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COLD RESISTANCE IN WOODY PLANTS[1]

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[1] The word "cold" is used in preference to "frost" since in America frost does not refer to very low temperatures as it does in Britain or Germany. Chilling injury, on the other hand, is not the subject of this review, although mentioned. "Woody plants" include here especially forest trees and some horticultural woody plants.

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INTRODUCTION

Woody plants, as a result of their life-form, must grow year after year in the same location and they must, therefore, be able to withstand great temperature variations in some climates. Since these sessile organisms survive only under conditions favorable to them, they become standing indicators of the environmental conditions in any particular place. Although soil may play an important role in plant survival, pronounced edaphic effects are comparatively local in the over-all picture.

It is generally agreed that climate exerts the most important controlling influence on the distribution of organisms on the earth, and to some extent, in the oceans. Of the various factors of climate, temperature often plays a leading role in its effect on the distribution of plants. There are many relationships between temperature and the natural distribution of certain plants but it is difficult to state which phase of temperature exerts the important effect.

Temperature by itself is perhaps an easy concept for the layman to grasp, since he feels heat and cold directly through his epidermal "punctiform" receptors and his nervous system. But it is not so easy to explain what temperature is to a scientist. The state of excitation or vibration of molecules is relatively simple, but the basic physics that causes vibration and caloric transfer indulges itself in a realm that advances quickly beyond our every-day standardized three-dimensional world (e.g., Conant, 1958).

Oddly enough, high temperatures themselves do not usually limit the distribution of organisms on the earth. Excessive heating of the atmosphere near the ground is usually prevented by displacement of warm air upwards by cold air and by turbulence produced by lateral air movement. Instead, low temperatures usually limit the organic reactions that constitute the processes of life. The reason why cold becomes so acutely limiting in some regions of the earth is not only the excess of outgoing radiation over incoming but also the fact that cold air tends to sediment or remain near the ground and thus to produce a relatively stable situation in which temperatures can fall far below the freezing point of water.

We have many reasons for studying the effects of low temperature on trees and other plants. Foresters and horticulturalists often want to grow an exotic tree in a particular location—or having done so, they want to know why it is injured or killed. A study of cold resistance in trees also interests zoologists, particularly in ecology, who want to know how climate affects plants, since the distribution of animals may be directly determined by the kinds of plant cover and food. Geologists are interested in the effects of climate on vegetation since ancient climates can sometimes be evaluated by the fossils in certain deposits. As pointed out, large plants are sessile organisms and therefore indicate climate better than fossil animals. Finally, our space age of "rapidly expanding horizons" demands that we know how life can withstand the great extremes away from the earth's atmosphere or on other planets and satellites.

CLIMATIC INFLUENCES AND THE ROLE OF TEMPERATURE

General Considerations on Vegetational Zonation

More as a curiosity than a basis for modern thought, one can find accounts of the effect of temperature on trees in the writings of Theophrastus in the third century B.C. (Jessen, 1864). Theophrastus wrote, for example, that trees that flowered and bore fruit in Babylon and Egypt could not do so in the cooler Greecian climate. He discussed various other temperature effects in his treatise on growth and reproduction, and he seems to have conceived the idea of habitats.

In more modern times the idea of vegetational zonations based on isotherms can be found in the voluminous writings of Humboldt (1817, 1832, 1843). But the first real attempt to define temperature zones exactly seems to have been made by De Candolle in 1874; he divided the earth into five temperature zones (Lundegårdh, 1938:148). Near the turn of the century, the zoologist Merriam (1890) propounded his scheme of climatic zones for North America and tried to relate these to temperature "sums". Since then a great deal of criticism has been leveled against these zones, but Merriam's nomenclature is still used. In the final analysis, it is not so important what we name these zones as that we have zones to name.

Most biologists agree that such zones exist. One can, of course, argue over the exact boundary of any zone where ecotones occur, and certainly different formations may grade into one another without sharp boundaries. Many gradations or true ecotones resemble parts of frequency diagrams or "binomial curves", with an optimal area or peak towards the center for each species optimum (maximum numbers of plants per area for a particular species). The number of individuals per area then grade out on each side to form the tails of the frequency diagram as one moves away from the center. This situation has been demonstrated in the eastern deciduous forests of the United States by running continuous transects (Whittaker, 1951), and can also be shown in rather confined situations in mountain country (Parker, 1952a), especially if the transects are continuous.

In the Rocky Mountains, where moisture is often a local controlling influence, one is impressed by the complexity of species mixtures, so that a breakdown into "habitat groups" can be helpful in understanding these mixtures. On the other hand, gradations from place to place nearly always exist, and the habitat system appears to ignore the intergradation phenomenon. Larger zonations also show such intergradations, and this makes the very existence of zones questionable. Even though recognizing their lack of clarity, zones can be defined and usually appear to follow the plan of isotherms in a very rough way (Lundegårdh, 1938). Of course, mere association with a climatic norm does not prove that the norm controls the range of a species, but suggests a relation between some phase or phases of climate and the limitation.

Livingston and Shreve (1921) may be best known for their attempts to relate plant formations or zones (in the broad sense) to climatic factors. Yet their many attempts to relate a specific moisture and temperature factor or "index" to plant distribution appears to have ended in failure; in fact, their own discussion leads one to this conclusion. Nonetheless, some sort of relationship is sometimes discernible. Some of their poor "correlations" may be explained by their heavy reliance on averages of monthly temperatures and grouping monthly means together, especially when confined to only one part of the year (Daubenmire, 1956). Schimper likewise recognized the limited value of mean temperatures in making correlations to vegetational zones (Lundegårdh, 1938:149). At the same time it is difficult to doubt that climatic areas are fairly well related to certain vegetational formations. The climatic areas developed by Köppen and Geiger (Goode, 1939) obviously relate in a general way to certain kinds of plant formations. The Köppen-Geiger method may not fit the narrower geographical limits of certain areas of the earth, but this does not deny the over-all picture. It does show the difficulty of trying to draw up general formulae for all sorts of situations.

One concise way of summarizing the effects of moisture and temperature on vegetational formations was presented in a triangular diagram by Dansereau (1949). Temperature forms one axis, moisture another, and the main vegetational formations—in South America—appear in the areas between the crossing of temperature and moisture lines that run parallel to one side or the other of the triangle. This, of course, over-simplifies the situation, but like Holdridge's (1947) diagram, it illustrates the interaction of moisture with temperature and emphasizes that temperature as well as moisture plays a leading role in the distribution of plants on the earth.

A more applicable system for our North American forests, at least in the east, may be Thornthwaite's revision of an earlier system discussed by Shanks (1954). In areas colder than the taiga, vegetational boundaries fall along horizontal lines, that is, paralleling the isotherms on mountain sides. Below the taiga, in warmer areas, lines drawn vertically divide the vegetational zones into moisture regions. These concepts agree with the conclusions of Pearson (1931) for mountain vegetation and of Boyko (1947) for continental vegetation.

Although easy to conclude that temperature plays some controlling influence over vegetation, it is much more difficult to show which phase of temperature is the important one in limiting this vegetation. Shreve (1914), basing his ideas mainly on experience in the southwestern deserts, considered the winter phases of temperature, particularly winter minima, to limit the northward ranges of many plants. Hutchinson (1918) thought the northern limits of many tree species undoubtedly related to low temperature and that migration of many plant taxa had not kept up to the rate of glacial retreat accompanying the warming trend. Firbas (1949), in his extensive review of forest history in Europe after the last ice age, felt that winter cold was an important factor in limiting northern ranges of woody plants, but that in some cases, as with fir, late frost and drought also played an important role (Firbas, 1949:259). Recently, remains of spruce and larch of Pleistocene age have been found near the Gulf of Mexico in Florida and suggest that all climatic zones were moved southward, not merely compressed between the glaciated area and the sub-tropics of today (Dorf, 1959, 1960). Of course, the cold-climate spruces are not necessarily indicators of a severely cold climate and may have been limited in their range as they were in Europe in post-glacial times by lack of moisture, not by cold (Firbas, 1949:226). But the presence of spruce near the Gulf suggests a much cooler climate than occurs there at present and further suggests that temperature played some role in limiting forest vegetation in glacial times as it does today.

DIRECT EFFECTS OF COLD

Some ecologists have considered the evidence insufficient to indicate that low temperature extremes limit the northward extension of various native trees. Many examples can be cited to show that species have been grown successfully well north of their natural range. Loblolly pine has been planted over 100 miles west and north of its natural range with good success during the past few decades (Parker, 1950). This species has even been grown to mature trees in Ohio, although the foliage is often winter-injured (special communication from Central States Forest Experiment Station). But in small seedlings of loblolly pine grown out-of-doors in northern Idaho, leaves did not survive the winter, although the buds did (Parker, 1955c). The same occurs in New Haven, Connecticut.

In spite of evidence against a relationship between the natural range of trees and their apparent physiological range, a great mass of data shows that the direct effects of cold (low temperature extremes) may affect the range of forest trees (e.g., Ernst, 1934). Damage can occur in any season, and frosts may occur commonly in summer in some forested locations. But in temperate climates, damage occurs most commonly in autumn and spring, of which spring frosts are the more serious (Zon, 1904).

In the following paragraphs, spring, autumn, and winter injury are considered. Such a division may be artificial, since sometimes both autumn and spring damage occur, the one injury being additive to the other. Such a case was observed by the writer on Cape Cod in 1944-1945. An unusually warm November following a cold October brought the exotic *Aesculus hippocastanum* into full leaf at Woods Hole. Leaves were then, of course, soon killed by winter weather. In early spring, buds farther back on the branches emerged, but a spring frost killed most of them. The trees then appeared "diseased" and dying, and were felled a few years later. Local native tree species did not leaf out in November or the following spring and were thus unaffected.

SPRING DAMAGE. A warm late winter followed by a severe frost in early spring may cause damage to native trees as well as to exotics. Harris (1926) recorded a case in Illinois in which ends of terminal branches of trees were killed and cambial tissues farther back sufficiently injured to result in "frost-ring" formation. Possibly the "bark sloughing" of elms (Humphrey, 1913) in Illinois was the result of spring frost, although he described the "diseased condition" as resulting from winter damage. Late winter warm spells followed by cold waves are also not uncommon on the U. S. west coast and can result in bark injury to Coulter's pine in California (Wagener, 1949).

A great deal of damage is frequently caused by cold air waves in late spring when foliage is succulent, tender, and non-hardy. Of all sorts of records of late spring frosts, a few should be mentioned here. While Shreve (1914) thought winter minima important, Rubner (1921) held the opinion that late spring frosts limit the northward extension of many native trees along the northern tree-lines of Europe.

A late spring frost in the Wasatch Mts. of Utah injured not only exotics but native trees as well (Korstian, 1921). Native blue spruce and fir (*Abies lasiocarpa* and *A. concolor*) were injured to various degrees, while lodgepole pine (*Pinus contorta*) was mainly uninjured in spite of two inches of new shoot growth. While the exotic *Picea excelsa* was undamaged, *Picea pungens* was badly damaged. Such frost-damage can be mistaken for weevil damage (Belyea and MacAloney, 1926), and a similar case of injury was described by Kienholz (1933) to red pine in New Hampshire. Beal (1926) described killing of entire oak trees by late spring frosts in the Appalachians, and Childs (1961) mentioned girdling of Douglas fir trees by frost.

Frosts as late as early summer (late June) have occurred in East Anglia, Britain (Murray, 1955), which badly damaged seedlings of native beech, oak, sweet chestnut, grand fir, eastern larch, and Douglas fir. Moderate damage occurred in poplar, sycamore, and Scotch pine. Birch, Norway spruce, Lawson's cypress, and two-year-old shoots of Scotch pine were mostly cold-resistant. Damaging spring frosts apparently occur fairly commonly in Europe. Garavel (1956) stated that regions were rare [in Europe] where the young shoots of walnut had not been destroyed [at one time or another] by spring frosts. Tubeuf

(1904) described some curious injuries to horsechestnut leaves as a result of late frost. In an entirely different part of the world, frosts occurring on June 5th, 6th and 10th at 27° F. (—3° C.) caused great damage to *Pinus lambertiana* seedlings in Mariposa county, California (Schubert, 1955). A plantation of these pines 1000 ft. higher on the mountains was severely damaged, whereas *Pinus jeffreyi* and *P. ponderosa* suffered little. This particular race of *P. lambertiana* was native to the region. Another spring frost injurious to forest vegetation was reported by Reeks (1958) on April 29th in Manitoba, after a dry early spring that may have aggravated the damage. Bud-killing occurred in aspen and some conifers, but winter-drying appeared to be the cause of most of the damage to balsam fir. In the mountains of the Southwest, trees over 100 feet tall were injured as well as seedlings of Douglas fir (Phillips, 1907). This frost, occurring in late April, had no effect on trees at higher elevations, apparently because they had not yet started new growth.

The "barrens" in Pennsylvania may result from cold air sedimentation into the lowlands in late spring. Clarke's (1946) data showed how much lower the temperature was in the barrens than elsewhere during a frost; many scrub oaks were killed by the frost. As a result of such frequent frosts, the oak never gets beyond the scrub stage. Another case of tip-killing in "frost pockets" was described by Hedgcock (1913) in Colorado. Hemenway (1926) cited von Schädelin who believed that pines were generally especially resistant to late frost, while most deciduous European trees were more susceptible. But Hemenway's results showed more severe damage by late spring frost to two species of pine than to various broad-leaved tree species.

AUTUMN DAMAGE. Frosts in autumn can be quite serious, especially with plants that have not become dormant. Apple tree breeders recommend varieties that become dormant before the first autumn frost. But even native trees and shrubs can be injured by autumn frosts. Schubert (1955), for example, mentioned serious frosts in October in the Stanislaus Experimental Forest Nursery that killed all terminal shoots of 1-0 stock *Pinus lambertiana* seedlings. *Pinus ponderosa* and *P. Jeffreyi* seedlings were undamaged. These results remind the writer of damage he observed in the Bavarian nurseries near Munich. Many Douglas fir, a few months old, were damaged if not killed by late autumn frosts, but mature trees were unaffected.

As will be discussed later, suitable biochemical preparation for the development of hardiness in the colder climates is necessary for the survival of overwintering plants with above-ground parts. Although a lowered moisture content may be beneficial, drought, instead of hardening plants to cold, may actually prevent the development of cold hardiness, as is suggested by the results of Fraser and Farrar (1957) in Canada. Seedlings of *Picea glauca* and *Pinus resinosa* grown in a greenhouse until early October were put out in the field just before some heavy frosts. Part of the plants had been frequently droughted and part kept amply watered. The seedlings in drier soil were much more injured by frost in both species and in all varieties than those kept moist. This may have been the result of greater cooling of the drier soil, but, as the authors point out, this was not definitely known.

Branches of apple trees, not suitably hardened, can be killed by January cold. Brierly (1942) described a case in which the lower tree branches, buried by a November snow, were killed when they were pulled out of the snow and exposed to —10° F. (—23° C.) in January. Other branches, not buried in the snow, were uninjured; branches left under the snow also suffered no damage.

A tree's cessation of growth in autumn, even in cold climates, is controlled, in many cases at least, by day-length; if the plant is not suitably adjusted to changing day-length it may go on growing or resume growth in late summer or autumn, and the old foliage as well as the new may not harden and therefore be injured. Some varieties of *Pinus strobus* in New Hampshire are known to put on a second length of shoot growth in autumn which may then be frost-injured.

Kramer (1937) observed that an abelia hedge along the driveways at Duke University continued to grow each autumn near the street lights on the campus. The lights provided a long-day effect, and in spite of the cooler autumn weather, the abelia did not become dormant and was injured by autumn frosts in the vicinity of the lights. But about 20 ft. away from the lights, it ceased growth and became hardy.

