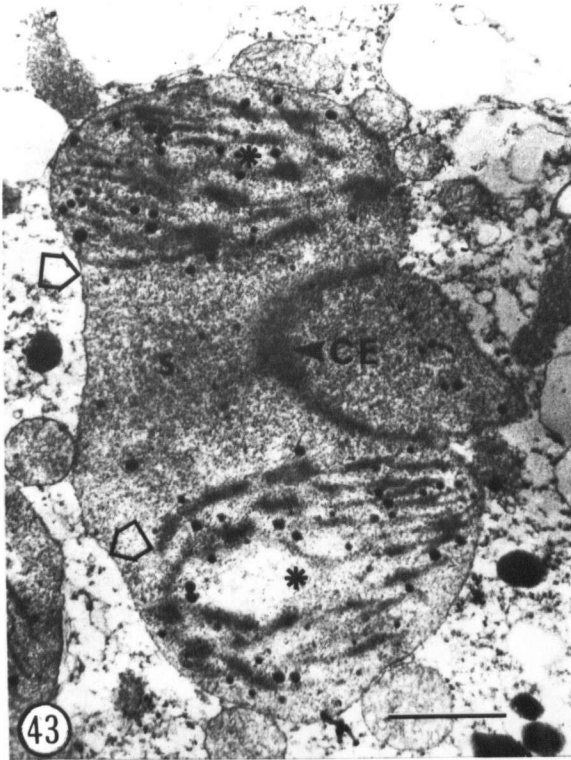
    A 1 μm scale is shown: 27,000x.
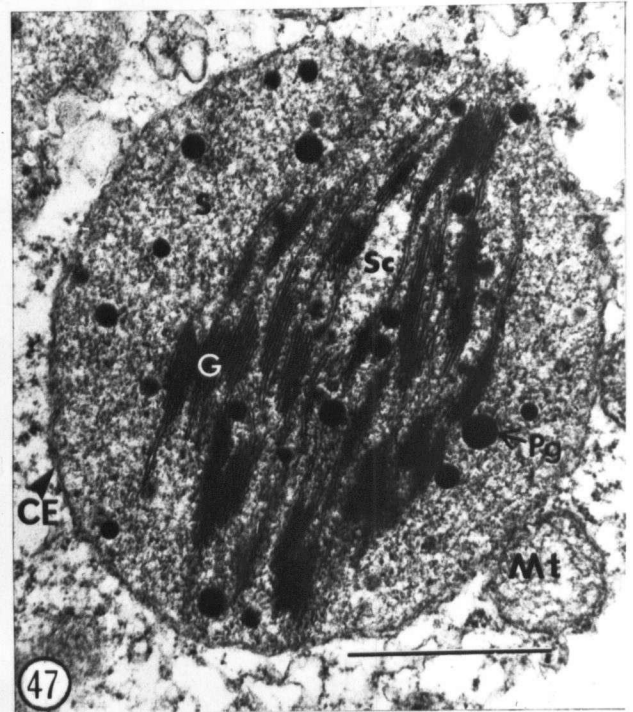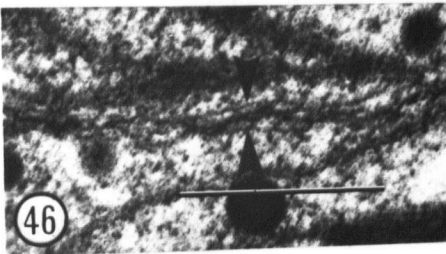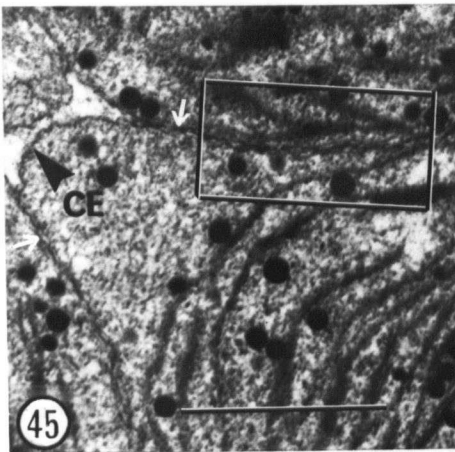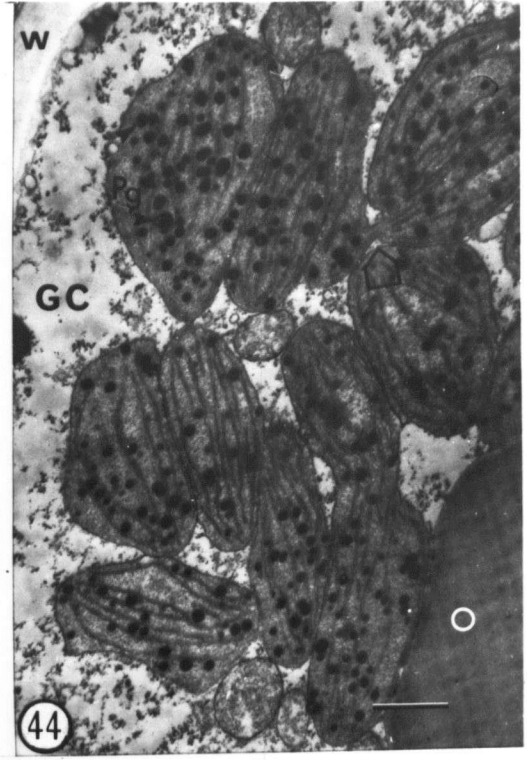
PLATE XV

Electron Microscopy of Winter chlorenchyma: continued

Figure 48   The appearance of parts of two cells from ten month old
(March) needle tissue. The single asterisk (upper right) in-
dicates a region of extensive cytoplasmic vacuolation asso-
ciated with the osmiophilic bodies (OB). In the double ast-
erisk area (center right), little osmiophilic material is seen
with the cytoplasmic vacuoles. Mitochondria (Mt, arrowheads)
are found in the single asterisk region but not in the double
asterisk region. Mitochondria are also localized in the non-
vacuolated cytoplasmic regions about the nucleus (N) and the
chloroplast clumps (CC). The homogeneity in texture and stain-
ing of the tannin vacuoles (TV) can be seen from the large
area shown. Trabiculae (T), cell walls (W), and extracellular
space (ES) are also shown.

A 5 μm scale is shown: 6,000x.

PLATE XVI

Electron Microscopy of Winter Chlorenchyma: Continued

Figure 49   A cytoplasmic vacuole (CV) is shown, lined with the mat-
erial (arrowheads) from the osmiophilic bodies, which in turn
line the cytoplasm adjacent to the cell wall (W) in an eight
month old needle. Part of a characteristic winter nucleus (N)
and chloroplasts are also shown.

A 1 μm scale is shown: 10,500x.

Figure 50   A series of cytoplasmic vacuoles starting from the cell
wall (W) and extending to the tannin vacuole (TV) and separa-
ting two chloroplasts clumps (CC) in an eight month old needle
are shown. Note the vacuoles are associated with an osmiophi-
lic body (OB, arrowhead). There is dense ground cytoplasm (GC)
immediately about the chloroplasts.

