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FROST HARDINESS OF SPRING RAPE: EXOTHERM AND LIPID STUDIES

by

Darryl Stout

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ABSTRACT

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During a five week hardening period, the freezing point of spring rape decreased and remained lower than the nonhardened value, in both cotyledons and leaves. The cotyledons reached their minimum value in one week, whereas the leaves required two weeks. The supercooling point and freezing point were independent of the percent moisture of cotyledons, whereas they were positively correlated to the percent moisture of leaves.

Germination of plants in the coldroom as opposed to in the greenhouse, resulted in the cotyledons and incompletely expanded leaves having lower freezing points, which were equivalent to those found for cotyledons and leaves directly hardened. The supercooling point of completely expanded leaves of plants germinated in the coldroom was constant during maturation, whereas it decreased in plants germinated in the greenhouse showing that the cold treatment had an effect which extended throughout the growth period. The amount of supercooling was negatively correlated to the weight of water per leaf disc in both the plants germinated in the coldroom and those germinated in the greenhouse.

Nugget cotyledons had a lower freezing point, supercooling point and less supercooling than Target cotyledons, suggesting genetic control.

The quantity of complex lipid and lipid phosphorus of leaves increased during a 35 day hardening treatment, indicating that the quantity of membrane per cell increased. The mole percent unsaturation of fatty acids of the neutral and complex lipid decreased during the hardening treatment, which was a change opposite to reports in the literature.

INTRODUCTION

Spring rape production in Western Canada has increased from two million acres to four million acres in the last two years. Spring rape crops are often killed or severely damaged by late spring frosts. This necessitates reseeding or results in a lower yield due to inadequate density of stand. This ranks frost resistance as an important production problem in maximizing the economic returns.

During a late spring frost in 1969, it was observed that plants from coated seed planted the previous fall survived while plants from seed planted that spring were killed. This observation and reports by farmers that early seeded crops are more resistant to spring frost than later seeded rape suggested that the low temperatures experienced during germination induce hardening of the rape plants.

Exotherm measurements which indicate the supercooling point and freezing point of the tissue were performed on the leaves of the spring rape plants. Although the freezing point values of grapefruit leaves were not directly related to the frost hardiness of the leaves, they did change as a result of hardening (Young and Peynado, 1965). Therefore even if the exotherm values do not measure the degree of frost hardiness of the rape leaves, it was considered advantageous to use this method in the preliminary experiments, because of the large number of measurements that could be made in a relatively short time. Thus an approach with quite a wide scope was possible.

Membranes have been implicated in the mechanism of frost hardiness. They have been identified as the site of frost injury (Heber, 1967; Greenham, 1966; Lovelock, 1957; Persidsky, 1970). Changes in cell mem-

branes during hardening have been observed (Siminovitch, et al. 1968; Pomeroy and Siminovitch, 1971; Gerloff, 1966) and these changes are believed responsible for the increase in frost hardiness. Complex lipid, lipid phosphorus, lipid hexose and complex lipid fatty acid measurements were used as a measure of the membrane properties of cells in spring rape leaves.

The objectives of these studies were to establish if spring rape plants could develop frost resistance and if the frost resistance had a genetic basis, thus making a plant breeding program feasible. It was also hoped that the chemical studies would reveal some information on the mechanism of frost resistance operative in the spring rape plant.

LITERATURE REVIEW

Some Current Ideas In The General Area Of Frost Hardiness

Low temperature as an environmental factor affecting plant growth.

Climate is generally considered the most limiting factor in distribution, succession and migration of plants (Alden and Hermann, 1971). Subfreezing temperatures however, may not limit natural altitude and polar migration of plants in cold climates (Daubenmire, 1959). Daubenmire (1959) believes, for example, that inadequate heat during the growing season is more limiting to plant distribution. Dunbar (1968) suggests that large seasonal oscillation of food supply, low productivity and the youth of the ecosystem in polar climates are more limiting to adaptation of plants. Parker (1963) agrees that many factors limit plant distribution but rates low temperature extremes and drought as very important. Weiser (1970) cites Parker and suggests that cold is one of the major factors limiting cultivation to 7.6 percent of the earth's land surface.