Even under normal day-length conditions, severe cold waves in late autumn can injure native plants if they are not winter-hardened. A great deal of damage was recorded in the northwestern United States in the November cold wave of 1955. Certain characteristics of the wave are shown in a graph by Parker (1957a). Assessment of the damage the following summer revealed that it was not confined to a few exotics and plantations of apple trees, but that it had injured the foliage of a number of native conifers (Duffield, 1956; Daubenmire, 1957).

Under such circumstances it appears that cold weather extremes play some role in the growth and development of forest trees. Mere northern boundaries of plants are possibly not so much affected as extensive areas, including much of the extreme parts of a plant's natural range; then, if the trees are seriously injured (especially if overtaken by diseases following weather-injury), their migration northward proceeds again from the area of survival. Since we are living in a period of warming climate, as of about the past three centuries, such wholesale injury and sometimes killing of trees by weather extremes might not be so obvious to us during our relatively brief lives. Many forest trees in the northwest are over 500 years old, but the oldest complete weather records in the United States are only about two centuries old, *i.e.,* those of New Haven, Connecticut, starting about 1770; the oldest temperature records in the United States probably began in Philadelphia in 1731 and somewhat later at Cambridge, Massachusetts (Havens, 1958).

WINTER DAMAGE. Mid-winter cold waves can also be quite damaging, and such injury is not necessarily confined to Mediterranean-type climatic regions. Schreiner (1937) described the effect of a sudden drop in temperature in northern Idaho in December that did great damage to a plantation of ponderosa pine from the west coast. Entire trees of Douglas fir and Engelmann spruce, 40-60 feet tall, may lose their leaves as a result of winter injury (Keithly, 1931). Oppenheimer (1949) described a mid-winter cold period that occurred in two waves in the eastern Mediterranean. This did great damage to exotics and even native plants, entirely wiping out a woody vine which had established itself as a weed in orchards and gardens.

In southwestern Utah, Cottam (1937) found widespread damage to *Covillia tridentata, Stromocarpa odorata,* and *Prosopis glandulosa* after a cold wave in which —11° F. (—24° C.) was recorded. Cottam concluded from this that Merriam's methods of using heat sums for determining vegetational boundaries was probably at fault. Fosberg (1938), on the other hand, resurveyed this same country and concluded that many of the shrubs described by Cottam as seriously damaged would recover. Fosberg therefore concluded that extremes in temperature had nothing to do with geographical limitations of plants. He admitted, nevertheless, that some individual shrubs of *Larrea divaricata* were killed to the ground and that the fruit crop was generally much lower than normal in this species.

In Texas a cold wave or "norther" may follow a warm period in winter. Zobel et al. (1958) described one in which exotic conifers were killed by only 25° F. (—4° C.), whereas they had previously withstood 15° F. (—9° C.) in other seasons. Winter damage to longleaf pine in Virginia seems to result from frosts in early December (Allen, 1961). Even in Haiti there have been reports of damage to pine by mid-winter frosts (Pederson, 1953). Similar winter frosts injured Acacia, Grevillea, and Eucalyptus trees in southern California (Skinner, 1938).

Exotic palms often act as winter-temperature indicators and cannot be grown very far inland from the Gulf of Mexico in the United States (see later). Date palms cannot withstand much below 20° F. (—6° C.) for any length of time and cease growth if maximum daily temperatures are much below 49° F. (+9° C.) for a number of days (Mason, 1925).

Even in very cold regions, trees can be injured by mid-winter cold. Watt (1956) described winter damage to *Pinus ponderosa, Juniperus virginiana, J. scopulorum,* and *Picea glauca* in North Dakota. Damage was suspected to have been caused by a cold period in winter following warm, bright days, a situation made worse by late spring frosts. On the other hand, it appears from Stoeckeler's (1952) observations that Douglas fir can extend itself far into eastern Montana, where temperatures can fall as low as anywhere in the United States. There have been newspaper reports of —70° F. in western Montana, but so far as we know these are unofficial. The lowest temperature on earth seems to have been —127° F., recorded by a Russian expedition to Antarctica in a mountain basin 700 miles from the south pole (Maher, 1958). But many plants can withstand even lower temperatures.

Another peculiarity of winter damage to trees that is evidently related to low temperature is that of "red belt" formation. Hubert (1918) was one of the first to report this phenomenon in the higher mountains of western North America. Red belt usually occurs as a horizontal band a quarter to a half mile in vertical dimension at about 7,000 ft. altitude in the Rocky Mountains. In many cases the trees die, and where they do not, their subsequent growth is retarded. Discolored leaves soon drop off (Hubert, 1932), which must curtail the photosynthetic activity for much of the season. In one instance, Hubert concluded that the situation was brought on by —23° F. (—31° C.) on December 12th followed by +44° F. (+7° C.) on December 14th during a warm wind or "Chinook" (Foehn). It would appear that bright sun favors the development of red pigment, since Hubert (1930) was able to produce the color artificially by cooling branches to 18° F. (—8° C.)

and then putting them in the sun. Those kept in the dark after cooling did not become reddish.

Henson (1952) described a case of red belt at 5,800 to 6,200 ft. elevation near the Bow River, Alberta. According to his theory, radiant cooling during winter nights results in cold air production and drainage into the valleys. Then Chinook winds blow over this sedimented air. At night the cold air pool is higher than during the day; there is therefore a zone of fluctuation of the cold air and this is where the most intense red belt occurs. One is inclined to conclude that the temperature fluctuations together with the effect of the sun cause the red belt. That excessive transpiration plays a role seems doubtful to the reviewer, since we have observed reddened trees in the mountains of Alberta which had quite fresh and flexible leaves. It is probable that the red color is the result of anthocyanin production which in turn is favored by bright light and a certain rather high sugar content. The high sugar content may be the result of heightened photosynthesis and/or retarded translocation from the leaf.

Some ecologists have become discouraged with the importance of the role of temperature and cast about for other factors. One of these was day-length. It was suggested that the distribution of many plants is influenced by proper adjustment to the day-lengths of a certain locality. But this appears to be only one of several adaptations necessary to survive in a particular climate. Again, some have thought that respiration might be related to latitudinal distribution. But Susan Scholander and Joan Kanwisher (1959) showed there were no significant respirational differences between certain northern and southern plant species when measured at similar temperatures. Perhaps another physiological factor which should be investigated is the relative ability of different species to grow at rather low temperatures. Growth of spruce stems and roots, for instance, is more rapid in the lower range of growing temperatures than is that of pine in Finnland (Aaltonen, 1948:259).

It is apparent that a particular species survives because of a combination of factors, including interaction with parasitic organisms which are themselves influenced by climate, and not because of a single one. It may therefore be quite impossible to develop a general rule or complex "index" which will explain the distribution of different plants in various parts of the earth when we realize that each plant has its own peculiar kind of adaptations. It seems possible that over long periods low temperature extremes play a role in limiting the northern range of certain plants, although this may involve spring or autumn extremes as well as winter ones. In a given species there is only a certain genetic amplitude of change or "reaction range" possible (if it is to remain that species), so that, even allowing for readaptation to a changing climate, there are limitations to a particular species. Over shorter periods of time, other factors may play a more important role in survival. Once the organism is weakened, other factors may play a role in its decline, as fungi and insects, and competition with other organisms cannot, or should not, be ignored in the over-all picture.

THE TREE-LINE

The normal geographical limitations of plants appear in sharper focus along visible "tree-lines" on mountain sides where one can frequently find clear examples of the effect of climate on vegetation, provided, of course, that a simple topographic or soil effect is not involved. Perhaps high mountain climates are not strictly comparable to those of the subarctic at sea level because of different radiation, wind, and moisture conditions (Gaussen and Barruel, 1955), but mountain climates are more accessible to us than most of the far regions of the north and do show certain similarities in their higher altitudes to the barren lands of the subarctic and arctic.

From studies in Switzerland and Austria it appears that not all upper tree-lines are controlled by the same factors; different species whose inter-mountain migration seems possible, do not all equally inhabit the upper reaches of the tree-line in different places in the Alps. In some cases the moisture regime is important; in others, that of temperature. Assuming that soil lack is unimportant (which is not always the case), the most important factors limiting vegetation are (*a*) desiccation of the leaves during cold weather (Tranquillini, 1957); (*b*) a short growing season and lack of photosynthesis in much of the year (Pisek and Winkler, 1958); (*c*) lack of snow in winter, exposing plants to winter-drying or winter cold (Pisek and Winkler, 1958); (*d*) excessive lingering of snow in summer, observable in the Cascades and Rockies in early August; (*e*) mechanical effect of high winds (Aulitzky, 1958); (*f*) rapid heat loss at night (Gaussen and Barruel, 1955); (*g*) excessive heat at soil level during the day (Schimper and Faber, 1935); and still others, as low maximum daily temperatures during the growing season, and drought. Drought is mentioned because many tree-lines occur above maximum areas of orographic precipitation, and because summer drought, particularly on south slopes, probably becomes acute in certain years in many high mountain areas. Extremes of low temperature are evidently not so crucial as popularly believed, and many valley minima are much lower than those of mountain sides. Although temperatures in high mountains fall quite low in winter, they are usually at least no lower than in the adjacent valleys (Shreve, 1914; Parker, 1952a).

In the high mountains of subtropical and tropical regions, rather different conditions prevail at the tree-line than in northern latitudes. Although diurnal temperatures fluctuate widely at elevations of about 4,000 meters in central America, they rarely fall below freezing. Monthly means do not actually diverge widely from one another in these locations (Weberbauer, 1945), which shows that trees in these areas do not have to be very cold-resistant nor develop seasonal hardiness; yet they must be able to grow under widely fluctuating day and night temperatures. Seedlings of *Abies guatemalensis* and *Cupressus lusitanica* have been raised in the greenhouse at New Haven from seed brought from the high mountains of Costa Rica, but neither was able to withstand even the early autumn weather of Connecticut. The *Abies* seedlings were killed outright by early October frost, and those of *Cupressus* were much injured (see Table 1). These high Costa Rican mountains obviously differ from those of the American Rockies where cold air masses from central Canada and Montana may pass entirely over them in winter.

In conclusion it appears that temperature limits plants in more and devious ways than is at once obvious. Although low temperature extremes do not appear to produce most tree-lines on mountains, they may conceivably influence the continental ranges of many plant species. Every forester knows that the selection of seed by its geographical origin for planting in a particular place is important, and usually one of the most important factors to be considered is susceptibility to low temperature.

FREEZING OF CELLS AND TISSUES

LIVING CELLS AND TISSUES IN GENERAL

As the analytical approach to science gradually overcame the alchemical methods of the 16th and 17th centuries, the question arose: why does cold kill plants? According to one of the oldest ideas, heat retention is necessary for survival in winter, but observations in early times associated ice formation with the damage. In some trees ice was observed between the bark and the wood, which suggested that the cracking of trees is the result of expansion of the ice. This expansion, believed to crush the living cells, thus allegedly accounted for their death (Hamel, 1758; Senebier, 1800; cited by Wartenberg, 1933).

Other theories also appeared in early times. According to Schulz (1823, cited by Wartenberg, 1933), low temperature causes the movement of the sap of a plant to stop, and the plant dies because of sap coagulation. Another idea current nearly two centuries ago was a continuation of the heat retention idea. Respiration—since it produces heat in animals— was also believed to produce heat in plants, which can then be a protection against cold. Göppert (1830) argued against this idea. One of the real pioneers in the field, he contended that the "Lebenskraft" or literally "power of life" is destroyed by ice formation; other effects were considered merely post-mortal. But the exact cause of death was not clear. The troublesome philosophical point involved here might not be appreciated today. Aristotle's old idea of the plant's soul departing on death was being questioned, and a material cause of death seemed to be indicated by the effect of the cooling treatment (Wartenberg, 1933).

More recently, the idea of a tissue being crushed by ice passed into greater disfavor, and the notion that a plant can be killed by an inner cellular physiological disruption or disturbance became more acceptable. Sachs believed (1860) that damage can come to the cells at the moment of ice formation, but that, once cells are frozen, entire or local killing can come about during thawing. His experiments showed that rapid thawing can be more damaging than slow thawing, which has often been observed in the writer's own work with evergreens. Sachs believed that slow warming results in a chance for the protoplasm to take up water again without damage and return to its original condition, an idea still held by many today, although the term "slow" should be defined (Luyet, 1954).

Some thought, as Nägeli did (Wartenberg, 1933), that, when ice forms, the plant dies and that is the end. Whether ice forms rapidly or slowly, or how long the ice persists, seemed of no great importance. Göppert agreed that death takes place when ice forms in the cells, for he was able to demonstrate that during freezing, flower petals change color, and that this change occurs only when the tissue dies. He therefore could not agree with Sachs that death occurs during thawing. Possibly this disagreement can be partly reconciled by the fact that during warming, ice may form and kill the cells, whereas many cells are not previously ice-invaded. Also, the rate of reinvasion of water on thawing is important to survival, as already mentioned above, and was further established by Iljin (1934), so that death can in some tissues result from too rapid thawing. One difficulty in ultimate solution is that the process of death in a plant is usually not clear-cut, but may take several minutes, hours, or even days after thawing. Our own experiments showed the respiration rate of pine leaves to undergo a climb on thawing, then a decline; the rate of decline evidently depends on the severity of damage, the more rapid declines following the more severe damage (Parker, 1953). This situation can be verified by the tetrazolium test, although the test may show a decline in dehydrogenase activity when the respiration rate rises (Parker 1952b). Death, as we measure it, will not normally occur until half an hour after thawing, and sometimes in certain tissues only after several weeks.

Sachs seems also to have originated the concept of ice-dehydration, although Müller-Thurgau (1880) probably did the most to forward this idea which finds favor today by most workers. Although Sachs argued in favor of the theory of damage by melting of ice crystals and return of water into the cell, it was clear in his day that extracellular ice often forms (Sachs, 1860). His illustrations show fringes of ice crystals in the intercellular spaces of some evergreen leaves. Müller-Thurgau concluded that death from cold is due to dehydration of the cells brought about by the formation of extracellular ice. Prilleux (cited by Wartenberg, 1933) also favored this idea, and Wiegand (1906) described results which further strengthened the concept. Wiegand found that the crystals arranged themselves parallel to one another as if they were growing in only one direction, although Sachs' drawings show the crystals growing out in all directions, tending to be perpendicular to the surface of formation. This situation appears to be similar to the formation (although on a macroscopic scale) of ice crystals in certain damp soils, where crystals continue to grow from a point of origin and sometimes protrude several inches off the ground. Water for such crystals can evidently migrate as vapor to the site of crystal formation (Gurr. et. al., 1952).

Wiegand further localized the frozen condition in poplar buds within the bud scales and bud leaves, but not necessarily between the scales and leaves of the bud. He noted that willow twigs, when frozen, become shrunken (at —20° C.), but that on thawing, the shrunken appearance disappears and they become turgid again. Twig shrinkage was located mainly in the bark; more than half of the contraction was in the bark, the rest in the wood. Buds of many species, except those of *Populus* and *Acer*, expand during freezing because, Wiegand thought, wood contraction is not involved and ice tends to bow out the bud scales. Frank (1880, cited by Wartenberg, 1933) also concluded that ice forms between the cells and can sometimes split the tissues apart.

The extent and position of ice and the effect of speed of cooling were extensively examined many years ago by investigators of frozen foods. Experts believed from early times that rates of thawing determine the subsequent condition of frozen meat (Richardson and Scherubel, 1908). But also, the speed of cooling was considered important, and it is generally supposed that the success of the latter-day freezing process depends on its speed. This idea may date back to Plank (1918) who stated that ice crystals grow at a rate depending directly on the rate at which heat is withdrawn from the tissue, and the final size of the crystals is therefore a function of speed of heat withdrawal from the tissue. As the crystals grow, the concentration of sap near the crystals was believed to be raised and water was supposed to move out from the cells to the region of higher osmotic pressure. Birdseye (1929), applying this lesson, found that haddock and beef muscle quick-frozen between —4 and —6° C. in 25 minutes had nearly the same histological appearance as fresh muscle when thawed. Comparatively slow freezing resulted in swollen and firmly-pressed fibers. Nevertheless, the speed of cooling some tissues, such as those of beans and peas, does not make a great deal of difference to their subsequent appearance, taste, or vitamin content (Lee et al., 1946). Yet they found that larger crystals result from slow freezing (to 0° F. in 100 hours) than fast (to 0° F. in 1 hour).