A 1 μm scale is shown: 12,000x.

Figure 51   A section of a six month old needle at right angles to the
cell wall. Note the osmiophilic bodies (OB, arrowhead) predom-
inently distributed in a tier in the cytoplasm adjacent to the
cell wall (W). The cytoplasmic vacuoles between the chloro-
plast clumps (CC) and the tannin vacuole (just out of the low-
er left of the figure) appear stretched in the direction of
the double ended arrow.

A 2.5 μm scale is shown: 6,000x.

Figure 52   A section tangential to the dome-shaped end wall (face C,
figure 2) of an ten month old cell. It can be seen that the
osmiophilic bodies (OB, and arrowheads) are found predominently
near the cell wall (W). Cytoplasmic vacuoles (CV) are associat-
ed with the osmiophilic bodies and also deeper in the cell
where there is extensive cytoplasmic membranes, many of which
appear like smooth endoplasmic reticulum (SER, arrowhead). Two
chloroplasts (Ch) are also shown.
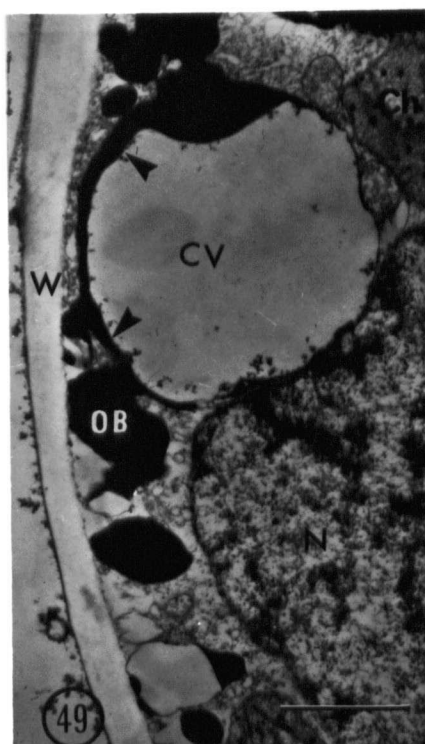
A 2.5 μm scale is shown: 6,000x.
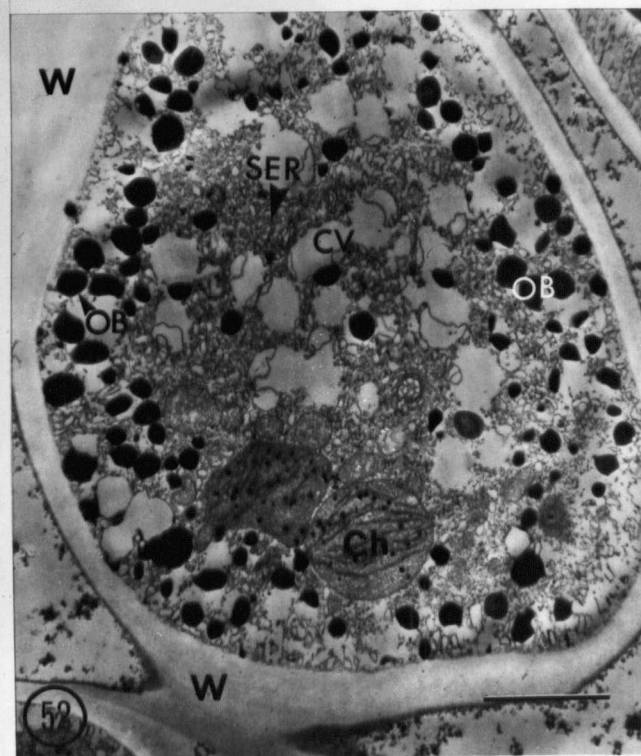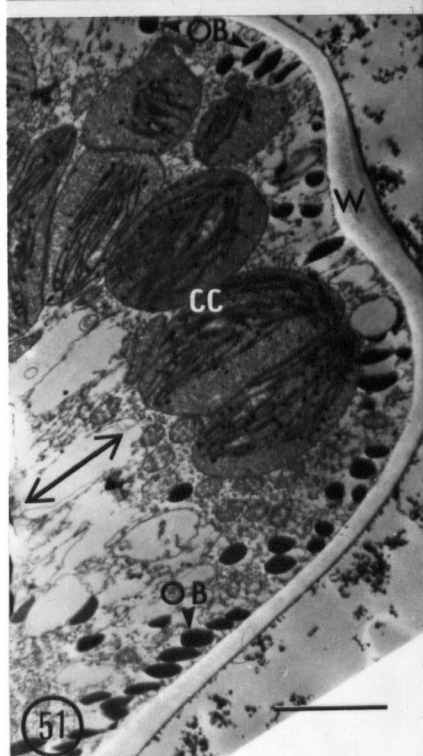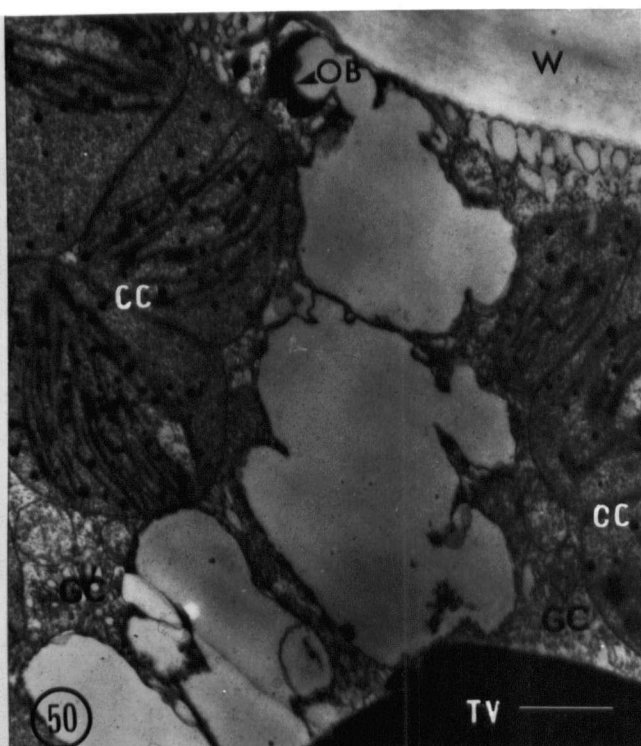
PLATE XVII

Electron Microscopy of the Winter Chlorenchyma: Continued

Figure 53   A mitochondrion (Mt) of an eight month needle is shown.
The mitochondrial envelope (arrowhead) appears as a single irr-
egular dark line. Tubular cristae (Cr, arrow) are indistinct
and not as numerous as in summer.

A 0.5 μm scale is shown: 38,000x.

Figure 54   A dictyosome (D) from a nine month old needle is shown.
Note that the budding viscles are all attached to the organel-
le and there are few dictyosome associated vesicles in the sur-
rounding cytoplasm.

A 1 μm scale is shown: 12,500x.