An experiment by Sakai (1965) using willow suggests that subfreezing temperatures may not be the limiting factor. He found that willow from warm climates have an inherent mechanism for cold adaptation that is never fully developed because the plants are not exposed to hardening temperatures in their natural habitat.

Weiser (1970) indicates that clones of dogwood from different latitudes have the potential to develop a high degree of tolerance to freezing under favorable hardening temperatures, but they must be timed to the environment for maximum expression to occur. Thus factors controlling the

timing or induction of hardening may be as important or more important than the potential minimum temperature, to which the plants can harden.

Some Definitions.

Plants in a subfreezing environment may suffer winter injury, which can be due to ice formation in the plant, excessive water loss, low temperature diseases, mechanical pruning, etc. (Levitt, 1966). When damage is due to ice formation in the plant it is referred to as frost or cold damage (Levitt, 1966). Some types of plants are damaged by low temperatures without ice formation, this is referred to as chilling injury (Levitt, 1966). Chilling injury can be divided into thermal shock which is due to rapid chilling and results in immediate death or suboptimal temperature which is lethal only after hours or days of exposure (Mazur, 1969). Plant species vary in their ability to resist these types of injury. Simon (1969) refers to the temporal characteristics which are acquired after exposure to a cold environment as cold acclimation and to the inherited traits that determine the ability and extent to which an animal can cold acclimate and undergo cold stress as cold adaptation. I believe this terminology is also appropriate when referring to plants. Cold hardening is often used in plant science and is synonymous with cold acclimation.

Ice formation in plants.

Winter hardiness levels as established by field observations are very closely correlated with results from controlled freezing studies, which suggests that ice formation in the plant is the main factor controlling winter survival (Levitt, 1966).

Freezing can be defined as solidification of a liquid (Luyet, 1966).

It may be solidification involving an orderly arrangement of molecules or atoms, which is crystallization or solidification without such orderly arrangement which is amorphization or vitrification. These two phases may be mixed in various proportions. The primary factors controlling crystal formation and growth are temperature, duration of exposure to this temperature, rate of heat transfer (cooling, warming, freezing and thawing velocities) and the nature and concentration of the solute. A study of crystalline formation of 15 solutions of crystalloids and colliods frozen in thin layers at various concentrations and temperatures led to classification into four types of crystallization. At slow cooling rates well ordered hexagonal units occur. At higher cooling rates some of the elements of hexagonal symmetry are lost and irregular dentrites occur. At still higher rates of cooling coarse spherulites occur in which spears deprived of branches radiate in all directions from centers of crystallization. At very high cooling rates, the process of crystallization is incomplete and the products formed are referred to as evanescent spherulites. The presence of birefringence showed that ice (solid water) once thought amorphous is actually crystalline. However, the lack of birefringence, after freezing concentrated solutions, does not necessarily prove that there are no crystals formed. An example of the difficulty in obtaining vitrification is the impossibility of preventing the formation of evanescent spherulites in a solution of about 20 percent gelatin cooled at a rate estimated to be 100,000 ^{o}C per second. On slow warming of ice, water molecules may move from small ice crystals to form larger ones which have a lower surface energy, this is called recrystallization (Mazur, 1970). Recrystallization can occur in pure water at temperatures as low

as -100°C and is an example of a reaction that occurs in the solid state (Mazur, 1970).

Ice formation within plants may be modified from that formed in pure water. Interference with freezing as evaluated thermodynamically is made up of two components (Olien, 1967). One component involves the effect of the solute on organization of the liquid water molecules. Nonpolar solutes increase the structure of water and polar solutes decrease the structure of water. This component is determined by the equillibrium relationship and is affected by changes in concentration during freezing. The second component involves inhibition of crystal growth by interaction at the ice-liquid interface. It is a kinetic factor. For example, large polymers extracted from hardy plants, act as competitive inhibitors of freezing, resulting in an extremely imperfect ice mass. Hydrophilic polymers in plant cells may cause water to vitrify (Sterling, 1969). Sterling found, using X-ray difraction, that concentrated gels frozen at rates from 1°C per minute to 50°C per minute contained amorphous ice. Luyet (1966) however, reported that solutions containing organic solutes cannot be converted to a completely amorphous state by rapid cooling. Complete vitrification of biological materials, with rapid freezing, may be impossible because nucleation cannot be prevented (Alden and Hermann, 1971). In plant cells, vitrification may be based on a temporary delay of ice formation (supercooling) in cells, promoted by either an accumulation of protective substances in the protoplast that hampers ice crystallization or dehydration of the cellular contents by extracellular freezing at the critical temperature (approximately -30°C) and then rapid freezing to -80°C or below (Alden and Hermann, 1971). The critical temperature is in