Some 25 years ago it was believed that ice crystals cause much of the damage to frozen foods. Woodroof (1938) found that in fruits and vegetables, holes were left on thawing. These holes evidently represented spaces occupied by ice when the tissues were frozen. Particularly large holes occurred in tissue near vessels, evidently because ice forms first in the vessels where water is relatively pure, then this ice seeds adjacent cells. This observation seems supported by our work with

Rhododendron leaves in which killing in autumn is confined to the region near the veins. Yet, in winter, damage is more generally distributed over the leaf surface.

During the 1930's the causes of death to plants by low temperature appeared to be a matter of great mystery. Some workers felt that no ice can form at all in thoroughly hardy tissues such as pine leaves when cooled to —30° C., an idea based on flexibility of leaves when cooled to such temperatures (Clements, 1938). On the other hand, Tranquillini and Holzer (1958) concluded that ice formed at lower temperatures in conifer leaves in March than in May, but in either case some ice was formed if temperatures fell low enough. In yeast cells as much as 87% of the water is frozen at only —22° C. (Wood and Rosenberg, 1957). Ice formation occurs in mulberry buds at —1 to —2° C. in summer and —6 to —10° C. in winter (Kawano, et. al., 1960). But the temperature at which ice forms in buds depends on their water content (Johnston, 1923). The data of Dorsey (1934) agree generally with those of Kawano et al., and in the course of a winter in Illinois, ice formed and melted in buds as many as 41 times. *Rhododendron* leaves are fairly flexible at —15° C. but curl up in such cold weather (Fukuda, 1932), presumably because of ice-dehydration and shrinkage of the cells. Since the upper epidermis is stiffer than the lower, the leaf curls downward. Ice crystals can be quite small under these circumstances (Asahina, 1956) and the leaves can then still be somewhat flexible at this temperature. When such leaves are plucked and brought indoors, they quickly unroll as they warm up, showing that this is not a winter-drying effect resulting from an excess of transpiration over water absorption.

The question of crystal size in frozen plant tissue was further examined by Luyet (1954) who found that if moss plants were cooled quite rapidly (by direct immersion in liquid air) and then warmed rapidly (direct immersion in water at 20° C.), no visible ice formed at all under the microscope and the plant survived. If warmed slowly, however, ice crystals were likely to form during this process and the plants died if their water content was over a certain level. These results point clearly to the importance of ice formation as the cause of death, not mere cold.

But the question repeatedly arises: does the ice form inside or outside the cells? Studies by other microscopists support Sachs' idea that protoplasm is dehydrated by extracellular ice. But Molisch (1897) found that ice could form either inside or outside the cells, and in the former case this was usually killing. Iljin (1934) also concluded that ice, on forming extracellularly, dehydrates the protoplasm. Some cells shrank so much that opposite walls sometimes touched one another. Even when ice formed in the protoplasm itself, as in the slime mold plasmodium, the effect appeared to be one of dehydration (Chambers and Hale, 1932). Iljin, like Siminovitch and Scarth (1938), felt that quick freezing could result in intracellular ice and that this was killing to the cells. If high rates of cooling are used, crystals may form uniformly and at random in the tissue, and predominantly intracellularly. But at slow rates of cooling, crystals form exclusively in the extra-(inter-) cellular spaces in many kinds of tissues (Meryman, 1957).

Ice frequently does not form evenly throughout a piece of tissue all at once, but advances from place to place over several minutes or longer. Unlike a simple solution in a breaker which shows a fairly sharp single temperature rise during cooling, there is often only a rather indefinite rise in plant tissue and then sometimes a secondary change in the shape of the cooling curve with time (Levitt, 1956: 16). Dead potato tissue, when frozen, shows only one freezing point, but living tissue may show "double freezing", perhaps because first the extracellular liquid freezes, then the cell contents (Hatakeyama, 1961).

Ice formation may be uniform in its advance through muscle tissue from the point of seeding (Bergh, 1948). But in vegetable material it is usually altered in its direction of growth by the different kinds of cell walls. In some cases, ice can be seen forming all around the cells, i.e., in extracellular spaces of leaves; then suddenly a single cell darkens as ice forms by the "flash" type freezing. When this happens in beets, vacuolar anthocyanin is clumped into small droplets within the ice. Gas bubbles may also form in the ice (Asahina, 1956). During thawing Asahina (1956) observed much flowing of water to and fro, and when frozen a second time, the ice appeared to have formed in new locations.

With the use of a low temperature room for microscopic work, the Institute of Low Temperature Science at Sapporo, Japan, has clarified many of the points mentioned above. Terumoto (1958b) concluded that intracellular freezing in onion cells is much more destructive than extracellular freezing. Asahina (1956) verified the idea that, whereas intracellular ice ordinarily kills the cells, extracellular ice usually does not, although the latter causes cell shrinkage. Asahina brought out a number of other points, some of which are:

1. Supercooling is common in plant cells. On quiet nights, substances like sugars, that tend to encourage supercooling, should somewhat delay damage by ice formation. [The importance of supercooling also appears in the findings of Lucas (1954) in lemons, although Chandler (1954) thought that supercooling was rarely more than a degree or two except on quiet nights. Apparently the ability of a leaf to supercool depends on its water content. When infiltrated with water, it cannot be supercooled, but turgescent or wilted leaves can (Ullrich and Mäde, 1940). Few biological materials supercool more than 10° C. (Lusena, 1955), but Salt (1961) stated that some insects supercool to —40° C.]
2. The protoplasm, and not so much the cell wall, prevents ice-seeding into the cell interior.
3. Hardy cells resist ice-penetration more than non-hardy because rapid water penetration results in more ready crystal formation on the cell surface.
4. Intracellular freezing, which is nearly always lethal, is common in non-hardy cells. This might explain the remarkable reproducability of results of the killing point of evergreen leaves in summer (Parker, 1959c).
5. Since ice can penetrate cell walls relatively easily, "frost plasmolysis" often occurs in hardy cells between the protoplasm and the cell wall, and results in protoplast contraction as ice forms in the remaining space. Cells can recover from this.
6. The vacuolar membrane is also resistant to penetration of ice in winter-hardy cells, and ice may grow in the cytoplasm without entry through the tonoplast.
7. Ice grows more slowly in the cytoplasm than in the vacuole.
8. Actual killing of a cell by intracellular freezing is believed to be caused sometimes by mechanical tearing [of membranes], but in other cells there appears to be a dehydration of the "fine architecture" of the cytoplasm. [Stiles (1930) also considered that drying is not the whole cause of protoplasmic disturbance, but that ice formation has some other effect apart from this.]

The final cause of killing remains today somewhat of a mystery. At least there is no general agreement on it, as brought out recently by a controversy between Meryman (1956) and Levitt (1957) in "Science". Levitt argued in favor of the membrane rupture concept, while Meryman, drawing his information mainly from zoological and medical literature, argued in favor of the salting out of protein theory, in which an increased solute concentration, particularly of electrolytes, causes protein precipitation during ice formation. But Levitt felt that the effect of changing electrolyte concentration was disproved some time ago by Maximov. Perhaps investigators should consider more carefully protein denaturation resulting from water-withdrawal from around the molecule. According to Heber (1959), certain protein fractions can be designated as "frost-sensitive", and certain enzymes are more sensitive to freezing than others (Ullrich and Heber, 1957). Work with virus proteins, collagen, and so forth, shows that water in association with protein is of great importance to its structural continuity, some of this water being in the crystalline (ice) state and arranged in order with the amino acid residues (McCulloch and Berendsen, 1960). Removal of a certain amount of the protein-associated water can result in the protein's alteration, but sometimes denatured protein can be renatured. Denaturation may involve breakage of the hydrogen or even s-s bonds which are important to configuration.

Certainly the presence of relatively free water in the cell appears to be of importance in freezing damage. Injury to conifer leaf cells in winter has been related to leaf water content, the drier leaves being more resistant (Pisek, 1950). Seeds are notoriously low in water when kept air-dry, and it is therefore perhaps not surprising that they can be cooled to very low temperatures without much injury. In our laboratory air-dry *Pinus resinosa* seed cooled to —25° C. lost one-third of their germinability, while those cooled to —63° C. lost two-thirds. Seed in the cold-treated lots also grew more slowly than control seeds.

Occasionally one hears dissenting notes on the ice-theory of cold injury. Genevès (1955) concluded that ice formation, dehydration, or membrane rupture has nothing to do with cold damage at all, and that cell colloids are irreversibly coagulated by temperatures of —5 to —180° C. But too much evidence exists that ice formation is related to damage by cold, and it seems likely from the foregoing review that ice formation results in dehydration that injures the cell by distortion, by protein precipitation, or by altering protein structure somehow through water withdrawal.

FORMATION OF ICE IN TREE TRUNKS

ICE IN WOOD. Recent years have witnessed a revival of interest in frost-cracking in trees, which, although a very old problem (Wrangell, 1844), brings up again the question of whether there is an expansion or contraction of wood on freezing. Siminovitch (Thimann, 1958:122) drew the example of apples in a box which, on being frozen, rattle about, thus showing that they had shrunk. Scholander, however, argued that it was the other way around: ice must expand on freezing, especially so since formerly-dissolved air was included in the ice.

Partly as a result of this discussion, the problem was approached by one of our students at Yale (Herrington, unpublished). He made use of an extremely sensitive strain gauge, ordinarily employed to measure wood shrinkage, which was attached to a recording milleameter. A piece of air-dry wood (about 14% H_2O) contracted as the temperature was lowered, following a hyperbolic curve when contraction was plotted linearly over time. However, if a water-saturated piece of wood was used, at first a small expansion appeared as freezing began, then a contraction from there on down to —80° C., the lowest point studied.

Perhaps as Hartig (1896) suggested long ago, when ice forms, the mycelles of wood are dehydrated and the water migrates to the crystals by diffusion, resulting in wood shrinkage. Ice and water have the same vapor pressure at freezing (0° C.), but at temperatures somewhat below this, they do not. If ice can dehydrate living cells by an osmotic process, it seems possible that it could also dehydrate cellulose by a similar process operating through gradients in diffusion pressure deficit set up by the differences in vapor pressure of ice and water. Water in wood is rarely, if ever, pure water and often contains sugars, amino acids, and salts so that it would not be expected to freeze at exactly 0° C.

Cracking can be artificially produced in a cross-sectional disk cut from a conifer tree trunk. The disk, about six inches in diameter and an inch thick, is cut half way through with a one-eighth inch saw cut in the plane perpendicular to the cross-cut and parallel with the tracheids, as one would cut a pie from the center out to the edge. This disk is then cooled to —80° C. and removed to the laboratory table where an ordinary light bulb is placed directly over it. While the disk is warming, it suddenly cracks open with a loud snap, the crack being opposite the saw cut and mainly in the heartwood. Apparently the heartwood is unable to keep up with the expansion of the sapwood during warming.

On the other hand, much evidence shows that cracking of trees in nature occurs during cooling (Wartenberg, 1933). He pointed out that Caspary, Sachs, Sorauer, and Göppert agreed that frost cracking of trees results from the greater tangential shrinking of the tree than the radial shrinking, and that this shrinking is greater in the outer part of the tree than the inner as heat is lost from the tree. During thawing, cracks tend to close. More recently, Kramer and Kozlowski (1960), in their review, concluded that cracking comes during cooling. Yet they cited others to the effect that apple tree cracks are common on south sides of trees, and we have noted that most cracks on city trees of New Haven occur on southeastern or southwestern sides of the trunks. This suggests that the sun warms the frozen tree on one side and expands the sapwood; then possibly the heartwood cracks, unable as it is to expand too; the crack extends to the outside of the tree. Bruce (1902) mentioned that cracking could come on either cooling or warming and that the sound was sharper on cooling, but more muffled when cracking happened during warming.

Schulz (1957) pointed out that the coefficient of expansion of wood, ice, or the two together could not account for the enormous contractions observed. He thought that cracking could occur at night, and Busse (1910) concluded that it occurs most commonly just before sunrise. Reopening of the cracks occurs in cold weather, the increase in crack width following somewhat behind the descent in temperature (Schulz, 1957). Many cracks in trees open up each winter and close again in spring (reviewed by Boyce, 1961; Vasil'yev, 1961), and they can occur in either conifers or hardwoods (Boyce, 1961).

Very similar cracks, either following the rays or annual rings, can be of drought origin, or even of bending due to wind ("wind shake"). If a crack starts in the late part of a growing season, as can be seen in some tree sections, it is likely to be of drought origin and not cold. Such cracks (usually following rays) are sometimes referred to as "Hitzerisse" or heat cracks (Wartenberg, 1933). These should not, of course, be confused with drying "checks" appearing at log ends as they dry.

Another problem in trees resulting from ice is the one of air bubble formation. As mentioned elsewhere, air bubbles may form in ice (Chalmers, 1959), and when the ice thaws, such bubbles might retard further water translocation (Preston, 1959). Scholander (1962) concluded that these bubbles would have to be removed from the tree's xylem by a positive pressure, yet he pointed out that only certain species seem capable of producing adequate positive stem pressures for this. Nevertheless, bubbles can be seen in xylary tissue under the microscope to shrink rapidly and disappear within about a minute as the gas dissolves back into the water, although this is in thin sections. On the other hand, we have observed rhododendrons wilting in spring when there was ample soil moisture. Some sort of retardation of water translocation is suggested, but this might be the result of root-tip injury in winter (Filippenko, 1958).

ICE IN CAMBIUM. When the cambium dehardens in spring, it passes from a condition of great resistance in most temperate-climate trees to one of extreme sensitiveness. This can happen in about two weeks. If a frost or brief cold period occurs after dehardening, the cells may be slowed in growth, injured, or killed. If only injured, "frost ring" may develop. This consists in most cases of the development of thin-walled parenchyma cells sometimes called "Parenchymholz". Such cells occur in an otherwise normal ring and may give the gross appearance of an extra ring or year's growth. This differs from "drought ring" mainly in the time of its appearance, the latter being often associated with known droughts later in the summer. But cambial injury can also result from lightning, girdling, or severe bending (Mix, 1916a), and according to Rhoads (1923), frost rings may arise anywhere in the growth ring. But Rhoads felt that injured tissues develop different structures, depending on cold intensity and cambial susceptibility. Uniseriate rays near the frost ring may become 2- or 3-seriate. Possibly this is related to changes in oxygen tension as a result of injury.

Cankers may result, particularly on conifers, from frost injury; other effects, such as resin cavities and fungal attack, may follow (Day, 1928b). In fact Day (1931) was able to relate canker formation to years in which frost rings occurred. Orr (1925) suggested that cankers could also develop from frost cracks.

Frost ring formation itself may take various forms: (*a*) collapsed cells, (*b*) parenchymatous wood, (*c*) gummosis, (*d*) traumatic parenchyma, and (*e*) widening of xylem rays (Harris, 1934). Sometimes the ring is similar to that resulting from fungal attack, but in frost injury the ring is more often all around the tree, not only part-way (Harris, 1934). Frost rings can sometimes be diagnosed with the help of weather records.

Frost rings are supposed to be more common on conifers than broad-leaved trees and shrubs, but may occur on either (Day, 1928a). They have been found in longleaf pine (Stone, 1940), and they are common enough in various species that they form a means of determining chronology in different trees (Bailey, 1925).