Figure 55   This is a 1 μm plastic section stained with osmium, tolu-
dine blue and Safranin, and photographed with phase contrast
optics. A winter nucleus (N) is shown with an oil body (O)
and the tannin vacuole (TV) nearby, in an eight month old nee-
dle. Note the evenly dispersed granularity of the darkly stain-
ed heterochromatin and also a single nucleolus (Nu) is shown.

A 5 μm scale is shown: 5,500x.

Figure 56   The nucleolus (Nu) and part of the nuclear envelope (NE,
arrowhead), containing numerous dense nuclear pores are shown.
Tissue is from an eight month old needle. The heterochromatin
(HC) appears to have a granular substructure.

A 1 μm scale is shown: 28,000x.

Figure 57   An entire winter nucleus (N) is shown between two chloro-
plast clumps (CC) in an eight month old needle. A small nucle-
olus (Nu, arrowhead) appears in this section and the small
clumps of granular heterochromatic (HC) appear evenly distribut-
ed in the nucleoplasm. Several cytoplasmic vacuoles (CV) are
shown.

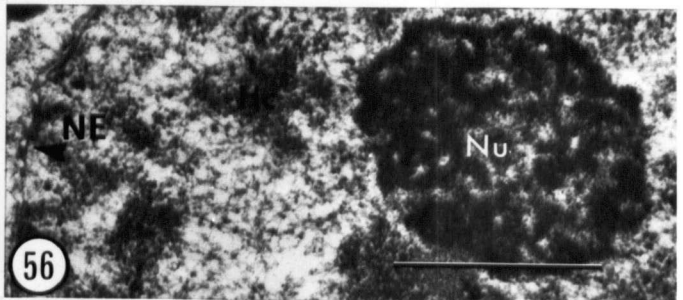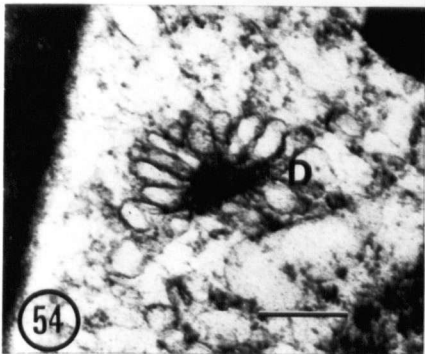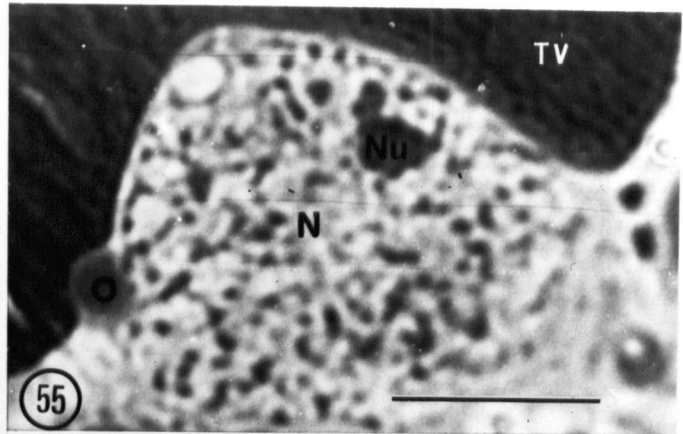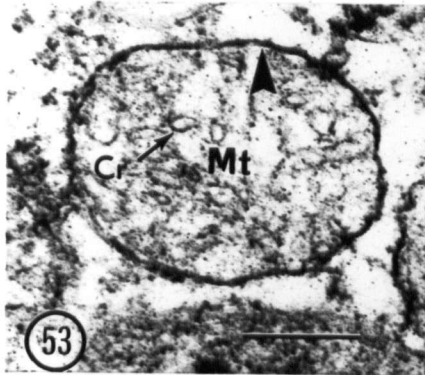A 2.5 μm scale is shown: 9,000x.
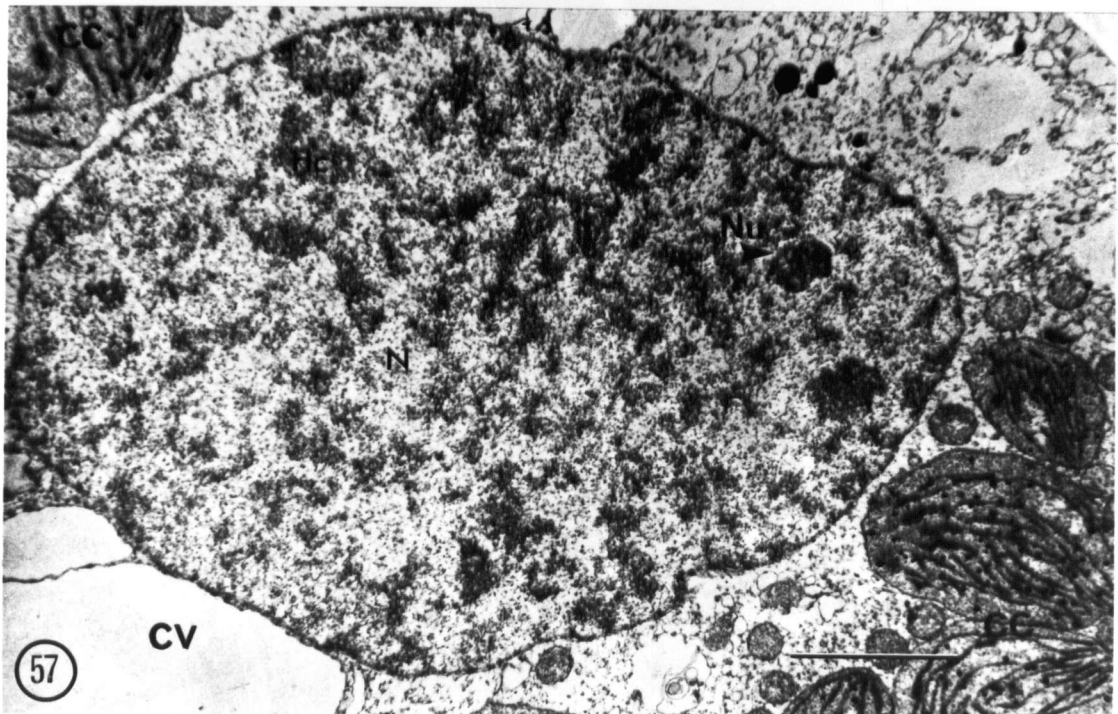
PLATE XVIII

Electron Microscopy of the Winter Chlorenchyma: Continued

and Winter Variations in Preparative Procedures

Figure 58   A large oil body (O) is shown next to the nucleus (N) in
a nine month old needle. Several mitochondria (Mt) are usual-
ly found near the oil bodies in spring. But in winter, this
observation is unusual. Note the more extensive tubular cris-
tae than usual. TV = tannin vacuole.

A 2 μm scale is shown: 12,500x.

Figure 59   The variations in winter fixations between cells are shown
in this electronmicrograph of a thirty -two month old needle.
Cell A shows a clump of chloroplasts in the normal winter fix-
ation. In cells B and C (they may be the same cell), the cyto-
plasm is filled with tannin material possible due to lysis of
the tonoplast. In cell D, all protoplast structures are brok-
en down. Cells B, C and D may be the winter correlates of
type III cells.