a zone where water was found to be most rapidly converted to ice (Tumanov and Krasavtzsev, 1966, cited by Alden and Hermann, 1971). The critical temperature is very close to Sakai's prefreezing temperature (Alden and Hermann, 1971). Sakai (1965) froze twigs of several woody plants to temperatures between -15° and -30°C. This produced varing amounts of dehydration. The twigs were then immersed in liquid nitrogen. The amount of dehydration required to survive immersion in liquid nitrogen decreased with increasing hardiness. Sakai proposes that if any freezable water remains in a cell following prefreezing, the intracellular crystallization nuclei originally formed when cooled in liquid nitrogen (20°C/sec) will recrystallize forming large crystals during the subsequent slow rewarming and will damage the cells.

A glycoprotein (polymer of a unit containing alanine, threonine, N-acetylgalactosamine and galactose) which has an antifreeze effect has been isolated from the blood serum of antartic fish (DeVries, 1971). On a weight basis it is as efficient as sodium chloride in lowering the freezing point. DeVries hypothesizes that it acts by being adsorbed on the surface of ice crystals and prevents water molecules from settling into the ice lattice of the crystal unit until a much lower temperature is reached.

Ice formation in plants occurs in three basic patterns (Olien, 1967). The first is typical of tender tissue. Ice crystals grow within the protoplasts destroying them and releasing their contents. The other two patterns involve redistribution of water molecules and are characteristic of hardy tissue. The first, an equilibrium process, involves a continuous exponential decrease of water in intercellular spaces as the temperature

decreases. The second, a nonequilibrium freezing, involves a sudden drop in content of intercellular water during which the amount frozen is not a function of temperature. All of these patterns may occur simultaneously in different regions of a single plant. Each may cause histological disruption. However, histological disruption rarely occurs in hardy tissue which tends to have a low moisture content (which favors equilibrium freezing), gas filled spaces (which provide room for crystal growth) and less rigidly structured cell walls. Thus injury to hardy tissue is related to desiccation of the protoplast. The protoplast is desiccated because supercooled water has a higher vapor pressure than ice at the same temperature, thus energy is available for transfer of water from the liquid of the protoplast to intercellular ice crystals. These intercellular ice crystals are not histologically disruptive and only become deleterious at low temperatures when the critical size of a stable ice nucleus decreases to a size that can penetrate the cell membrane. Mazur (1970) states that membranes may contain water filled pores. Ice crystals small enough to pass through such pores are unstable above -10 to -20°C because of the high water activity produced by a small radius of curvature.

Intraprotoplasmic ice crystals ordinarily kill plant cells (Parker, 1963). However, large cells of the fat body and labial gland of some insects survived intracellular ice (Salt, 1962). Insects that survive rapid freezing to extremely low temperatures also tolerate intracellular ice (Losina-Losinsky, 1967, cited by Alden and Hermann, 1971).

Idle (1968) suggests that intercellular ice is not deleterious to hardy plants because it forms in only a few locations and away from vital

tissues. He found that ice forms near the outside of the stem and leaf stock or just beneath the epidermis of the leaves, but not in vital tissues such as the vascular system or the palisade layer which is responsible for photosynthesis. In non hardy tissue ice first nucleates in the vascular system resulting in the growth of large ice crystals which cause cracks in the tissue. This is called glacier formation. Hardy plants escape this type of damage due to the presence of other sites of nucleation. Idle states that a patch of extra leaky cells may cause enough of a local increase in water to form a site of nucleation or the death of a cell due to supercooling could provide a site of nucleation. Thus he hypothesizes, that hardy plants owe their success to the imperfections of a small minority of cells.

Cells are characterized by an optimum freezing rate at which maximum survival occurs on freezing (Mazur, 1970). Below this rate death increases due to the increased period of time cells are exposed to solution effects during dehydration. Faster rates increase death due to formation of intracellular ice. Mazur's hypothesis was developed for yeast and red blood cells, but he believes it also applies to nucleated mammalian cells and cells from higher plants.