ICE IN BARK. Injury to bark in spring has already been mentioned. Whether the "bark sloughing" of elms (Humphrey, 1913) is a winter or spring effect is hard to say, but frost injury to Coulter's pine bark seems to be a result of cold following a late winter warm spell (Wagener, 1949). Breuer (1951) distinguished between two kinds of "frost plates" on trees. One type develops on stone fruit trees on their south sides on a sunny day after a frosty night. Blister formation then occurs, followed by drying and falling off of the bark affected. This seems to differ from Breuer's "damp frost plates" which originate after mild winters when the sap has begun to run and there is then a hard frost. Cavities between the bark and wood of yellow poplar are probably also the result of frost (Tryon and True, 1952), but bark lesions may result from cold winds or from ice coatings, according to Pomerleau (1944).

Blisters or "Frostblasen" may occur on apple tree leaves as a result of frost (Wartenberg, 1933, citing Sorauer). Parenchymatous growth then ensues, leaving air spaces of somewhat unusual size under the epidermis, a situation probably resulting from tissue warping during freezing.

Bark cracking, as in wood cracking, sometimes results from freezing of the tissues, which causes shrinkage in the tangential direction (Wartenberg, 1933). According to Wartenberg, Sorauer was able to produce artificial cracks on apple tree twigs. Such cracks could develop, apparently by proliferation of the cambium in spring, into tumors. The cracks often occurred near dormant buds in the bark.

VARIATIONS IN RESISTANCE WITH THE SEASON

It was suspected from an early time that plants are not so cold-hardy in the growing season as during the dormant. Hales (1727) mentioned the appearance of fat in trees in connection with a possible development of cold resistance. Exposing plants to cold under artificial conditions was known to increase hardiness (Göppert, 1830), but tropical plants could not be induced to harden. Rein (1908) likewise found that some plants could be cold-hardened but others could not. Chandler (1913) concluded that exposure to cold as well as the condition of maturity of tree shoots had its effect in producing hardiness.

Perhaps the earliest seasonal cold resistance study in woody plants is that of Winkler (1913) in Germany. Resistance of tree buds sharply increased in November and decreased in March. The lower the temperature that trees were exposed to, the more rapid their "accommodation" to cold. Some trees showed an increase in resistance, even in summer, from —30° C. to —10. "Accommodation" of evergreens to cold was not believed so great as that of broad-leaved deciduous trees, but this rule mainly applies to the species he studied.

Hardiness in Elberta peach bark may vary from —16° C. in winter to only —5° C. in summer (Chandler, 1913). Fergus (1926) and Hildreth (1926) showed that cold resistance in apple twigs increased steadily from July to January. In late March the curve changed sharply as tissues lost hardiness into early May. Seasonal changes have even been demonstrated for cortex, pith, and bracts of tung twigs in Louisiana (Fernholz and Potter, 1941).

Seasonal variations in resistance of peach buds to cold was also studied by Cullinan and Weinberger (1934) but with uncertain results. Meader and Blake (1943) found a steady increase in cold resistance from November to February in New Jersey in per cent of live peach buds surviving treatment. Then in March there was a decrease. This resembles results of Chaplin (1948) with peach fruit tree buds in Illinois. Chaplin thought that there were mid-winter hardiness variations depending on temperature. In Austria, apple bark can start losing its hardiness in February (Larcher and Eggarter, 1960), but this depends on temperatures in the year in question (Kohn, 1959), showing that such changes are not simply day-length controlled or endogenous. Pisek (1958) found Ontario apple bark reached a resistance of about —33° C. in January in Austria, while cherry and apricot were resistant to —25 and —21° C., respectively. They then began to decline in hardiness.

Turning to forest trees, among the earliest studies were those of Pfeiffer (1933) and Schmidt (1936), but these were for only part of the year. Pfeiffer's important contribution was to demonstrate clearly the importance of rates of cooling to the results obtained for individual tests of conifer leaves. Ulmer (1936) made the first thorough study of the hardiness of several species of wild woody plants. He found that species near the tree-line in the Austrian Alps had resistances somewhat different from one another at almost any time of year. For example, *Pinus cembra* in December could not be killed at about —45° C., while

Rhododendron was resistant to about —28° C. and *Loiseleuria* was resistant to about —35° C. His curves show that the spring decline in hardiness is steeper than the autumn increase, a situation that now appears to be common in woody plants of cold climates.

Clements (1938) carried out what appears to be the earliest study on cold resistance in forest trees in North America, although results were confined to only a part of the year. Ponderosa pine seemed somewhat less hardy than fir, the fir dehardening from —26° F. (—32° C.) to —3° F. (—19° C.) from early March to early April. That the pine is less hardy than the fir is an observation made by foresters in the field, according to Clements. But winter killing may be the result of cold or drying, and the two are sometimes very hard to distinguish. Winter drying ("parch blight") is not the subject of this review, but the following authors are listed for those interested: Munger (1916), Bates (1923, 1924), Ivanoff (1924), Goldsmith and Smith (1926), Meyer (1928), Schmidt (1936), and Curry and Church (1952). Winter declines in leaf moisture have been found in a number of evergreens (Clements, 1938, in the conifers mentioned; Schmidt, 1936, in Norway spruce), and various seasonal changes in moisture content of evergreen leaves have been observed by Meyer (1932) in *Pinus rigida,* by Cartellieri (1935) in various ericaceous plants and *Pinus cembra,* by Ulmer (1936) in species of *Rhododendron, Calluna, Arctostaphylos,* and *Pinus,* and by Reifsnider (1961) in *Kalmia latifolia.* Detailed studies on *Rhododendron* leaves revealed a slight winter decline in two separate years, but mountain laurel, and Austrian pine underwent no appreciable winter decline (Parker, 1963). All species, however, declined in moisture in spring as transpiration increased concomitantly with the opening of stomata.

The findings of Ulmer (mentioned above) on cold resistance were verified by Pisek (1950) and Pisek and Schiessl (1947): while *Pinus cembra* could withstand —47° C. in their apparatus, *Rhododendron ferrugineum* never tolerated more than about —15° C. The latter is therefore susceptible to winter killing if snow does not cover the leaves. At the same time seasonal changes in cold resistance of Mediterranean evergreens are exceedingly small (Larcher, 1954), and many are not hardier than —13° C. in winter (Table II).

In North America, up to 1955, very little was undertaken on seasonal changes in cold resistance of forest trees, except for the work of Clements (above) and Siminovitch and Briggs (1949) on black locust.

A series of studies (Parker, 1955b, 1957a) showed that northern Rocky Mountain conifers undergo the same sort of autumnal changes in hardiness exhibited by the Austrian trees. None of the native Rocky Mountain conifers could be injured by treatments in winter down to —60° C. (Parker, 1955b). Further studies with eastern white pine indicated that hardening takes place in a series of steps, not in a smooth downward curve (Parker, 1959c), but we have never verified this. White pine leaves were hardy even to —196° C. in late winter. Further seasonal studies indicated that a southern species like longleaf pine *(Pinus palustris)* hardens only slightly in autumn and is practically incapable of responding to low temperatures by hardening further beyond its endogenous capability (Parker, 1961b). The hardiness changes of eastern white pine, on the other hand, are fairly typical of other native northern conifers (Parker, 1961a). Deciduous broad-leaved trees, in turn, go through much the same seasonal changes as do conifers in the colder regions of the world (Tumanov and Krasavcev, 1955, in Russia; Till, 1956, in Germany; Parker 1962a in Con necticut). But the Russian work suggests that hardiness may vary appreciably during the three coldest months.

One of the astonishing facets of these seasonal studies is that there appears to be no lower limit down to absolute zero for the hardiness of some tissues of certain woody plants. Sakai's (1958b, c) mulberry bark withstood —183° C., Parker's (1962a) hardwood twigs withstood —196° C., and Krasavtsev and Khvalin's (1959) and Tumanov's (1961) woody buds survived —253° C. (—423° F.). This is perhaps not too surprising in view of Lipman's (1936) moss protonema which survived liquid air temperatures, Becquerel's 1953, 1954) spores which survived —272° C., and Sun's (1958) germinating peas which withstood —183° C.

The environmental causes of seasonal hardiness trends are also of interest here. Alfalfa (Dexter, 1933) and wheat (Khitrinskii, 1957) indicate that hardiness is influenced by day-length. Short days are more effective in hardening than long days. Moshkov (1935) believed that hardiness of some woody plants is directly influenced by day-length. It became apparent in our work, in cooperation with F. Mergen at Yale, that day-length can influence hardiness development in *Pinus strobus* kept in a heated greenhouse (Parker, 1961b). But hardiness developed indoors was much less than that developed in the field in autumn.

Maximum winter hardiness is brought on by a few nights near freezing (Meyer, 1928; Harvey, 1930), but we have wondered whether low temperatures during the day are not even more effective. Illumination sufficient for active photosynthesis is also believed beneficial for developing the necessary foods that may be directly involved in hardiness development. Considering these two factors—day-length and temperatures near freezing—it is possible that some slight increases in hardiness in autumn might result from day-length change and that when there is weather approaching 0° C., hardiness increases markedly. It appears that this temperature induction is ineffective in spring or early summer in most woody species.

Many tables and curves of absolute cold resistance of species in the literature are open to question, since any of several factors can influence results, and tables are made from results obtained under different conditions. Speeds of cooling and warming seem important (Pfeiffer, 1933), although Chaplin (1948) found no differences in results at different rates of thawing. Many workers do not specify speeds of cooling and warming, and sometimes claim this is of no importance.

Pre-treatment of tissue can also influence results. Mulberry bark cooled to —30° C. and held there several minutes can then be cooled down to —185° C. without damage (Sakai, 1958c, 1960b). But to make a general rule that —30° C. is a kind of "ultimate" level beyond which it does not matter how cold it gets, seems doubtful to us (Parker, 1960b). At a cooling and warming rate of 4° C. per hour, this ultimate level may be closer to —70° C. One can get all sorts of resistance levels among species from about —6° C. to at least —60° C. (Table I, next section). Buds of arctic birches cannot withstand temperatures of —180° C., but they can survive —60° C. (Scholander, et al., 1953). Of course, such twigs cooled and warmed more slowly might have survived —180°C., as others with black birch have shown. There is some evidence that hardiness varies during the coldest part of winter and well below resistances of —30° C. (Tumanov and Krasavcev, 1955).

The question sometimes arsises as to the validity of the cooling treatment in determining field hardiness. Most workers realize that different effects can be obtained by different treatments. Repeated freezing and thawing versus one freezing and thawing, or prolonged freezing versus very brief freezing can give different results. Day and Peace (1937) cited conflicting evidence on this problem by other workers and pointed out that for conifers, the time elements must be specified as well as the temperatures considered. They concluded that if temperature is sufficiently low, maximum damage occurs in only 15 minutes, but if a temperature approximating the critical temperature is used, injury increases with time, at least up to about an hour. Likewise, repeated freezing, if it approximates the injurious temperature, is more damaging than one such freeze. Two exposures of 90 hours each caused more damage than one exposure for this same length, but these two exposures were less damaging than a continuous 180-hour exposure. On the other hand, at "super-low" temperatures (about —185° C.) Sakai found that bark tissues could be kept viable for over 100 days. It is reasonable to assume that still longer periods of survival could be obtained.

Finally, the methods for determining viability can be in error. It is possible for plasmolysis to occur, yet enzymes may be largely inactive, although ordinarily the plasmolysis test is considered reliable by many workers in judging viability. Possibly electrical resistance or conductance gives a more immediate measure of protoplasmic changes and may indicate not only the extent of damage to frozen cells (Wilner, 1961) but also the state of hardiness in uninjured cells (Wilner, 1960). One advantage of this method is that the tissue does not have to be destroyed for testing. The tetrazolium test, originally suggested about 1941 to be a general test for viability, seems to us the simplest and yet most accurate method for determining viability in dormant tissues of woody plants (Parker, 1951, 1952b, 1953, 1955a). Tetrazolium salts are also reduced by living cells in bark and xylem, as in *Pinus ponderosa* (Parker, 1955d, 1957a), and have been found apparently reliable in testing viability in apple twigs (Larcher and Eggarter, 1960) and in forest tree twigs (Parker, 1962a). But a serious source of error in any viability test is the time factor. If a plant tissue survives three days, it may still die on the fourth; this is also true of frozen and thawed animal tissues (Smith, 1958). It is therefore sometimes desirable to keep the tissues under favorable conditions for several days if not weeks before applying the tetrazolium test following a certain cold treatment.

VARIATIONS AMONG SPECIES AND WITHIN SPECIES

Variations Among Species

All gradations of cold resistance occur in the plant kingdom, but since resistance varies widely and variously with the season, it is difficult to generalize. Many tropical plants die when cooled to a few degrees above the freezing point of water (chilling injury). Others, normally flourishing in the tropics, can withstand light frosts. Some of the date palms seem to endure a short season when night temperatures fall to nearly 20° F. (−7° C.) (Mason, 1925). On the other hand, date palms in the southern U. S. are frequently injured by frosts and depend largely on subsequent growth to recover, as is usually true of bromeliads, palmettoes, and other such plants. Winter temperatures in some southern locations near the Gulf of Mexico are well indicated by the presence of date palms, since they are widely planted and then killed back during the harder winters to certain northern limits. This is also true of other plants like the Royal palm or banana which are more sensitive to cold. In contrast, the native trees of, say, central Alabama are quite capable of withstanding fairly hard frosts in winter. Then as one proceeds north, native trees generally become more and more cold-hardy until we encounter the trees of northern Canada. The pines appear in Table I to be the most resistant conifers, but we have, of course, not tested all the varieties of *Abies* and *Picea* which prevail in those northern regions.

A study of seedling injury in the severe winter of 1955/56 in southern Germany indicated that species of *Tsuga, Larix,* and *Pinus* suffered the least, while those of *Cedrus, Cephalotaxus, Sequoia,* and *Cryptomeria* were quite damaged. Although *Abies* was listed in this latter group, several species of it were quite resistant, including *A. siberica* (no damage) and only slight damage to *A. grandis, amabilis, subalpina, arizonica, veitchii,* and *holophylla.* Although Douglas fir lost its leaves, it recovered quite well. Broad-leaved species were only a little damaged (Dimpflmeier, 1957).

Other species differences have been discussed, but exact inter-species comparisons have rarely been made, and when they have, they are not very revealing. We have assembled in Table I a number of species obtained in the New Haven area which were cooled under fairly exact conditions. It was hoped that the cold resistance of the various plants could be related to their northern latitudinal limits, but this appears to be only roughly possible. The top two species in Table I proved to be totally frost-killed in October in New Haven; the group which is hardy to the −20s from *Cryptomeria* through *Ilex* can be badly injured by winter cold in our latitude and longitude; those hardy to the −40s are likely to be injured, but rarely if ever entirely killed; those hardy to the —50s, perhaps including *Tsuga canadensis,* are only rarely winter-injured from cold; the last two are quite resistant so far as we know within the varieties tested.

Broad-leaved deciduous trees native to our area are generally more cold resistant than the conifers, judging by freezing results in our laboratory. Some seem capable of the lowest temperatures obtainable (see elsewhere in this review), but this is also true of some of the pines. Of course in the field, plants may be subjected to repeated freezing, while in our laboratory tests they receive only one cold treatment.

TABLE I

COLD RESISTANCE IN JANUARY AT NEW HAVEN, CONNECTICUT, IN DEGREES CENTIGRADE OF LEAVES OF VARIOUS WOODY PLANTS.

PLANT	ORIGIN	LOWEST TEMPERATURE WITHSTOOD***
*Abies guatemalensis**	Costa Rica	—6° C.
*Cupressus lusitanica**	Costa Rica	—10
Cryptomeria japonica	Japan	—20
*Pinus palustris***	No. Florida	—24
*Pinus taeda***	No. Carolina	—25
Hedera helix	unknown	—25
Ilex opaca	prob. native	—25
Chamaecyparis pisifera	Japan	—42
Rhododendron catawbiense	unknown	—43
Taxus baccata	unknown	—45
Juniperus virginiana	Brandford, Conn.	—52
Kalmia latifolia	Guilford, Conn.	—54
Picea excelsa	Europe	—58
Tsuga canadensis	New Haven, Conn.	—60
Pinus sylvestris	Europe	—62
Pinus strobus	New Haven, Conn.	—70

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*Greenhouse-grown seedlings.