A 5 μm scale is shown: 4,000x.

Figure 60 to 62 are from material stored within a freezer for several months
at temperature -18°C after collection at below freezing temperatures in mid-
winter.

Figure 60   A clump of chloroplasts (CC) from a nine month old needle fixed
in sea water buffer is shown. Note the consistently elongated
form of the chloroplasts and the reduced areas of close cont-
act (arrowhead) between adjacent chloroplasts. The tannin
vacuole (TV) and two cell walls (W) are also shown.

A 2.5 μm scale is shown: 7,000x.

Figure 61   Part of a cell fixed in the sea water buffer from a nine
month old needle showing the change in the shape of osmiophilic
bodies (OB, arrowhead) to a stellate form. Two chloroplasts
(Ch) are shown, one with a small starch grain (St) which is
unusual in winter material . W = cell wall.

A 1 μm scale is shown: 12,000x.

Figure 62   The appearance of substructure in the form of darker cores
(arrowheads) in the osmiophilic bodies (OB) after this nine
month old winter tissue had been immersed into liquid nitrogen.
Some cytoplasmic vacuoles (CV) are associated with the osmio-
philic bodies and dictyosomes (D), a chloroplast (Ch) and a
trabiculum (T) are also shown.
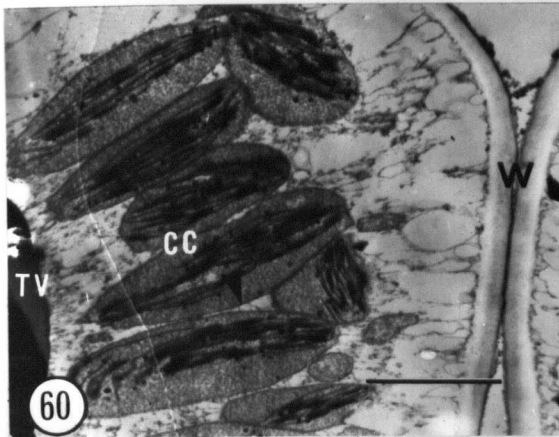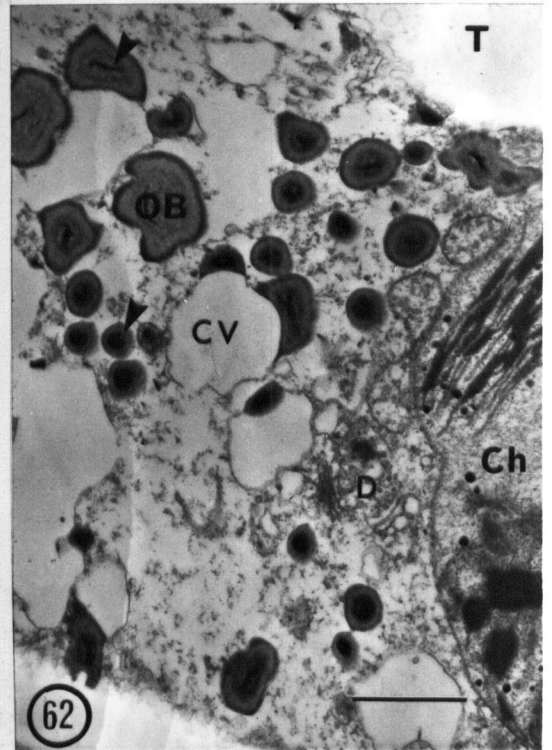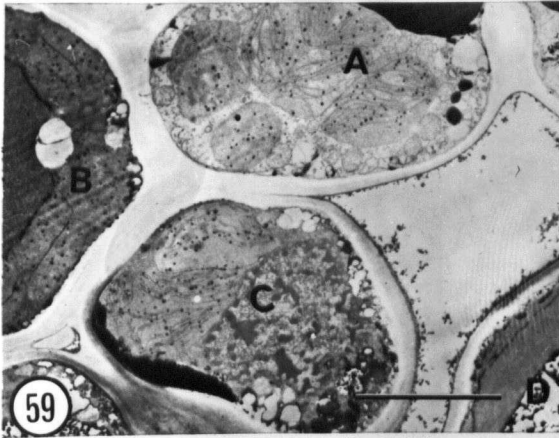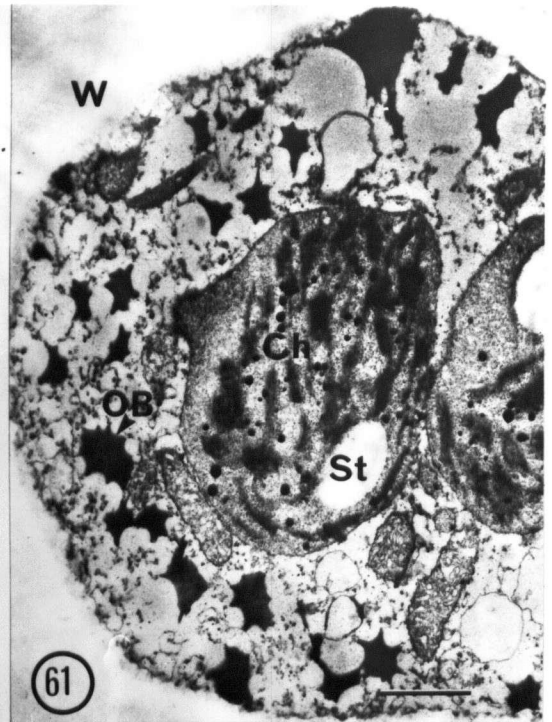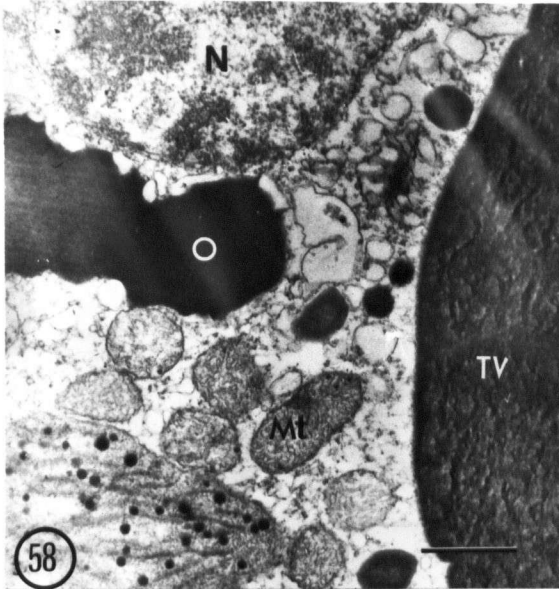
A 2 μm scale is shown: 15,000x.

PLATE XIX

Electron Microscopy of the April 27th 1973 Fixation

Figure 63
Several chloroplasts (Ch) are shown along the cell wall (W). One chloroplast (with the starch grain (St)) is not near the wall. Note occasional dilations of the thylakoids and the even distribution of stroma about the thylakoids. A 25 to 50 nm gap is always observed in this tissue (solid arrowheads) between chloroplasts. Note the lobed mitochondria (Mt, arrowhead) and the cytoplasmic vacuole (CV) without any associated osmiophilic material in the dense ground cytoplasm (GC). (open arrowhead). PM = plasma membrane. TV = tannin vacuole.