Sakai and Yoshida (1967) obtained survival curves of cortical parenchymal cells of mulberry trees which differ from Mazur's. Their curve had a maximum survival at a moderate freezing rate and a second maximum at freezing rates that produce vitreous ice, provided that rewarming is also rapid. I believe that their curves would agree if Sakai and Yoshida had used slower cooling rates which would produce the solute effect demonstrated by Mazur and if Mazur had used cooling rates great enough to

produce vitreous ice. Rowe (1966) presents a survival curve as a function of cooling rate for biological specimens which would support this. At moderate freezing rates that provide maximum survival of plants cells, survival is higher at slower rewarming rates (Sakai and Yoshida, 1967).

Moore, et al. (1970) studied movement of water out of cells at cooling rates from 0 to 10,000°C per minute. At low freezing rates they found that the rate of water leaving the cell is at first moderate, then twice as fast and then drops to zero. The cell shrinks to a size at which there is little free water left even though the membrane permeability decreases with temperature. At the highest cooling rates, the permeability of the membranes decrease so rapidly that water transport is prevented and the volume of free water in the cell after cooling is little different from the initial volume.

Not only is ice formation affected by the cell constituents, but even the structure and properties of water are believed to be affected. Ling (1970) has evidence that supports the association-induction hypothesis according to which cell water exists as a polarized multilayer on the surface of cell proteins and membranes. Olien (1967) believes water close to a surface has an ordered structure. The degree of order decreases to that of bulk water as the distance from the surface increases.

In biological materials, water has been found to maintain its liquid like properties at temperatures well below freezing. Dehl (1970), using nuclear magnetic resonance, found that when the water of hydration of the fibrous protein collagen is relatively high (0.45 g of water/g of collagen) water remains unfrozen at temperatures as low as -30°C.

Essentially all the water that does not freeze remains in a state of high mobility (liquid-like) even at -50°C. The unfreezable water amounts to approximately 2.6 molecules per amino acid residue, based on an average amino acid molecular weight of 90. Krasavtsev (1967), found that intercellular ice continued to form from intracellular water down to -60°C in woody plants.

Possible sites of freeze-thaw injury.

The fact that Mazur (1970) found an optimum cooling velocity for maximum survival, suggests that freezing stress consists of two components oppositely dependent on cooling rate. One component is intracellular ice formation and the other is related to protoplast dehydration due to extracellular ice formation.

As stated previously, intracellular ice is normally fatal. The ease with which ice can be induced to form within the protoplast is an index of the transition between the tender and hardy state (Olien, 1967). However, even hardy plants can develop intracellular ice if cooled rapidly enough (Olien, 1967).

When only extracellular ice is formed injury may result from histological disruption or dehydration of the protoplast (Olien, 1967). Hardy plants can withstand extracellular ice and because natural cooling rates usually are not sufficient to cause intracellular ice their critical freezing temperature is related to the amount of dehydration they can withstand (Olien, 1967).

Evidence that dehydration is important is shown in an experiment of

Williams and Meryman (1970). They found that spinach grana are injured when their volume decreases to a critical minimum of about 25 percent granum volume. Grana from hardy plants or artificially protected grana survive by allowing a reversible influx of solute which forestalls excessive dehydration and shrinkage. Williams (1970) believes this leak mechanism will also operate in intact cells, provided sufficient extracellular solute is available.

Injury during cell dehydration may be due to physical stresses, which deform cell constituents, thus destroying their function or due to chemical changes such as pH or ionic changes, which denature cell components, thus destroying their function. Most of the theories explaining injury suggest that denaturation of macromolecules such as enzymes or lipid membranes are the ultimate cause of injury.

Johansson and Krull (1970) measured cell contraction at the frost killing temperature of two winter wheat cultivars. Cell contraction is measured as the ratio of cell volume in an unfrozen plant to that in the frozen state. Levitt (1956) used it as a measure of the stresses placed on the plant protoplasm. The degree of cell contraction at killing temperature expresses the greatest stresses the plant protoplasm can endure. They found that the degree of cell contraction was inversely correlated to the frost hardiness of the plant. The degree of cell contraction at a given killing temperature was the same for both cultivars, thus they conclude that protoplasmic changes during hardening were not important in explaining the differences in the frost hardiness between the two cultivars.