**Greenhouse-grown, but put out-of-doors in October.

***These temperatures are far from final; for example, resistances can vary from year to year. All temperatures were determined with the same apparatus at rates of cooling and warming of 4 to 8° C./hr. Those marked "unknown" had been growing in New Haven several years. At certain other times, many of these species were cooled much lower without damage.

In summer, leaves of various cold-climate deciduous forest trees may appear to vary in cold resistance from one species to another, judging by field observations after early summer frosts (Bailey, 1960). But in our study of several common species of deciduous Connecticut forest trees, repeated cooling tests did not reveal consistent differences among species in hardiness during the summer (Parker, 1962a). There was, however, an increase in hardiness from June to August of 2 to 3° C. in leaves of all species tested.

VARIATION AMONG VARIETIES

Much has yet to be learned of this subject, but a number of field observations have yielded results which can be discussed here. Richardson (1960) stated that ecotypic variation in "frost" hardiness within a species is widely known, and cited several Swedish and German authors. Foresters can also learn much from the horticultural literature in this field.

Hardiness in apple trees is not controlled by a single genetic factor

TABLE II

SUMMER AND WINTER RESISTANCE OF SOME EVERGREENS OF THE NORTHERN MEDITERRANEAN REGION IN DEGREES CENTIGRADE. ADAPTED FROM LARCHER (1954:621).

PLANT	SUMMER RESISTANCE	WINTER RESISTANCE
Nerium oleander	—5° C.*	—9° C.
Laurus nobilis	—6	—10
Arbutus unedo	—6	—11
Viburnum tinis	—6	—11
Olea europea	—6	—13
Pinus Pinea	—6	—13
Quercus ilex	—6	—13
Chamaerops humilis	—13	—13
Trachycarpus Fortunei	—13	—14
Cupressus sempervirens	—8	—16
Cedrus atlantica	—7	—16
Cedrus Deodara	—7	—18
Citrus trifoliata	—14	—20

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*These temperatures are estimated from Larcher's graphs.

but is polygenic (Lantz and Picket, 1942). Besides this, more than one physiological character is often involved in cold hardiness itself, so that the genetic study may be further complicated. Lantz and Picket crossed Delicious apples, which are not very hardy, with a hardy apple strain, and obtained a higher percentage of resistance in the F_1 than in either parent. When tender trees were crossed with the Delicious, the progeny were heavily damaged by winter cold, as might be expected, yet some of the offspring were resistant. They concluded that hardy apple varieties can transmit their hardiness to a relatively high percentage of trees when crossed to a tender variety and that crossing two tender varieties can result in a few offspring that are hardier than either parent, although most of the offspring will be tender. That the resistance of trees to winter injury is controlled by multiple factors or polygenes is also indicated by results of Rudolf and Nienstaedt (1962) in conifers.

Studies of various strains of *Pinus sylvestris* were made in Europe over 30 years ago and clearly revealed geographical varieties which changed color in autumn at different times. The eastern European races of pine lost their green color most readily and became yellowish, while the western races (France and Belgium) largely retained their green color (Kienitz, 1922). This change seemed to be a "protective coloration", since Kienitz found that the French races, which remained largely unchanged in color, were injured by a late spring frost, while the others were evidently uninjured. Langlet (1936) also noted varietal differences in hardiness of Scotch pine: those from the north or from high altitudes were the hardiest.

Discoloration in Scotch pine was further studied by Gerhold (1959) in southern New York State. He concluded that, in spite of the genetic variation producing different yellowing effects, intensity of illumination as well as length of day influenced results. Long-day treatment tended to prolong the normal green color into December, while short-day treatment (shorter than normal for that season) tended to speed up discoloration. It was suggested by Gerhold that discoloration might be related to the development of hardiness. Gerhold further conjectured that discoloration is the result of chloroplast clumping. Clumping, however, can occur in conifers without a yellowing effect, according to our own observations. Clumping and shrinkage of chloroplasts may sometimes be related to discoloration, but one does not necessarily follow the other.

A study of color change to purplish in jack pine *(Pinus banksiana)* was made by Stoeckeler and Rudolf (1956) at Rhinelander, Wisconsin, with stock obtained from various locations in and near Wisconsin. They found good correlations between latitude and extent of discoloration of foliage: the more northerly ones changed to a purplish color the most. A correlation existed also between this discoloration effect and the average mean temperature of January in the place of origin.

Many broad-leaved evergreen leaves, as those of *Hedera helix,* tend to redden in winter because of anthocyanin production. Formation of such glycosides is related to the general increases in sugar with colder weather, provided there is good illumination (Parker, 1962b). However, the reddening of deciduous tree foliage in autumn does not appear to be associated with any appreciable increase in hardiness before the leaves fall.

In Scotland different ecotypes of *Larix decidua* could be distinguished among 12 provenances (Day, 1958). Seedlings were cooled three days at 13-19° F., 19-25, or 22-28. Some provenances were relatively resistant to frost, especially during the early stages of flushing.

Picea excelsa has also been found to consist of different altitudinal ecotypes in the Swiss alps (Fischer, 1949). But Mirov et al. (1952), in seeking an answer to the question of whether low-altitude seed of *Pinus ponderosa* could be used at high altitudes in the Sierra Nevada of California, concluded that there was no relationship between seed origin and foliage injury. Their observations extended for 12 years. They did, however, find differences in growth rates of trees obtained from different altitudes.

There is also information on the resistance of some broad-leaved deciduous trees, and these may be mentioned here. White ash *(Fraxinus americana)* from a southern source, grown at the Harvard Forest in Petersham, Massachusetts, was usually killed to the ground in the seedling stage (Wright, 1944a). Even native ash seedlings were unable to become entirely dormant in autumn and were frequently somewhat damaged by autumn frost. As the trees age, they seem more able to become dormant and hardy in autumn. Red ash *(Fraxinus pennsylvanica),* when brought to Petersham from various parts of its natural range, appeared to consist of three ecotypes: one from the northern States which was slow-growing, winter-hardy, and lost its leaves in autumn; one from the southern coastal plain down to South Carolina which were fast-growing, sensitive to cold, and retained its leaves through two killing frosts; and a third type from New York which was intermediate between the other two (Wright, 1944b). One added fact which may be an indication of biochemical differences, is that deer preferred the southern variety to the other two.

In silver maple *(Acer saccharinum)* Wright (1949) found three biotypes, each of similar genetic make-up. These were all in a relatively small geographical area and, according to Wright, could be existing distributed at random in the population without being sorted into geographically distinct areas. One of these types was very winter-hardy, another moderately hardy, and a third not very hardy. Even in semi-tropical trees there may be varietal differences in cold hardiness (Halma, 1942).

Kriebel (1957) studied varieties of sugar maple *(Acer saccharum)* in Ohio from various parts of the eastern United States. Although day-length response was about the same in all varieties, winter hardiness showed a certain amount of variation among varieties. Shoots of young trees from the south (sub-species *floridanum*) were very sensitive to autumn frost injury. Trees from an origin more northern than Ohio were subject to stem-killing from late spring frosts because of premature bud-break. This resembles the findings of Ozol (1953) who reported that hardy *Juglans mandshurica* and *J. cinera* were poorly adapted to southern Russia, since they tended to bud out too early in spring and were then injured by frost. Kriebel cited Heikinheimo who came upon this same phenomenon in a northern variety of *Picea excelsa* which was injured by spring frosts in a southern location. Kriebel also found that trees from the Gulf coastal plain continued to grow in autumn until killed by frost in Ohio, while trees from northern sources were the first to stop growing.

In spite of the difficulty of northern trees budding out too soon when moved south, a chilling requirement to break dormancy undoubtedly serves to prevent many northern trees from leafing out in unusually warm early winter weather. Perry and Wang (1960) found that geographical races of *Acer rubrum* from southern Florida had no chilling requirement at all, while the farther north one went, the longer was the chilling requirement necessary to obtain bud break.

RESISTANCE OF ORGANS AND TISSUES

COMPARISON OF ORGANS AND TISSUES

The most sensitive part of a tree to cold, at least during the winter, is the roots, as pointed out many years ago by Göppert (Chandler, 1913). Göppert found tree roots killed in winter at —10 to —15° C., although Engler about 1903 concluded that tree roots were killed by temperatures only a little under freezing (Chandler, 1913). Engler's conclusions would seem supported by those of Junack (1954) on roots of *Quercus robur* and *Q. borealis* in winter as well as those of Graves (1919) on beech. Wartenberg (1933) gave —2° C. as the killing temperature of alder roots. This agrees with Mitchell's (1944) results on guayule in which roots were killed at 28° F. (—2° C.), while tops developed a resistance to as much as 5° F. (—15° C.). But *Pinus ponderosa* roots, dug sometimes from under a snow cover, attained a winter hardiness of about —12°C., and there was only a small seasonal change in hardiness (Parker, 1957a). Avdoshin's (1959) results reveal somewhat wider seasonal flucutations in forest tree roots, and a summer minimum of —2.5 to —3.5° C. occurred in most species.

Apple tree roots are usually killed at —9 to —12° C. in winter, although they can barely withstand —3° C. in summer (Chandler, 1913). Peach roots, according to Chandler, parallel apple roots in resistance. Small apple roots are less resistant than older ones. As a result of this tenderness of roots, root tip killing may occur every year near the soil surface in climates like those of Wisconsin (Chandler, 1913, citing others). Nevertheless, according to Mann (Kramer and Kozlowski, 1960), killing does not occur if there is a good snow cover. In our experience in Idaho, frozen soil can thaw out underneath a snow cover, and soil temperatures then remain near 0° C. (Parker, 1952a).

Some variation in hardiness of apple root stock occurs from one variety to another (Anderson, 1935, cited by Kramer and Kozlowski, 1960). Craig (1900) found root-killing in apple roots in Iowa most severe in light soils, and he concluded that deeply-planted trees are more likely to escape damage than those under a thin soil layer. There seem to be variations in cold resistance in roots of various forest tree species, the more northern species having hardier roots in winter (Avdoshin, 1959).

Chandler (1913) reported a gradual change of cold resistance from the non-resistant roots in winter up to the more resistant trunk, with no sharp boundary. During summer, the most tender parts of apple trees are the cambium, young cortex, and young sap-wood cells (Chandler, 1913). When winter comes, however, the pith of the young twigs remains relatively unresistant, and browning of the twig wood and part of the cortex is common, whereas the cambium appears to be more resistant. Such injury may not, however, spell the end of the twig, and because of cambial survival, new wood may be formed over browned older wood. This situation occasionally develops in bark as well, with new bark underlying the outer dead bark which has been frost-injured. This agrees with findings of Larcher and Eggarter (1960).

In pines of northern climates the resistance of leaves can vary several degrees from one side of the tree to the other, or between shade and sun leaves (unpublished data), but differences at various heights of trees have not been detected in our work.

In general, fruit buds appear to be less resistant than wood or bark tissues in peach trees (Chandler, 1913). On the other hand, after cold waves that came on slowly, more damage has been observed in young woody tissues than in flower buds. Bud scales seem to be of no protection against cold, although they may retain water and retard gas exchange.

Flowers that have developed and young fruits that have begun to form are quite sensitive to cold as is generally known. Fruits of some peach varieties are killed at 28° F. (ca. —2° C.), while others are largely undamaged (Chandler, 1913). Sometimes no injury occurs to the fruit flesh, yet seeds inside can be killed. A thorough study of freezing injury in apple tree parts was made by Pisek (1958), and he seems to agree with Chandler's general conclusion that fruit buds are usually more sensitive than bark to frosts. Pisek found that in June in opened flower buds, pistils were most sensitive (—4° C. damaging), whereas other flower parts were killed at about —5° C. in his freezing apparatus. Kohn (1959), however, gave —1.3 to —1.8° C. for temperatures of killing apple flower pistils. Perhaps it should be mentioned here that leaf or petal temperature may be lower than that of the air at night in the field because of radiational heat loss.

EFFECT OF NATURAL FROSTS ON FLOWERING AND FRUITING

Even though micro- and megasporangia may form in abundance on conifers, there is no assurance of high seed production, although chances are better than with a poor set. A frost in late spring can be disastrous to a future cone or seed crop. A severe frost occurred in the area near the Gulf of Mexico, March 27th, 1955, after a period of warm weather which had brought megasporangia of *Pinus palustris* and certain other pines nearly to a receptive condition. Longleaf pine megasporangia were so injured that by that autumn of 1956 we understood that there was hardly a single mature cone in all the stands of the South. A similar situation may account for the periodic failure of *Pinus echinata* seed crops (Campbell, 1955).

The flowering of oaks may be likewise overtaken with disaster. Acorn production depends largely on late spring frosts in Michigan (Gysel, 1956), and variation in acorn crops from place to place seems related to the occurrence of late spring frosts. Local experience in the deep South suggests that frost also plays a role in both acorn and pecan crop production. But late spring frosts are believed to account only in part for poor beechnut crops in England (Mathews, 1955), and good correlations were found between beechnut crop size and per cent of average sunshine for the previous July. In pear trees there is frequently no correlation between per cent of flowers frost-injured and the subsequent fruit yield (Perraudin, 1955), but experience with many kinds of flowering fruit trees in New England shows that occasionally, severe late frosts can ruin a subsequent fruit crop.

In the western U.S., Maguire (1956) found it possible to predict ponderosa pine crops with some accuracy 27 months in advance of seed fall, on the basis of mean monthly spring temperatures. Abnormally high spring temperatures correlated well with successful megasporangial development and usually resulted in large seed crops 27 months later.

In considering these results, the writer made a similar study in southern Alabama. But a comparison of temperatures and records of long-leaf pine seed crops from tables of Wahlenberg (1946) revealed no correlations of any kind. Apparently the 1955 frost was unusual, and other factors, among them rusts, drought, rain during pollination, insects, and rodents, all play a role in the success of the final cone crop. Although we found a relationship between numbers of megasporangia per tree and the numbers of fallen cones from previous years, seasonal variations in cone crops appeared to be influenced by factors not yet fully assessed (Parker, 1957b).

Prevention of injury to fruit tree flowers has been achieved by making use of the very mechanism that causes their injury, i.e., the freezing of water. Trees are sprayed before and during frost with water, then as it freezes on the flower surfaces, the latent heat of ice formation released prevents the flower tissues, which are not frozen at 32° F., from undergoing freezing injury (Rogers, 1962). Obviously, this would not be effective in severe freezes.

There are, of course, various other methods for preventing injury by light frosts which are not the subject of this review. These include smoke or smudge pots and fans which circulate ground-sedimented air. Chemicals are being worked on which might increase hardiness. One of these, tested in our laboratory for the Naugatuck Chemical Co., N-dimethylaminomaleamic acid, increased the hardiness of developing peanut plants some 2° C., but this may have been the result of arrested development and smaller cell size. Thiouracil is known to increase protein nitrogen in olive trees (Kessler (1956) and seems to increase, but only very slightly, cold hardiness in alfalfa (Jung, 1962).

More effective in reducing frost injury in the field is the method of starting seedlings under an overstory (Amann, 1930). Hollows, where cold air might collect, should be avoided (Hough, 1945).