A 1 μm scale is shown: 10,000x

Figure 64
This electron micrograph is of the boxed area in figure 63. Note the consistent gap of cytoplasm (Cy, and between arrowheads), between the two chloroplasts. Grana in these chloroplasts consists of only two or three thylakoids (arrows).

A 0.5 μm scale is shown: 30,000x.

Figure 65
When the spring chloroplasts (Ch) are sectioned in the plane of the thylakoids, large numbers of plastoglobuli (Pg) are occasionally seen to be associated with the thylakoids. Note the irregular shape of the chloroplasts and the consistent gap between adjacent plastids (open arrowheads).

A 5 μm scale is shown: 20,000x.

Figure 66
A nucleus (N) in spring tissue is shown. The heterochromatin (Hc) is in an irregular dense formation typical of the summer condition. The tannin vacuole (TV), an oil body (O) and a trabiculum (T) with chloroplasts (Ch) along it are also shown.

A 5 μm scale is shown: 6,000x.

Figure 67
A low magnification of part of a cell showing the extensive areas ground cytoplasm (GC) and occasional cytoplasmic vacuoles (CV). Note that the tannin vacuole (TV) barely enters this section. The cytoplasm contains large amounts of osmiophilic material ranging in size from less than 0.5 μm bodies (usually found along the cell wall, open arrowheads) to larger irregular bodies (solid arrowheads) to still larger oil-like bodies greater than 2 μm in diameter. Chloroplasts (Ch) are often irregular in shape but do not have large stromal areas. They are generally found close to the cell walls and many have starch grains (St). Mitochondria (Mt) frequently have lobed and irregular sectional profiles.
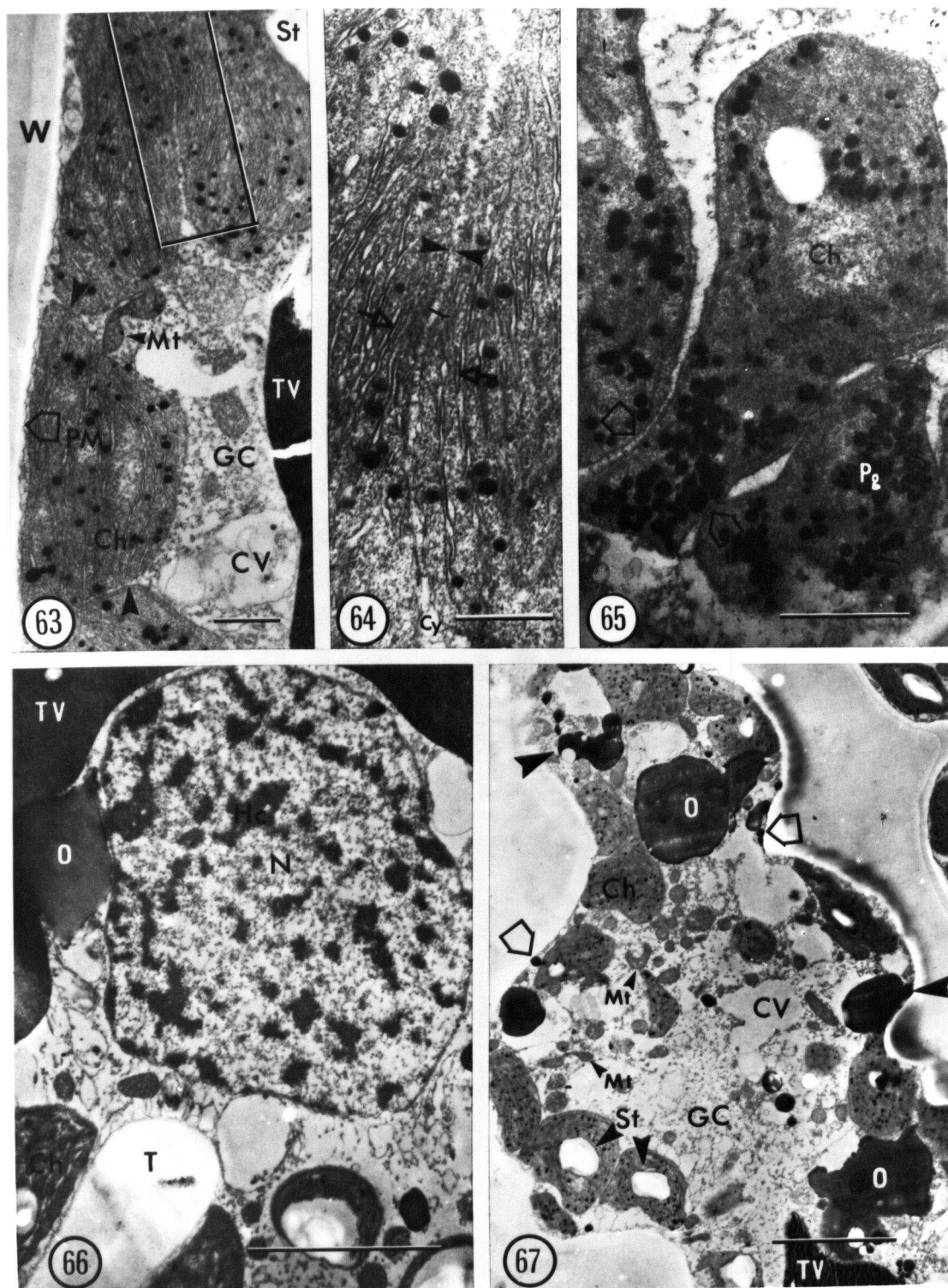
A 5 μm scale is shown: 4,000x.

63

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PLATE XX

Electron Microscopy of Chlorenchyma: Spring Fixation
and Material Prepared by Various Procedures

Figure 68   Two types of osmiophilic cytoplasmic inclusions seen in the
April 17th, 1973 fixation are shown. At the right, a large oil-
like inclusion (O), and cluster of even more osmiophilic mater-
ial to the left (O). The latter material has an appearance sim-
ilar to the winter osmiophilic bodies. Cytoplasmic vacuoles
(arrowhead) were occasionally seen associated with the latter
structures. W = cell wall.

A 5 μm scale is shown: 6,000x.

Figure 69   The nucleus (N), chloroplast (Ch), and tannin vacuole (TV)
are shown in a three year old summer needle. The nuclei in old-
er needles are characterized by the rarity of nucleoli in sect-
ions, and extensive, dense heterochromatin (HC). Acrolein fixa-
tion.

A 5 μm scale is shwon: 4,500x.

Figure 70   Part of a chlorenchyma cell from a five year old needle
fixed in the acrolein fixative solution. The tannin vacuole (TV)
and a large osmiophilic oil-like body (O) are shown. The cyto-
plasm is poorly stained but chloroplasts (Ch), containing numer-
ous plastoglobuli and starch grains (St) can be seen. W = cell
wall.