Johansson (1970) extended the study to rye, wheat and turnip rape,

using several cultivars of each. In this experiment he no longer found the absolute correlation between cell contraction and frost hardiness that was previously reported. The correlation is affected by external conditions such as temperature. However all cultivars show the same correlation when exposed to identical external conditions. He also found that hardened and nonhardened material are now represented by two curves instead of one. The relative content of unfrozen water (mg of unfrozen water per 1000 mg total water) calculated from percent tissue moisture and calorimetric determinations at the killing temperature (tk) gave a better measure of the protoplasmic factor of the plant frost hardiness. For example, in the series of rye cultivars all plants were killed when between 12.2 and 14.8 percent of their original water remained unfrozen. Plants with the higher relative content of unfrozen water having lower killing temperatures.

Levitt (1966) suggests that dehydration results in a decrease of intermolecular distances, so that SH groups of different proteins can be oxidized to S-S bonds. Then, on rehydration, the proteins cannot separate at the S-S bond resulting in protein denaturation. Evidence for this is; the SH content of cabbage protein decreases during hardening, so that less S-S bonds can form to cause denaturation and the SH content of cabbage protein decreases as a result of frost death suggesting formation of S-S bonds.

Lovelock (1957) showed that lipoproteins can be denatured by high salt concentration, unfavorable pH or removal of water to the eutectic point. When a solution is frozen, water crystallizes as ice. A temperature (the eutectic temperature) exists where all the water that can

crystallize freezes leaving only the solute and its water of hydration. Further temperature decreases result in solidification of the solute and water of hydration (Meryman, 1966). Any one type of lipoprotein has a different degree of resistance to each of these factors and different lipoproteins show different resistances to any one of these factors. Lovelock attributes the sensitivity of these lipoproteins to denaturation, to the weak association forces which hold them together.

Fishbein and Griffin (1969) hypothesize that freeze-thaw damage is due to macromolecular dissociation during freezing followed by abnormal reassembly during thawing.

Enzyme denaturation is another source of injury. Freezing inactivates lactic dehydrogenase, catalase, lipoxy dehydrogenase, etc. (Levitt, 1966). However, membrane bound enzymes appear to be much more susceptible than most soluble enzymes (Mazur, 1969). Santarius and Heber (1970) using the loss of ATP synthesis as an indicator of membrane inactivation, found that freeze-thaw injury depends on temperature, extent of dehydration and the time during which low temperature and increased electrolyte concentration act on the membrane.

DNA has also been suggested as a site of injury. Henderson (Savard, 1969) hypothesizes that dehydration damages the double protein sheath that surrounds chromosomal DNA. His hypothesis is as follows. During the early stages of freezing and thawing the electrochemically active radicals H+ and OH- exist for a longer than normal period of time. These radicals react with protein from the damaged sheath and bond it unnaturally to the DNA. He believes that if a cell is not too severely damaged (one in which pro-

tected or repaired DNA can function) it can repair the nucleus and mitochondria and then carry on normal cell function. Gusta (1970, cited by Weiser, 1970) found rapid destruction of nucleic acid components by ribonucleases after freezing and during freeze drying.

Freeze-thaw damage could also be due to an imbalance of metabolism. Some soluble enzymes are inactivated by freezing (Mazur, 1969) which would impair normal metabolic functioning. Korovin and Bakumenko (1968) found that freezing corn or wheat for two hours resulted in an increase of inorganic phosphorus and a decrease in organic phosphorus. Organic phosphorus of the acid soluble fraction and RNA-P decreased, while phosphorus of lipid and DNA changed insignificantly. Thus they conclude that freezing inhibits assimilation into organic compounds. Levitt (1956) postulated that toxic compounds accumulate at low temperatures due to a change in metabolism and are the cause of chilling injury.

Lyons and Raison (1970) provide an interesting example of how low temperature can change metabolism. They found that in chilling sensitive plant tissue and homeothermic animals, the activation energy of the respiratory enzymes increase below a transition temperature, possibly due to a configurational change of the membrane associated enzyme proteins, induced by a phase change of the membrane lipids. This increase in activation energy did not occur in chilling resistant plants or poikilothermic animals.