PHYSICAL SEASONAL CHANGES IN CELLS AND TISSUES

GENERAL MORPHOLOGICAL FEATURES

Some years ago horticulturalists attempted to relate gross morphology of apple shoots to cold hardiness. In spite of many indications of morphological differences in hardy and tender apple varieties, it was believed impossible to name any one test by which the degree of constitutional hardiness of a seedling apple could be foretold. Beach and Allen (1915) felt that, for a certain variety of apple, the length of season necessary to mature it was of the first importance. If the twigs had not matured by the time of the first frosts, injury was more likely to occur. They observed, nevertheless, that tender varieties appeared to be more completely dehydrated by ice formation than hardy ones. Strausbaugh (1921) concluded that the number of lenticels in the bark of plum twigs could be related to their capacity to retain water, but whether this can be related to freezing resistance seems uncertain.

Cell size has been related to cold resistance of cells (e.g., Iljin, 1935; Siminovitch and Briggs, 1953). Höfler (1951), in considering the causes of drought resistance, mentioned cell wall flexibility as well as protoplasmic condition. Dorsey (1934) thought that vacuolar size plays a role, cells with larger vacuoles being more easily frozen.

PHYSICAL SEASONAL CHANGES WITHIN CELLS

VACUOME. Most studies of the physical nature of cells in relation to the development of hardiness have been directed at the cytoplasm. Vacuolar material, however, comprises the main part of most mature plant cells, and yet it has been relatively little studied.

For many years a popular technique was to squeeze juice from leaves, stems, or some other plant part. In spite of inherent errors, results indicate an increase in osmotic pressure (solute concentration) in winter (e.g., Gail, 1926; Sato et al., 1951), even though some data show the "cell-sap" to decline in osmotic pressure as winter comes on (Korstian, 1924; Larcher, 1954). Korstian's data might result from getting less total expressable material from the tissue in winter, and Larcher's data might be partly explained by the fact that the plants he studied do not become very hardy in winter. We are generally agreed that increases in osmotic pressure are largely caused by increases in carbohydrates, presumably sugars for the most part. Salts appear to play a relatively unimportant role in most conifer leaf saps (e.g., Kurimoto et al., 1954), at least as far as seasonal fluctuations are concerned.

It has been difficult, in the meantime, to relate these osmotic pressure changes to microscopic observations of physical cellular changes. Scarth and Levitt (1937) did, nevertheless, suggest that the increase in solutes is reflected in an increased tendency of the cell vacuoles to gelate in certain tree barks. In connection with drought resistance it was suggested many years ago that certain extremely drought resistant ferns have vacuoles which can be gelated during drought and on remoistening solated (Iljin, Rouschal, and others cited by Küster, 1935). When gelated, the cells were able to become air-dry and still recover. It seems possible that gelation could aid as a mechanical buffer against collapse of the cytoplasm during dehydration.

The idea of vacuolar gelation was favored by Clements (1938) in western pines and in certain crop plants. Meyer (1932) suggested that colloids, possibly in the gel state, occur in winter in leaves of pitch pine *(Pinus rigida)*, but no direct proof of this seems ever to have been offered by anyone. Meyer (1928) managed to show marked changes in quantity of expressable sap with the changing season, but Meyer (1932) later concluded that this was the result of total leaf water content changes. Priestley (1930) also mentioned gel to sol changes in the cambium of trees in spring.

Complete cessation of Brownian Movement in the leaf endodermis cells of various species of pine was found to occur in autumn (Parker, 1958b). Leaves that hardened most rapidly in autumn also ceased molecular motion in the vacuoles the soonest. The phenomenon could be most easily observed in endodermis cells because of the large number of visible particles in the vacuolar mass. The same could also be seen in transfusion parenchyma cells, but it was very difficult or impossible to observe in the chlorenchyma cells. Actually the term "gelled" should be used reservedly, since a substance with the thickness or viscosity of glycerine can show no Brownian Movement at all, yet still have liquid properties (Seifriz, 1920). By the word "gelled" we refer to the ability of a cell to retain its vacuome intact when cut open in an isotonic water solution.

Micromanipulation of vacuoles in pine bark was undertaken in hopes of being able to judge their consistency by mechanical means. Using a Chambers micromanipulator, we punctured phloem parenchyma cells in summer and caused them to lose their contents. In winter the vacuolar mass also was ordinarily lost on puncturing or cutting open the entire cells (Parker, 1960a). But it was soon realized that sections that were mounted in a water medium of low solute content were taking up water in winter and solating. When mounted in 2 M. sucrose, however, the vacuolar mass in winter could even be pried loose as a solid entity. In summer, too, a strong plasmolyzing agent was capable of gelating the vacuome, but the situation seemed different from that of winter in that the summer vacuome ordinarily coagulated into droplets during the staining previous to dehydration. Also in summer, the total solid (coagulated) vacuolar material appeared to take up much less space than in winter (Parker, 1960a).

The fact that vital stains, such as neutral red, can cause coagulation of the vacuome in summer but not usually in winter, offers another means of approaching the problem of vacuolar condition. Again using white pine bark phloem parenchyma cells, it became apparent that coagulation with vital stains was the same thing that was occurring when material was "fixed" during preparation for permanent slides. It had long been apparent in our work with the commonly-used elaborate ritual of fixation, embedding, staining, and microtome sectioning, that the vacuome of these cells ordinarily appeared coagulated into droplets in summer, although often homogeneous in winter. Apparently the Flemming's fluid coagulates the vacuolar mass even before alcoholic dehydration (Parker, 1960a).

The vacuolar condition thus appears to change physically with the season, at least in *Pinus strobus.* Further unpublished research by myself and a graduate assistant shows that the coagulation phenomenon on fixation occurs in all ten conifer species studied, but the winter change to a homogeneous vacuole occurs mainly in the Pinaceae, especially in the more cold hardy varieties of *Pinus.* Species like *Cryptomeria japonica,* which are not very cold hardy, seem incapable of altering their vacuolar condition. Possibly these vacuolar changes can be related to the phenomenon in apple twigs in which the hardier tissue takes up dyes like methylene blue more readily than the less hardy (Dunn and Bakke, 1926). The "hydrophyllic colloid content" of the cells was believed to be involved. But Dunn (1937) later concluded that this test was unreliable. Probably hardiness depends on other factors as well, which are not measured by this dye test. The reason why the vacuome should change seasonally or, in fact, take up neutral red as it does, is not understood, but Pop and Soran (1962) suggest that there are submicroscopic lipoidal droplets which tend to coalesce as they take up the dye. The lipoidal emulsion may be kept dispersed by saponins. Our electron microscope views of white pine cell vacuoles do reveal a mass of submicroscopic droplets joined by very fine threads (Parker and Philpott, 1961).

The change in vacuolar condition, especially the increase in sugars and sugar-like substances with numerous —OH groups, may involve a greater binding of water (e.g., Jones and Gortner, 1932). The problem of bound water has been reviewed by Kramer (1955b), and the general conclusion is drawn that bound water in plants is not very well related to either cold or drought resistance. Levitt (1959), however, found that in cabbage there is a slight increase in bound water on hardening, although this is rather small. Levitt argued that most workers have not measured bound water correctly. But even Levitt's method would not be considered very accurate by technologists who measure bound water in wood.

The information on bound water is full of conflictions and contradictions. Whipple (1912) concluded that water is held so tenaciously by apple and pear buds in winter that not enough water can be drawn out to injure the cells. On the other hand, Starck (1936) found that in the main, hardy and tender varieties of apple could not be distinguished on the basis of the amount of unfrozen water remaining on cooling to certain levels. Lebedincev (1930) reported less ice in frozen sap of hardy plants than non-hardy, but this might be the result of higher sugar levels in the hardy plants.

Using Levitt's method for determining bound water, the writer has not encountered significant seasonal variations in white pine or eastern red cedar leaves (unpublished data). We conclude that if bound water is of great importance to cold resistance in evergreen leaves, our methods of measuring it are incorrect. In conifers the loss of volatiles other than water is a serious source of error in the oven-drying treatment used in the method.

CYTOPLASM

Cytoplasmic Membranes and Overall Condition. Although frequently considered in the literature, the problems of the cytoplasm as they are related to cold resistance are far more difficult to unravel than many have suspected. Scarth (1944) concluded that several definite entities are involved in the cytoplasm that changes with the season, although many other workers (e.g., Kessler and Ruhland, 1938) treated the cytoplasm as if it were a single more or less homogeneous unit. Scarth (1941) concluded that ectoplasmic changes are in the direction of greater ductility in winter and that, as long as the point-of-no-return of stretching is not exceded, the plant cell can recover its shape on thawing. He pictured the endoplasm, however, as becoming more viscous on hardening.

One of the interesting controversies that arose before the last World War was that concerning the viscosity of cytoplasm. Kessler and Ruhland (1938) concluded from centrifugation of chloroplasts in vivo that sedimentation is slower in winter than in summer; therefore, the cytoplasm was considered to have become more viscous in winter. Levitt (1956), on the other hand, argued that this could be the result of a change in density of the chloroplasts themselves, and from his own studies and those of Levitt and Siminovitch (1940, 1941), it was concluded that the ectoplasm becomes more fluid in winter. The chloroplasts might not only be different in density as a result of the presence or absence of starch grains, but their centrifugability might vary with the characteristics of the endoplasmic reticulum (ER). Although Jeremias (1956) concluded that chloroplast sedimentation in his material was too unreliable for any definite conclusions, Franke (1962) found an increased amount of sedimentation in summer in ferns, suggesting a lower viscosity at that time than in winter. This situation was believed related to a greater hydration in winter, increased sugar protective substances, and an increased cytoplasmic volume (judging by results of others).

Some have objected to the term "viscosity" itself when dealing with protoplasm, but the word is agreed on by the Society of Rheologists and refers to the rate of change in stress when a shearing force is applied. Apparently the principal difficulty in the use of the word is that cytoplasm is a non-Newtonian fluid and that, although fluid at times, it may still have structure.

The problems of viscosity bring up the question of how cytoplasmic streaming takes place. Some suggest that in the slime moulds, streaming is the result of contraction of a gel portion, this contraction having the same source of motive force as that of muscle myosin (Seifriz, 1955). But in the cyclosis of certain plant cells, streaming seems to originate at the interface between the ectoplasmic gel and the fluid endoplasm (Kamiya, 1962). In either case (interfacial or contraction theory), many suppose that the rate of streaming is a measure of viscosity. But cytoplasmic viscosity is an elusive property (Heilbrunn, 1958). Even quick changes in temperature can alter viscosity (Weber and Hohenegger, 1923), or the application of certain electric currents (Bersa and Weber, 1922).

The use of cytoplasmic streaming rate as a measure of viscosity is invalid according to various workers (Seifriz, 1955; Heilbrunn, 1958) because, among other things, the motive force of streaming is not taken into account during the change in viscosity. Other forces may be involved, as well. Rate of streaming may be related to auxin content (Thimann, 1951), and a lowered rate of streaming in winter in cambial cells of white pine (Thimann and Kaufman, 1957) may therefore be the result of a lowered auxin content and yet may also indicate an increased viscosity. In any case, this streaming is probably taking place in the endoplasm, not in the ectoplasm with which Levitt and Siminovitch (1940) were concerned in trying to explain plasmolysis phenomena and cold hardiness. One can observe streaming in cells of pine leaves which bipasses the chloroplasts just as a river winds through a series of hills.

The criterion of Brownian Movement in the cytoplasm is also a precarious affair, since cytoplasm seems capable of reversing its sol-gel condition simultaneously in different parts of a cell (Weber, 1921; Seifriz, 1955). Furthermore, cells that are injured often show more active Brownian Movement than uninjured cells, and it is frequently difficult in fresh sections to distinguish between injured and uninjured cells.

Much has been written on the type of plasmolysis of cells in winter versus that in summer (e.g., Levitt, 1956). It is generally assumed that the kind of plasmolysis (concave or convex) is a measure of viscosity and possibly also flexibility or ductility. In vertical phloem parenchyma cells of white pine bark, plasmolysis is much more difficult to obtain in summer than in winter, and oftentimes, on addition of a concentrated sugar medium, there is a vacuolar contraction without any freeing of the cytoplasm from the cell wall in summer (Parker, 1960a). This suggests that the viscosity of the ectoplasm is higher in summer and thus supports the idea of the ectoplasm being less fluid in winter (Scarth and Levitt, 1937). Evidently there is a tendency for the protoplasm to withdraw from the cell wall in winter, at least in wheat, and this is related to the development of the "second" phase of autumn hardening and to carbohydrate increases (Genkel and Zhivukhnia, 1959). This work suggests that the plasmodesmata pull more readily free of the cell wall channels in winter, a conclusion also drawn by Genkel and Oknina (cited by Lupareva, 1958). But our electron microscopic pictures of white pine chlorenchyma cells stained with $KMnO_4$ reveal no fundamental differences in winter and summer in the appearance of the plasmodesmata (unpubl. data). The "concave" condition in plasmolysis may be merely the result of a greater cytoplasmic glutinosity or stickyness which causes it to adhere to the cell wall in certain places, according to Seifriz (1955), and he felt that there is no reason to suppose that this adherence tendency is a measure of viscosity. He also considered the summer condition might be related to the tensile strength of the Hechtian strands which attach the cytoplasm to the cell wall. But whether such strands are extensions of plasmodesmata or are points of adherence to the cell wall is uncertain (Seifriz, 1955). Recent electron microscopic pictures (Sun, 1960) indicate plasmodesmata to be more common in plant cells that was previously suspected. In white pine chlorenchyma cells plasmodesmata appear in electron microscopic views as discrete patches and thus to have a pattern which is not suggested by the rather random nature of the Hechtian strands seen with the light microscope. Whatever the strands represent, Scarth (1944) believed that they are more ductile in winter than in summer.

Further insight into the nature of cytoplasm is obtained from studies on the sinking of starch grains. In winter they can often be seen to have sunken to the bottom of ray cells of pine bark (Parker, 1958b, 1960a). In summer the cells have to be centrifuged to obtain starch grain sedimentation, indicating an increased summer consistency of that part of the cytoplasm which binds the starch grains (using "consistency" to mean viscosity, elasticity, and adhesiveness). This seems in contrast to results of Kessler and Ruhland (mentioned above) and of others who worked with "press-sap". Since press-sap includes vacuolar material, it would not be surprising if it were more viscous in winter because of vacuolar carbohydrate increases. Eventually the entire controversy over viscosity may be difficult to settle, partly in view of the elusiveness of the word's meaning. The complexity of the problem is indicated by Stretkova's (1961) finding that more hardy plants decline more rapidly in viscosity on cooling than the non-hardy.

It was hoped that the electron microscope might reveal some distinct cytoplasmic changes which could be related to viscosity changes, for Wohlfarth-Bottermann (cited by Franke, 1962) has shown that viscosity of animal cells could be related to a visible cytoplasmic structural aspect. At first we thought there might be seasonal differences in the extensiveness of the endoplasmic reticulum in pine cytoplasm (Parker and Philpott, 1961), but further work and better summer fixation makes this suggestion seem doubtful now. Especially in need of further study is the question of change in the osmiophyllic character of the vacuolar membrane. The use of osmium stain and the light microscope seem promising for such a study.

Although membrane systems appear easily destroyed by injurious freezing (e.g., Franke, 1962), enzyme systems may conceivably be injured without visible membrane injury. Meryman (1957), citing Joslyn, stated that dehydrogenases are more sensitive to freezing than certain other enzymes like catalase and lipase. Ullrich and Heber (1961) found that soluble enzymes which are not sedimented at 28,000 g and certain particulate enzymes like adenosine triphosphatase showed no loss of activity from in vitro freezing. But enzymes of the Hill-reaction and of photophosphorylation did show irreversible loss of part of their activity after freezing. Cytochrome oxidase lost some activity, but this could be partially restored by dispersion of the coagulate. Protein denaturation might result not only in membrane breakage (as to ER) but also in injury to the enzymes, some of which may be located on the ER.