A 5 μm scale is shown: 5,700x.

Figure 71   Part of a chloroplast (Ch), and mitochondria (Mt) are shown
from an acrolein fixed three year old summer needle. The chlo-
roplast is unusual in having a few plastoglobuli and some gra-
na (G, arrowhead) containing more than three thylakoids. The
cytoplasm above the organelles contains extensive osmiophilic
material. W = cell wall.

A 1 μm scale is shown: 22,800x.

Figure 72   Mature summer needle tissue is shown after severe freezing
(-18°C). All protoplast structural organization is lost in this
tissue. Tannin material (TM) is found right up to the cell wall
(W). The material shown may have been a chloroplast since struc-
tures resembling a starch grain (St) and possible plastoblobuli
(Pg?) are shown.

A 1 μm scale is shown 13,800x.

PLATE XXI
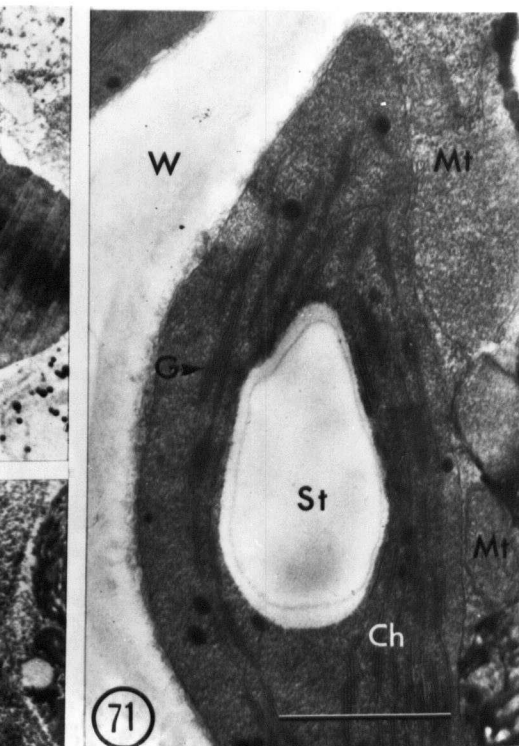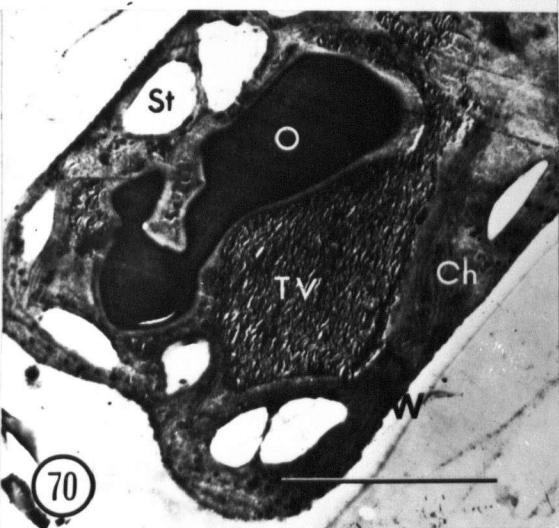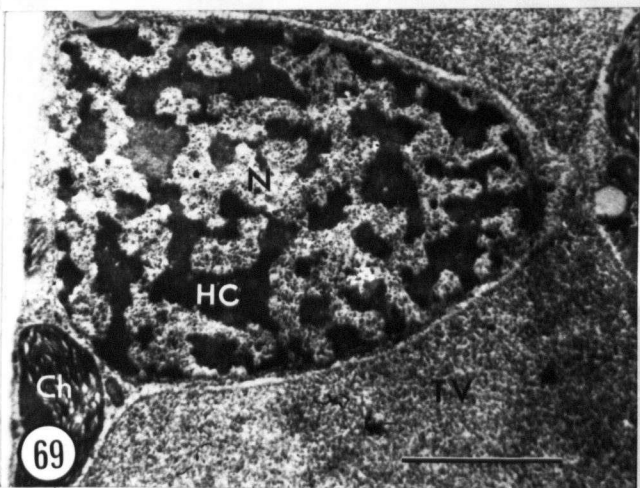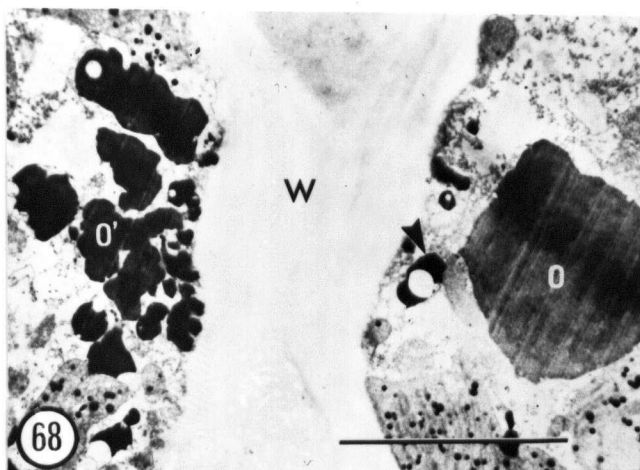
Studies on Pinus contorta Seedlings Subjected to

Various Treatments in Controlled Environment Chambers

Figures 73 and 74 are both of summer needles from a tree in which frost hardiness was partially induced. This tissue was fixed by the winter procedure at -4°C.

Figure 73   Part of the cytoplasm is shown. Cytoplasmic vacuoles (CV) are very numerous, and are associated with the osmiophilic bodies (OB, and arrowheads). Tannin material (TM) of the same density and texture as the tannin vacuole material is often scattered in the cytoplasm in small vacuoles. Mt = mitochondria.

             A 1 μm scale is shown: 26,000x.

Figure 74   Two chloroplasts (Ch, A and B) are shown which may be fused along the outer envelope membranes. Note the absence of starch in this material. The cytoplasm contains many vacuoles with no discernable contents (asterisk) Mt = mitochondria. W = cell wall.

             A 1 μm scale is shown: 18,000x.

Figure 75   Two of the trees (pot A) in which frost hardiness was artificially induced are shown in pots one week after exposure to -18°C. Visible damage to the trees consisted of the yellowing of the youngest needles (open arrowhead, shows lighter shaded needles), most of which eventually died. Second year needles (solid arrowhead) remained green and were used as the tissue shown in figures 73 and 74. Pot B contains a control tree which had no hardiness inducement treatments. The tree is dead and the needles are dried out and falling off. This tree was exposed to the -18°C frost at the same time as pot A.

             A 10 cm scale is shown: trees are one fifth natural size.

Figure 76 and 77 are of summer trees which have been exposed to warm temperatures and no water for 30 days.

Figure 76   Note that the chloroplasts (Ch) have detached from the cell wall (W). The plasma membrane (PM, arrowhead) is still along the cell wall. Osmiophilic bodies (OB, arrowheads) are more numerous than in normal summer tissue.

             A 1 μm scale is shown: 10,000x.