Persidsky (1970) has evidence that lysosome membranes are a target of injury during freezing, resulting in the release of hydrolytic enzymes.

There are also some physical processes associated with injury. Exotherm studies demonstrate a distinct exotherm at the time of injury (Weiser,

1970). Tumanov and Krasavtsev (1959, cited by Weiser, 1970) attribute it to restricted water movement through the membrane which results in temporary undercooling and then intracellular nucleation. Weiser (1970), on the other hand, hypothesizes that a critical (vital) level of water is reached, then when further water is removed to extracellular ice a chain reaction results consisting of denaturation, additional vital water release and death. Water serves both a structural and functional role in nucleoproteins and lipoproteins (Weiser, 1970).

A theory of chilling injury suggested by Lyons and Asmundson (1965) also has a physical basis. They showed that the mole percent unsaturated fatty acids has an important effect on the solidification temperature of a fatty acid mixture in the concentration range found in plants. Chilling sensitive plants tended to have a lower mole percent unsaturated fatty acid content than chilling resistant plants. They suggest that injury is due to crystallization of the hydrocarbon chains thus blocking some physiological activity of the lipid membranes.

It is likely that most of these mechanisms can be the cause of freeze thaw damage, but that the critical one depends on the particular tissue and its particular physiological condition. The time of injury may be during freezing, on thawing or during post thawing (Levitt, 1956).

There is also evidence that certain types of injury can be repaired under the proper conditions. Chilling corn at $0.3 \pm 0.3^{\circ}C$ for up to 60 hours produced visual injury as well as increased oxygen uptake and increased ion leakage (Greencia and Bramlage, 1971). Transfer to $21^{\circ}C$ resulted in recovery of these symptoms. However after 72 hours of chilling the plants would not

recover. Robinson (1970) found that mammalian cells can recover from freeze-thaw induced damage if placed in a properly conditioned medium immediately after thawing.

Chemical changes during acclimation.

Plants native to temperate regions can develop varying degrees of resistance to these types of injury. Many chemical and physiological changes have been observed during hardening. Attempts to correlate these changes to the degree of resistance have also been made. Since the exact site of damage is not known, it is difficult to find if the changes actually contribute to hardiness or if they are only the result of a change in growth habit. However, many hypotheses exist suggesting how these changes may contribute to hardiness.

Changes in carbohydrates often accompany acclimation. Large polymers in the cell walls (mainly xylans) are believed to represent a class of natural protective agents (Olien, 1967). These polymers may interfere with the structure of ice crystals growing along cell walls. Olien observed that resistant winter cereals develop more imperfect crystals than less resistant tissues.

Oligosaccharides such as sucrose and raffinose also accumulate. Sergeev (1964, cited by Alden and Hermann, 1971) suggests their function may be to prevent bud break during periods of warm weather in the winter after the cold requirement of the plant is satisfied since they are known to be growth inhibitors.

Simple sugars accumulate during hardening due to hydrolysis of starch. It is postulated that they may function by holding water of hydration more firmly through hydrogen bonding or by substituting for water of hydration

in structures sensitive to dehydration (Ullrich and Heber, 1957 and 1961, cited by Alden and Hermann, 1971).

Steponkus (1971) found that cold acclimation of English ivy takes place in two stages. The first requires low temperatures and allows the plant to respond to the presence of sucrose. Both stages must be completed for developement of cold hardiness. Steponkus suggests that the second phase may involve alteration of the protein complement of the cell, enabling frost sensitive proteins to bind the sugar. He bases this on the fact that a protein fraction from acclimated plants has a higher ^{14}C-sucrose binding capacity than protein from non acclimated plants.

Sugars probably also play an important role as an energy source for acclimation (Alden and Hermann, 1971). In tender plants, such as citrus, the osmotic effect of sugars is believed to be important to frost hardiness (Surkova, 1961) since a direct relationship was observed between osmotically bound water in the leaves and frost hardiness.

Lack of a direct correlation between frost hardiness and simple sugar content of many plants, led many investigators to consider simple sugars relatively insignificant (Alden and Hermann, 1971). Siminovitch, et al. (1967), suggested that the removal of the starch grains may be the important function and not the accumulation of sugars.