Chloroplast Changes. Chloroplast migrations in evergreen leaves have been known for about a century (Mohl, mentioned by Lewis and Tuttle, 1920). Mer (1876) also mentioned a condition of chloroplasts coming free of the cell wall in winter. Haberlandt (1876a, b, c) made a thorough investigation of chloroplasts in various plants and concluded that the effect of frost resulted in the clumping or balling-together of the chloroplasts, but not of their distruction unless the cells were injured. In plants frozen and killed, on the other hand, chloroplasts were observed to disintegrate. Haberlandt cited Kraus who found in 1872 that the plastids of pine leaves migrated to the "Innere" or central part of the cells. It was believed that as a result of this heaping together, leaves took on their characteristic dull appearance. This seems quite possible to us, although other changes may occur in winter, for example, total chlorophyll sometimes declines in winter without any apparent chloroplast clumping (Bourdeau, 1959). If carotene also declines, leaves may become quite brown, as in some species of *Juniperus*. But they usually recover in spring.

The question then is whether chloroplasts undergo internal changes in winter or merely change position in the cell. Lewis and Tuttle (1923) felt that chloroplasts "laked"[3] in winter in the native Canadian spruces, and this concept seemed accepted by Zacharowa (1929) who worked in the cold climate of Moscow, Russia. Zacharowa used the word "zerfallen" (fallen apart) to describe the chloroplast changes in conifers. She explained the marked inhibition of CO_2-absorption in winter by these chloroplast changes, although it is more probable that all gas exchange is cut down by stomatal closure in winter (Tranquillini, 1957) or that CO_2 assimilation by chloroplasts is retarded by sugar accumulations (Parker, 1963). In spruce, Zacharowa concluded that chloroplasts remain intact longer into the winter than in pine, and this seems to explain the greater net photosynthesis of spruce during the colder season than of pine. The idea of winter chloroplast destruction was also expressed by Schmidt (1936) who likewise used the word "zerfallen" to describe the disappearance of spruce chloroplasts around the first of January.

In the meantime others (e.g., Riasanzew, 1930) became convinced that conifer chloroplasts merely migrate to a different location in the cell in winter, and, although the plastids appeared to shrink, disintegra-

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[3] A term applied to red blood corpuscles when they lose the continuity of their outer membranes.

tion was not mentioned. Kessler and Ruhland (1938) observed chloroplasts throughout the winter in species of *Buxus* and *Ilex.* In wheat, Heber (1959b) concluded that chloroplasts swell somewhat in winter as they take up more sugar and water-soluble protein. Chloroplasts seem capable of shrinking or swelling as they take up or lose water (Kramer, 1955a).

It would certainly be remarkable if chloroplasts with their lamellae and double surface membranes could dissolve in autumn and then reform in spring as one would infer from certain of the foregoing as well as from the recent work of Tutayuk and Agaev (1961). Rather, it now appears that the chloroplasts in clumping and losing chlorophyll become harder to see in the light microscope. Guilliermond (1941) suggested that chloroplasts could in some cases retrogress to their original mitochondrial condition (nowadays this might be called a return to a proplastid condition), but we have not seen any evidence of it in our work. Genkel and Barskaya (1961) observed differences in spruce chloroplasts in summer and winter, but changes are difficult to see in the American (A. I. B. S.) republication of their pictures. Our studies indicated no fundamental changes in either the chloroplast envelope or the grana and intergrana lamellae as winter came on (Parker and Philpott, 1961, 1963). Chloroplasts can be seen in the light microscope in a "dividing" state (constricted in the middle) on occasion in white pine, but never fractionating, according to our observations. Their agglomeration into the cell-sides is evidently the main factor in making them difficult to see. The appearance of greenish particles about the size of mitochondria in winter cells may be the result of chlorophyll coming free of the chloroplasts and being taken up on oil droplets, and perhaps even on mitochondria. Chloroplast migration, in the meantime, may be a result of ectoplasmic changes, but may also be influenced by illumination, since we have observed migration in summer in some pine cells subjected to very intense light and heat. In many conifers no definite chloroplast migration may occur at all in winter, although they may become dislodged from their neat cell-side arrangement and become scattered in the cytoplasm.

NUCLEUS. Nuclear condition has usually been ignored in studies on cold resistance. If hardening involves changes in the cell's fine architecture, then one should study the nucleus as much as the cytoplasm, especially in view of the importance of the nucleus to cell metabolism. Strausbaugh (1921) found nuclear rupture in cells of plum buds after injury by freezing. Some investigators have noted that the nucleus is larger in cold-hardy tissue than in non-hardy; according to some, this may be related to a higher degree of hydration. Siminovitch (1959) noted the nucleus to be more prominent in winter in certain woody twigs. The nucleus of certain pine cells (Parker, 1960a) and of black locust bark cells seemed to us to stain less readily in winter with bromphenol blue-$HgCl_2$. But this effect could result from masking by the cytoplasm which itself takes up this dye to some extent, and the masking may be greater in winter. There is some question as to the specificity of bromphenol blue-$HgCl_2$ to proteins, and we have noted that it stains sieve tube callose readily, this callose being supposedly a carbohydrate. But it is possible that callose could have a nitrogen fraction.

In certain Russian work which I cannot cite it was suggested that there is a relationship between certain nucleic acid changes and hardiness. Of course, nucleic acids are not confined to the nucleus. Jung (1962), in this connection, found that thiouracil and guanine could slightly influence protein and cold hardiness levels in alfalfa.

Since proteins are affected by exposure to very low temperatures, it would not be surprising if the genetic material itself is disturbed by such treatment.

BIOCHEMICAL SEASONAL CHANGES IN CELLS

PROTEINS AND AMINO ACIDS

In the modern American literature of plant physiology there has been much discussion over proteins in connection with cold resistance. It is reasonable to suppose that since the basis of the living process consists of protein-constituted enzyme systems, nucleic acids associated with proteins, and cytoplasmic lipo-protein membranes, one should seek more information about proteins in trying to solve the problem of the causes of cold resistance.

Protein levels are known to vary seasonally in many species of plants, but in numerous cases, at least, this may be due to nothing more than changes in enzyme levels. The activity of amylase and catalase may decline somewhat in the bark of certain woody plants as cold weather comes on, but catalase seems to reach a high point in January (Manskaja and Schilina, 1931). During spring, catalase may rise momentarily, then decline in wood, bark, and buds. In the meantime, amylases appear to be active in bark, wood, and buds during the whole rest period.

These results are actually not very clear-cut, but those of Mulay (1931) are more so. He found that soluble, insoluble, and total nitrogen increased markedly from September to December in pear bark, then declined in spring. This agrees with results of Karmarkar (1934) who recorded a definite rise in protein nitrogen and total nitrogen in apple shoot bark in autumn, reaching a peak in winter. Piney (1929) noted that in January, protein nitrogen reached a maximum in beech trees and thereafter declined, especially just before buds began to burst.

Black locust *(Robinia pseudoacacia)* is remarkable for the large amount of protein in its inner bark (Jones and Phillips, 1937; Parker, 1958a), and it was suspected by Jones and Phillips that there are larger amounts in winter than in summer. Jones and Gersdorff (cited by Jones and Phillips) believed that this black locust bark protein consists mainly of globulin, albumin, and a proteose. Zacharowa (1925) also commented on the high protein content of meristematic tissue of various crop plants and its high resistance to cold. The inner bark of black locust is especially suited to protein studies because it is not precipitated by vacuolar substances on homogenation as well as being relatively high in quantity (Siminovitch and Briggs, 1949). They found a rise in total protein nitrogen in autumn which was largely accounted for by a rise in water soluble protein. Although Heber (1959a) suggested that protein results with black locust could be the result of changing extractability as pH changed with the season, Siminovitch (1959) continues to feel that there is a close relationship between protein synthesis and the development of cold hardiness. The effect of sugars, also increasing in autumn, was discounted; in fact, the disappearance of starch was believed to have the advantage of the loss of solid particles on which ice could seed, rather than the gain of sugars from starch hydrolysis (Siminovitch and Briggs, 1954). Although an interesting idea, it seems unlikely to us, since starch grains can be observed in white pine bark that is quite hardy and viable.

In spite of the evidence for the importance of water-soluble protein, it was known long ago (Müller-Thurgau, 1880) that potatoes show a gain in nitrogenous substances after cold treatment, and in fact undergo a marked rise in soluble protein at 3° C. (Levitt, 1954); yet this plant does not become appreciably cold hardy. But in fairness to protein-proponents it should be mentioned that potatoes sometimes increase in sugars, but do not harden (Levitt, 1954). Artificially-induced hardiness is better related in tree barks to sucrose increases than to those of water-soluble protein (Sakai, 1958a). Somewhat similar conclusions were drawn by Heber (1959a), although sometimes sugars did not show close relationships with cold hardiness. In fruit tree barks there is more of an increase in non-water-soluble protein than in water-soluble (Henze, 1959). In English ivy, although there is a slow autumn increase in water-soluble protein, its seasonal trend is poorly related to hardiness changes, in fact it increases in spring on dehardening (Parker, 1962b).

It has also been suggested that instead of increasing, proteins undergo splitting during hardening (Lidforss, 1896). Perhaps protein-splitting is suggested by Levitt et al. (1961), in which an increase in —SH groups occurs on hardening in a variety of plants. But it is important to know which proteins undergo these changes. Such —SH increases might be the result of increases in amylase and phosphorylase which, according to Ewart et al. (1953), contain —SH groups. It is possibly of some connection that additions of certain compounds with —SH groups to cells injured by irradiation retards development of injury symptoms (Svilvinyi et al., 1961).

If protein splitting occurs, this might involve enzymes of the respiratory system and thus alter the respiratory rate. It is known that respiration of apple twigs varies seasonally (DeLong et al., 1930), and evergreen leaves seem to have a lower winter than summer rate when measured at standard temperatures (Bourdeau, 1959). But such respiratory changes could be the result of changes in oxygen availability, pH, or, according to Tumanov and Trunova (1958), auxin content. Redox potentials may also vary seasonally (Lupareva, 1958; Konovolov et al., 1959). These potential changes may reflect —SH level changes which, in turn, could be affected by a shift in the cystine-cysteine balance, for example.

Both amylase and phosphorylase enzymes have been detected in black locust bark (Ewart et al., 1953) and could account for starch-sugar conversions in autumn. Sugar increases in beets in autumn are known to be paralleled by an increase in phosphorylase activity (Terumoto, 1957, 1958a). This might account for the autumn rise in protein. The end of the rest period in fruit trees is related to a decline in phenoloxidase activity while there is a heaping up of reducing substances (ascorbic acid, glutathione, and others) (Tomaszewski, 1957). Perhaps this would account for the early spring decline in protein nitrogen found by Sattler (1930) in *Hedera helix* and *Ilex aquifolium,* although this was followed by the annual peak in April in *Hedera* and in May in *Ilex*. Our data on *Hedera* indicate similar trends (Parker, 1962b) as in Sattler's for *Hedera*.

A clue as to what such protein changes might represent should be given by cytological investigations. But it has been difficult to find a stain that is exclusively for protein. Results with the bromphenol blue stain of Mazia et al. (1953) suggest that the protein is cytoplasmic and not vacuolar (Parker, 1959b, 1960a). Siminovitch (1959) described a clustering of opaque material near the nucleus in unstained black locust bark cells in winter. On the other hand, work with *Syringa* indicates that the protein increases in winter are vacuolar (Kern, 1923). But Kern's use of mercury stains following soaking with caffein may be misleading, since phenolic compounds can give a strong reaction with mercuric salts, as in conifer vacuoles (Parker, 1957a). Kern also found a ninhydrin reaction increase in autumn and certain other vacuolar changes which he thought might reflect protein increases, but ninhydrin is more of an amino acid indicator than one of protein. An extensive unpublished study by the writer of amino acids and amides in *Pinus strobus* bark indicated no consistent seasonal changes that could be related to cold hardiness changes. Amino acids are periodically resurrected as possible associates of cold hardiness but at best they are poor indicators of protein levels, and there is no evidence that they would have a protective effect against freezing.

One is, of course, confronted with the question of how protein increases in themselves could account for hardiness increases. Gortner (1938), in reference to heat resistance, suggested that when water is more firmly bound, there is a lowered metabolism and a strengthening of the intermicellar bridges. The presence of free water tends to weaken such intermicelllar bridges in protein. Possibly increased proteins could bind more free water. On the other hand, Stocker (1951) suggested that there might be an opening of the protein-lattice system, resulting in increased pore-permeability and stronger hydration on cold-hardening. But a decrease in [heat] resistance seems to be accompanied by an increase in total hydration of the protoplasm, owing perhaps to a more ready unfolding of micelles (Levitt, 1956, citing Bogen). Harvey (1918) found that cabbage proteins were changed on hardening to less precipitable forms, but this does not seem to have been entirely verified. Proteins from pea stems, however, become much less heat-coagulable when the pectin content of the non-particulate cytoplasm is induced to increase or is actually increased by pectin additions (Galston and Kaur, 1962).

It is questionable whether heat and cold resistance are as closely related as some would like to think. Some marine plants are quite cold resistant yet not very heat resistant, while others are not very cold resistant and quite heat resistant (Biebl, 1939; 1958). And certain agricultural crop plants decrease in heat resistance, but simultaneously increase in cold resistance when fertilized with nitrate nitrogen (Badanova, 1958). We have found *Juniperus virginiana* leaves are more heat resistant than *Pinus strobus* leaves, but the former are less cold resistant than the latter in winter (unpubl. data).

Whatever the protein structural changes, it seems reasonable to think that proteins — either of membranes or of enzymes — must be protected in hardy tissue from sudden water loss attendant on freezing. Sugars and sugar-like compounds might be able to stabilize this water without binding to proteins at all. Substances like sucrose markedly retard the rate of ice crystal growth as well as alter its pattern, although they do not depress the ice nucleation point any more than they do the freezing point (Lusena, 1955). But proteins are as effective as sugars in ice crystal alteration, according to Lusena. Possibly sugars "solvatize" the proteins, a process by which they protect the proteins directly by replacing some of the hydration water or serve to hold this hydration water more firmly (Ullrich and Heber, 1957, 1961).

It has been proposed that pentose and hexose sugars, at least in fruit tree barks, increase not only as free sugars on hardening but also as sugars chemically bound to certain of the protein fractions (Henze, 1959). That such bound sugars may not be contaminants was indicated by the fact that electrophoretically-separated proteins still had the sugars bound to them (Henze, 1959). We have repeated certain of his work, using black locust bark protein, and found the same two sugars (arabinose and galactose) with some ribose in the protein hydrolyzate. There did not appear to be galacturonic acid in the hydrolyzate, which would suggest the presence of pectin. Although the water soluble protein quantity increased in autumn in our black locust bark samples, the ratio of bound sugar to protein did not increase significantly. We also verified Levitt's (1954) finding in cabbage, i.e., the protein-bound carbohydrate is no higher in hardened than in unhardened leaves (unpubl. data). The only marked change we observed in cabbage was an increase in oligosaccharides like raffinose, a change which we have observed in many vegetables, e.g., parsley, which can withstand light frosts, but an observation which we have never seen published. Heber (1959a) also found no increase in protein-bound sugar in wheat as leaves were hardened. As a result of this, one is inclined to conclude that the effect of the sugars is not exerted through chemical binding to the proteins, but that, instead, increased sugar may alter the state of hydration of enzymic as well as membrane proteins, may change the condition of the water by sugar-replacement, or, as some have suggested, may act as a solvent together with the sugar-bound water during periods of cell desiccation attendant on ice formation.

LIPIDS AND RELATED COMPOUNDS

After the invention of the microscope, early work in the previous century suggested that as starch disappeared (indicated by a simple iodine test), oily or droplet-like substances increased which can take up such substances as the sudans. This phenomenon was reported by numerous writers in the late 1880's (Levitt, 1956:88). For example, Fischer (1891) concluded that some tree species produce a fatty material in winter, but that other species remain starchy. However, Fabricius (1906) questioned these generalities, and Niklewski (1906) concluded that the increase in fat does not follow the decline in starch. This agrees with Meyer's (1918) conclusion, and Kirchoff (1915) thought that starch was replaced by tannins.