Figure 77   The perinuclear cytoplasm contains endoplasmic reticulum (ER), circular mitochondria (Mt), an unusual constricted (open arrowhead) chloroplast (Ch) containing a small starch grain (St). The nucleus (N) contains the same type of chromatin arrangement seen in spring and summer. A small oil body (O) is also seen. W = cell wall.

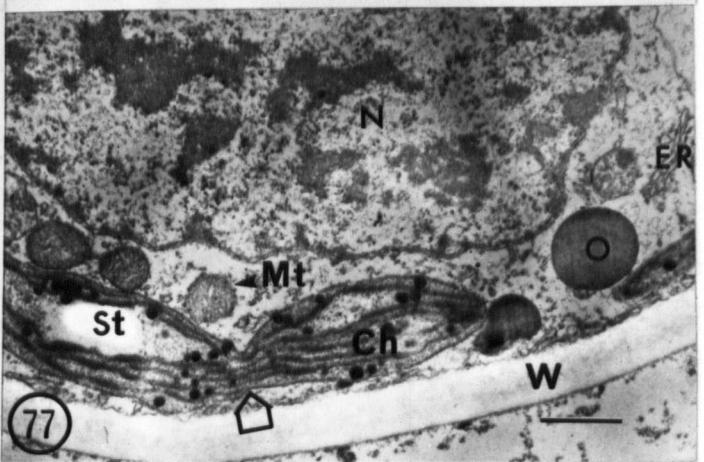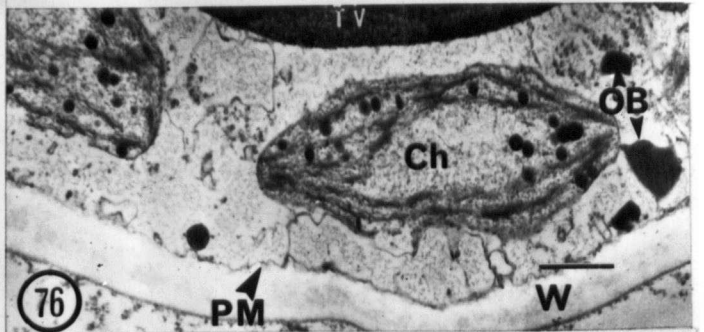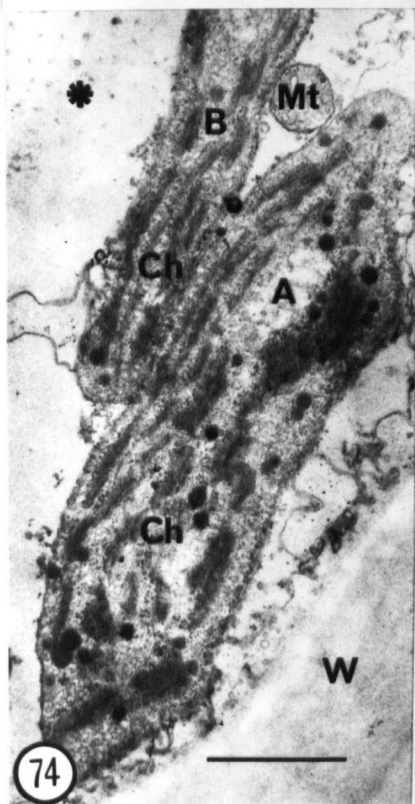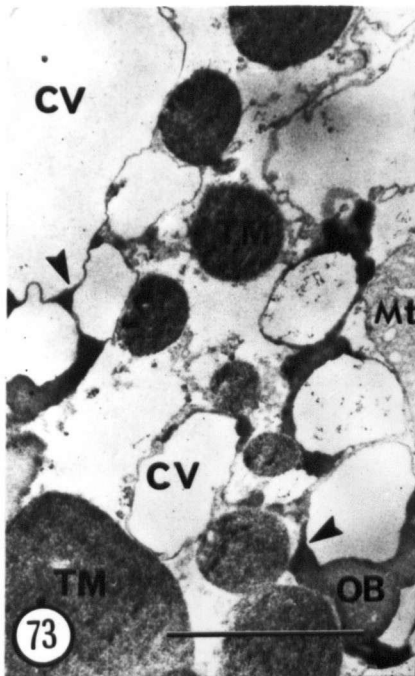   A 1 μm scale is shown: 11,000x.

PLATE XXII

Apparatus for Frozen Section Observation

Figure 78   The apparatus for the sectioning of material kept continuously frozen. Labelled items are: (A) - brine-ice cooling bath; (B) - CO_2 bottle; (C) - sectioning arm of cryotome with liquid nitrogen reservoir on the knife; (D) - foil ladel; (F) - mounting media (gum arabica); (G) - insulated forcepts; (H) - plastic coverslips cut into small squares; (I) - vacuum grease; (J) - steel cold sink; (K) - Dewar for liquid nitrogen storage.

A 10 cm scale is shown, apparatus is one fifth actual size.

Figure 79   The appartus for the observation of frozen sections. Objects not described above are: (L) - the cryomicroscope stage (which is diagrammatically shown in figure 80); (M) - liquid nitrogen reservoir; (N) - freon bottle; (O) - Dewar of liquid nitrogen to liquify freon gas; (P) - microscope camera.

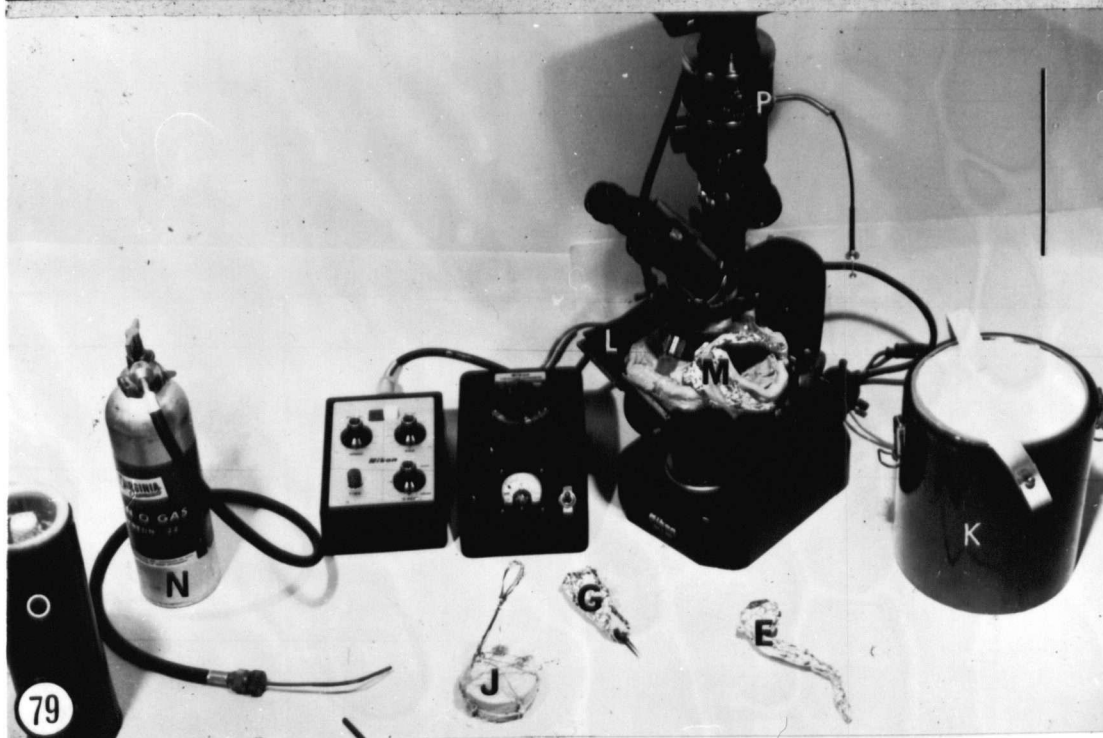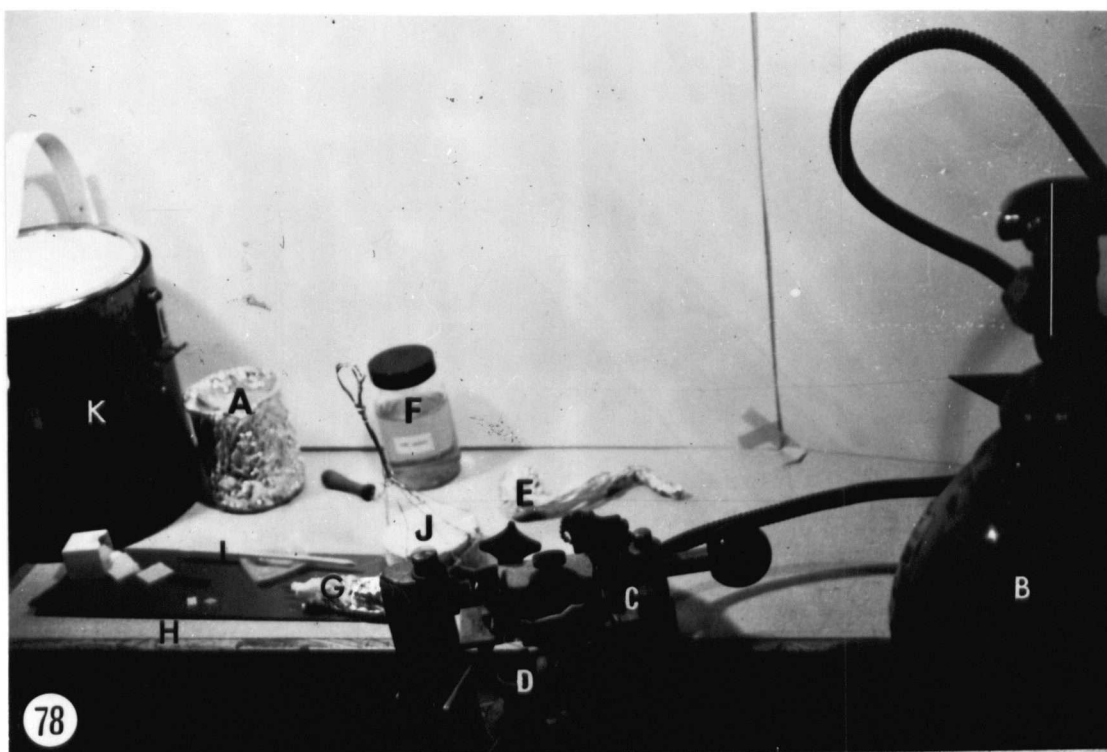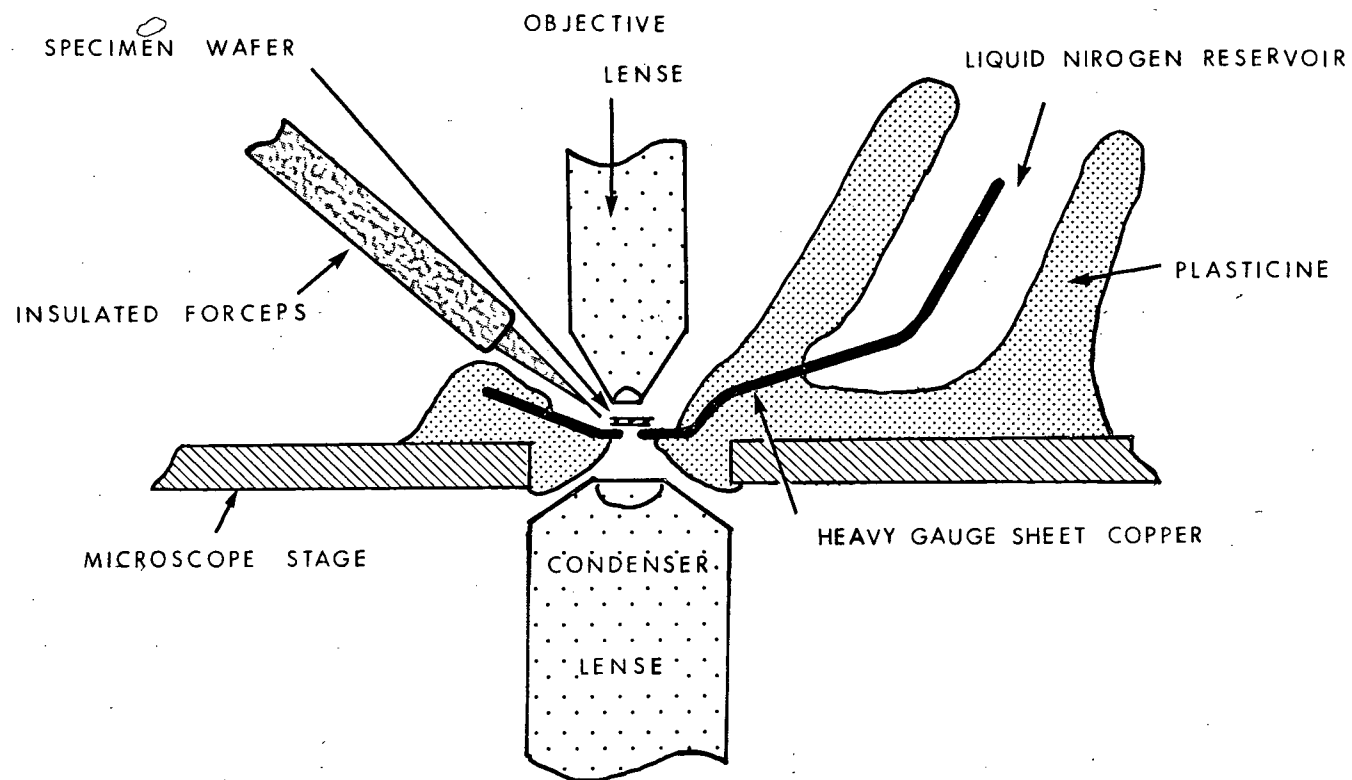A 15 cm scale is shown: apparatus is shown at one sixth actual size.

Figure 80   Diagram of the cryomicroscope stage apparatus. A cross-section is shown.

FIGURE 80: DIAGRAM OF THE CRYOMICROSCOPE STAGE

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