At the present time it appears that many trees, as species of *Tilia, Betula,* and *Robinia,* retain a great deal of starch in winter, but this starch is commonly not found so much in the bark as in the wood (rays, young tracheids), perhaps because of poorer enzyme activity in the wood. Furthermore, starch reversion or hydrolysis is not always complete in hardening cells, and in cases we have observed, some starch is frequently left in conifers in winter, as in trunk bark (Parker, 1959a). Nevertheless, such winter grains are normally smaller in winter than in summer.

Another feature that should be noted is that the sudan stains, applied in alcohol solution, can accumulate in phenolic compounds and not exclusively in lipidic ones. Numerous phenolic compounds are present in whole extracts of bark and they appear to be vacuolar, judging by results obtained with Ehrlich's reagent (Parker, 1957a).

In the cytoplasm itself there is usually an increase in sudan-black-staining particles in winter, evidently following the decline in starch in autumn (Parker, 1957a). These particles are sometimes only about 1 mμ in diameter and often appear to be hexagonal in fresh unfixed sections. The chloroplasts also take up some sudan black in summer, but this staining effect does not seem to increase in winter. Osmium stains indicate that the tonoplast is more osmiophyllic in winter (Parker and Philpott, 1961), but we feel that further work on this is necessary before definite conclusions can be drawn. That there is a fatty layer formed in autumn in the outer cytoplasm has been suggested by Russian work (e.g., Avdoshin, 1959). Such cytological observations may be related to changes observed by Siminovitch and Chater (1958) in phospholipid levels. Their data show a high in extracted phospholipid in winter and a decline to nearly nothing in spring.

Most recently a relationship has been suggested between the swelling capacity of mitochondria and the "chilling" (not involving freezing) capacity of the cells (Lyon et al., 1962). The chilling capacity of various species of plants has also been related to the level of saturated fatty acids, those more sensitive having a higher mole percentage of saturated fatty acids than the non-sensitive plants (Wheaton et al., 1962).

SUGARS AND RELATED COMPOUNDS

The presence of sugars in plants has been known since very early historical times, and the cultivation of sugar cane was practised in India, China, and later in Egypt at the beginning of the first millenium A.D. (Pigman and Goepp, 1948). It was not until the 18th century, however, that crystallizable substances were isolated from honey, grape juice, and raisins. With the advent of the microscope it was found that starch grains are commonly dissolved in trees in autumn and replaced in spring. This phenomenon was soon related to sugar changes. Kirchoff in 1811 was able to show that starch breaks down to a sweet syrup from which a sugar is crystallizable (Pigman and Goepp, 1948). It was, therefore, logical to assume that there is not only a starch decline in trees but simultaneously a sugar increase (Mer, 1876). Lidforss (1896) found a sugar maximum in winter in evergreens. DuSablon (1906) and later others cited by Levitt (1956) reported much the same. Miyake (1902) pointed out that near Tokyo there was some starch left in trees in winter, but that in colder climates farther north, there was hardly any. Pojarkova (1924) thought that the less starch left in winter, the deeper the winter rest and the hardier the plants. She believed that the starch is converted to oils, but, it is more likely that it is mainly converted to sugar (e.g., Worley, 1937).

If it could be clearly shown that sugars increase as hardiness increases, and decreases as it sinks, then this would at least be circumstantial evidence for a sugar-hardiness relationship, although of course not proof. Sinnott's (1918) study showed sugar increase in a great number of trees species as winter came on. The fact that starch was regenerated by warmth could be easily demonstrated by bringing winter twigs into a heated room. A winter starch minimum has been shown by more than 35 writers, reviewed by Levitt (1956:78).

It was realized at an early time that the vacuolar sap of living cells of, for example, bark, consist mainly of water and sugars, while electrolytes account for only a small part of the solutes (Chandler, 1914). In spite of this, he concluded from depression of the freezing point and dry weight determinations in apple twig cortex, that about half of the vacuolar material is a high molecular weight compound other than sugar. Our own studies suggest that in some trees this could be various phenolic compounds together with certain pentosan-hexosans and other compounds not yet analyzed. These do not seem to fluctuate seasonally as much as the sugars (Parker, 1962a).

Most of the older studies on sugars in plants involved simple methods of analyzing for reducing and non-reducing sugars. These were assumed to be fructose, glucose, and sucrose. Unknown until but a few years ago, there are a number of other sugars and sugar-like compounds in overwintering plants which have been revealed by paper chromatography. The writer found that *Pinus monticola* leaves from Idaho forests in winter contain an unidentified sugar in quantities as high as sucrose. At the same time, this unknown sugar is entirely lacking from leaves of the same species taken from a heated greenhouse. About mid-April this sugar disappears in the field trees, not only in white pine but also in ponderosa pine (Parker, 1957a). Analysis indicated that it is raffinose, a trisaccharide (Parker, 1957a). Together with stachyose, it had already been found in the bark of certain fruit trees in winter (Bradfield and Flood, 1950), but this was not related to seasonal fluctuations by these writers.

In *Pinus palustris* Parker (1957b) found that when starch largely disappeared from the leaves of mature trees in the mild winter of Brewton, Alabama, raffinose appeared in the chromatograms and rose to a concentration similar to that of sucrose. When the warmer weather of spring arrived in February, starch reappeared and raffinose declined to undetectable amounts. Sucrose levels did not change very much during this time. Of course, raffinose and even stachyose can occur in tree barks in summer in certain species (Zimmermann, 1957) or even in the exudate of certain pines in spring (McWilliam, 1958). But still, the question was not answered as to the general character of these seasonal sugar changes.

A general survey of bark as well as leaf sugars was made in southern New York for several species, native and exotic. In every kind of conifer, in both leaves and bark, an increase in raffinose and sometimes in its relative stachyose occurred in autumn and early winter (Parker, 1959a). In the bark of many common broad-leaved trees the same general picture appeared (Parker, 1958a). At the same time leaves of deciduous trees were incapable of this oligosaccharide increase in autumn even though they persisted on the trees well beyond the first autumn frosts in a green condition. The seasonal coming and going of raffinose has now been demonstrated in six families of evergreen trees (Hida et al., 1962). In *Hedera helix* raffinose also comes and goes with the season (Jeremias, 1958; Parker, 1962b), but sucrose in *Hedera* has the highest level of all other sugars, a situation which appears quite common, especially in tree barks (Parker, 1962a). Other sugar-like compounds or sugar-containing compounds like glucosides undergo seasonal changes (Neish, 1958; Sakai, 1958c). But these changes are relatively small compared with total sugars that may increase as much as ten times over the summer levels (Parker, 1962a). In the meantime total protein nitrogen barely increases by half of its summer level in tree barks of northern climates (Parker, 1958a).

Raffinose increase has also been found in wheat in Russia and was related to hardiness changes (Gunar and Sileva, 1954), but this relationship has been questioned (Liashchenko et al., 1958). Good relationships between raffinose levels and hardiness in white pine leaves are demonstrable (Parker, 1959b, c), but sucrose may remain at high levels in spring, possibly because of renewed photosynthesis. Although Siminovitch and Briggs (1953) encountered an increase in sugars on hardening in autumn, spring sugar-hardiness relationships were poor.

In our opinion sugars and their close relatives have been in a state of ill-repute too long and should be reinstated in the hardiness picture. Certainly the work of Heber (1958a, b), Sakai (1958a, 1960a), and Tamari (1962) would lead one to the conclusion that sugars and sugar-like substances must be important. Not only sugars, but their close relatives the polyhydric alcohols, increase in barks of some tree species in winter (Sakai, 1961). Studies with insects implicate glycerol as the protective agent in overwintering stages (Salt, 1961). Bull and human spermatozoa can be preserved at −196° C. by treatment with glycerol (Sherman, 1962), and blood and animal tissues for cancer research can be similarly preserved. This seems irrefutable evidence in favor of the idea of the protective effect of sugar-like substances for living cells and complex proteins.

Recent work on fishes suggests an increase in a non-protein nitrogen fraction, but this apparently serves only as an "anti-freeze", not as an antidote to ice injury; nor does it hold for all fish species (Gordon et al., 1962). In woody plants we have already mentioned that non-protein nitrogen seems to have little or no relation to cold hardiness. Modern Russian research, which has been extensive in cold hardiness, never seems to have taken protein changes very seriously; instead, it emphasizes the importance of starch hydrolysis to sugars in developing both the "deep" dormancy and cold resistance of winter (e.g., Khristo, 1961). Russian work also mentions lipids frequently.

Soaking cells in various kinds of sugar solutions is known to increase their cold hardiness (Tumanov and Trunova, 1957), but this usually amounts only to a hardiness increase up to 6° C. (Levitt, 1957b). The effectiveness of sugars also depends on the season applied. In some cases, of course, the effect may be merely the result of osmotic loss of water from the cells. Possibly the most convincing evidence for importance of sugar additions in increasing hardiness is that of Ullrich and Heber (1961). As mentioned elsewhere, they found that certain enzymes are protected from ice-coagulation by sugar additions in vitro.

Possibly the reason sugar additions to cells are usually ineffective in producing hardiness of any great extent is that insufficient sugar accumulates in the cytoplasm, and the reason why sugar analyses of whole cells or tissues may not relate well with hardiness changes is that what is measured may be largely vacuolar or sieve tube sugar which could have little bearing on the sugar content of the living cytoplasm. On the other hand, it has been suggested that the hydration of the cytoplasm, and hence its hardiness, is dependent on sugar levels in the vacuome (Levitt, 1956).

In view of the evidence at present, the hardening mechanism may be the result of a complex of factors, at least when hardiness is highly developed. Certainly the picture is not yet clear. For example, it is known that additions of indole acetic acid can reduce the ability of sucrose to contribute to hardening (Tumanov and Trunova, 1958). While some popular writers may imagine that glycerol metabolism could account for protection of Martian plants and animals from very low temperatures, it is hardly likely that evolution would be so simple as to have ended in a single one-compound mechanism. Yet it is interesting that the sugar-phenomenon in hardening plants is practically universal on this planet, even if it is not the only cause of hardening.

POLYSACCHARIDES AND COMPLEX SUGARS

One might suspect that the above-discussed changes in sugars could also be symptomatic of related carbohydrate cellular changes. While starch-sugar changes are fairly well understood, the process of converting sugars to other sugar complexes has not been so well worked out. Neish (1957, 1958) found that in spruce leaves both pinitol and pungenin increased in winter as well as raffinose. Shikimic acid varied very little with the season. We have never found consistent seasonal changes in organic acids e.g., citric, malic, and tartaric, in conifer leaves that could be related to cold hardiness. Neish (1958) also cited Tanret who found picein in Norway spruce in higher amounts in February than in May. It thus appears that there are a number of sugar-like substances in conifer leaves which increase in winter and which could influence hardiness. Anthocyanins, which of course contain a sugar residue, also may increase in winter and are sometimes an indicator of hardiness in comparing one variety to another (Tischler, 1905), but these pigments are confined to vacuoles. It is known that the more northern and hardier races of jack pine, as mentioned before, take on more purplish coloration in winter than the southern, less hardy races (Stoeckeler and Rudolf, 1956). In *Hedera helix* the appearance and disappearance of anthocyanins with the season have been directly related to increases and decreases in cold hardiness, possibly because anthocyanins are symptomatic of sugar level changes (Parker, 1962b). But such coloration is influenced by illumination intensity, and if the genetic factor is lacking for red pigment production, this does not preclude a plant's ability to harden.

Work with pectins and pentosans has been intriguing but mainly disappointing. Lott (1926) believed he found a correlation between hardiness in blackberry and raspberry varieties and both the per cent of bound water and per cent of pectin. But his method of estimating pectin with NaOH neutralization seems unreliable by modern standards. Pentosans, or as they are sometimes called, hemicelluloses, have been a likely candidate for importance to cold resistance for many years. Originally hemicelluloses were considered to be polysaccharides that could be removed from woody tissue by cooking with 5% H_2SO_4. Schellenberg (1905) concluded that a layer on the inner side of the cell walls of living cells of woody plants increases in autumn and decreases in spring, a fact originally noted by DuSablon (1904). Yet hemicelluloses in maple trees appear to decline in winter (Jones and Bradlee, 1933).

As far as cold resistance is concerned, the layers on the cell wall are probably not of great importance unless cell wall flexibility is more important than we suppose. But if soluble polysaccharides are accumulating in vacuoles or cytoplasm this is quite another thing. This, in fact, is the basis of the "pentosan" theory in which pentose sugars form into chains as pentosans (Hooker, 1920). Hooker was led to conduct his investigation as a result of a report by Spoehr that cacti increased in pentosan content when put under xeric conditions. The pentosan content of apple shoots seemed to Hooker related to their varietal hardiness. In his data seasonal pentosan changes are hardly impressive, and one is inclined to question his fermentation procedures for determining pentosan. Rosa (1920) also favored the idea that cold resistance is a function of pentosan content in certain vegetable crop plants. The higher pentosan content of the "protoplasm" was believed to allow greater water "absorption" in colloidal combination, and such water was supposed to remain unfrozen at ordinary winter temperatures. Hooker (1920) believed that strongly-bound water is never formed into ice unless the temperature falls below —73° C. He felt, nevertheless, that there is a smaller quantity of "less-strongly bound water" in winter (water frozen at temperatures between about —2 and —73° C.) than in summer, and therefore a decrease in most of the freezable water. This less strongly-bound water was supposed to be bound by pentosans to a large extent, although whether pentosans were located in the cytoplasm or in the vacuome could not be determined. Not very long afterwards, Murneek (1929) compared the terminology of hemicellulose, pectin, and pentosan, and expressed the belief that pentosans are effective in cold resistance. Doyle and Clinch (1926), on the contrary, reported an absence of relation between pentosans and hardiness in conifers, which were extracted with 1% HCl. Hildreth (1926) came to similar conclusions with apple trees. Levitt (1956) wrote only a short paragraph on pentosans in his lengthy review, mainly citing authors who made no claim for relationships to hardiness.

In recent years, however, the pentosan theory has been revived (Jeremias, 1956), at least for winter wheat. Henze (1959) also concluded that there are increases in both hexosans and pentosans in bark cells of certain fruit trees (we do not refer here to the protein-bound sugars). Nevertheless, his tables do not always show very consistent seasonal changes and more data would seem needed, especially since only three measurements per year were made per variety. We do not mean to deprecate the pentosan theory entirely, perhaps partly since tolerance is at least a sort of analgesic for the always-present possibility of being wrong, but it is not entirely out of place to remark in closing that one can be as dogmatic in rejecting a theory as in accepting it.

In this review we have followed the course of research in cold resistance in woody plants in its more important aspects. Starting with plant geography, we have progressed, or rather retrogressed, to the level of the molecular. Certainly here in the molecular realm lie many of the real secrets of how cold resistance works. The temptation is to continue searching into ever smaller aspects of the problem, and we might well quote Mommaerts (1962):

"So we open this box, with greater or lesser difficulty, only to find another one inside; and so on, until we are left with the smallest box we can handle. This, we think, must be the last one to keep the secret from us.... This is precisely the attitude of a child facing an unknown machine such as a cigarette automat, coming to the conclusion that 'there is a man inside'. Throughout the development of microscopy, polarization- and ultra-microscopy, and nowdays electron microscopy, we have been looking for that little man, in the cells, the nuclei, the mitochondria. Each time he seemed to be waiting in the next smaller box, but having arrived there now, we may as well realize that, meanwhile, he has quietly slipped away."

It is of course popular today to talk about RNA, draw spirals on the blackboard and call them proteins, and spray hormones on things to make them grow into new and stranger shapes. But the truth is that we should not lose sight of the larger picture, and, in the case of cold resistance, not forget that the grandiose problems of plant geography and synecology are as much a part of the great puzzle as are those of the molecule.

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