An increase in soluble proteins on hardening has been well documented with many plants (Alden and Hermann, 1971). Heber (1959, cited by Alden and Hermann, 1971) attributed it to a change in extractability of protein influenced by seasonal changes in pH. However, Siminovitch, et al. (1967) found that hardened tissue of black locust bark had an increased protein synthetic capacity. Hardy tissue incubated at 25°C for four hours incorporated more leucine-1-^{14}C than did non-hardened tissue. This suggests

that an actual increase in protein synthesis is envolved.

This increase in soluble protein could be an increase in the protein complement of the cell or a shift to different types of protein varying in stability or activity at low temperatures. A change in quality is shown in an experiment by McCown, et al.(1970). They found that extra peroxidase isoenzymes and fewer acid phosphatase and esterase isozymes were formed during hardening.

Bolduc, et al. (1970) observed that IAA oxidase activity increased tenfold during 40 days of cold treatment of winter wheat seedlings. They claim that virtually all work has confirmed that frost resistant plants have higher enzymatic activity than non-resistant plants and therefore the rates of enzymatically catalyzed reactions do not decrease as rapidly when the temperature falls.

Results relating amino acid metabolism to frost resistance are not consistent, thus changes in amino acids may only indicate something about protein metabolism (Alden and Hermann, 1971). However, some hypotheses relating amino acids to hardiness have been suggested. Proline accumulates in cold resistant trees and like oligosaccharides, it is an inhibitor of growth and respiration (Sergeev, 1964, cited by Alden and Hermann, 1971). It is also known that amino acids catalyze various processes and so may function as catalysts in frost hardiness (Alden and Hermann, 1971).

Changes in lipid metabolism during hardening are also well documented. The most consistent changes observed have been an increase in unsaturated fatty acids and an increase in phospholipids. The larger amount of phospholipids is believed to represent an increase in total membranes and possibly a change in quality of membranes (Siminovitch, et al. 1968).

Changes in nucleic acids have also been observed during acclimation. Chuvashina (1962) found that nuclei from bark cells of hardy apple cultivars became increasingly methylophilic from August to mid-winter, but nuclei of non-hardy apple cultivars remained pyronenophilic even in winter, indicating that they continue to be physiologically active. The procambial tissue of fruit tree buds and even the developed vascular bundles maintained a high content of nucleic acids which is believed to be a defence mechanism against frost injury in the spring (Barskaya and Oknina, 1959, cited by Alden and Hermann, 1971). No increase in DNA occurred during acclimation of black locust (Siminovitch, et al. 1968) or dogwood (Li and Weiser, 1967). In contrast, DNA of crowns and roots of alfalfa (Jung, et al. 1967) and excised twigs of apple (Li and Weiser, 1969) increased during hardening. However, all the above workers found an increase in RNA before, during, or both, the development of cold hardiness of plants. Kessler and Frank-Tishel (1962) postulated that a high guanine and cytosine content in RNA is related to cold and drought resistance in plants and the ability to synthesize RNA that is rich in guanine and cytosine is a basic part of the cold and drought-hardening processes in plants. However, in some species there is evidence that nucleic acids, unlike some proteins, are not altered by sub-freezing temperatures. Freezing calf thymus DNA to -192°C did not denature it (Shikama, 1965).

Induction of these chemical changes during hardening appears to be controlled by a translocatable factor (Weiser, 1970). Acclimation takes place in three stages. The first stage involves the translocatable factor and is initiated by a short photoperiod and warm temperatures. The leaves are the site of perception and the promoter moves from them through the bark. Only a small increase in hardiness results from this stage. The

second stage is initiated by low temperature and in some cases, frost. The maximum increase in hardiness occurs during this stage. A third stage induced at temperatures between -30 C and -50 C also exists, but it may not be important in nature. Full hardiness will eventually develop under long photoperiods and low temperatures. Plants exposed to short photoperiods and warm temperature will only reach the first stage of acclimation. The translocatable factor may be a growth retardant hormone, a sugar or, as Weiser believes, a hormone that is specific to the initiation of changes required for hardening.

Membranes

Analysis of thermal death curves of various organisms shows it to be a first order process (Brock, 1967). A first order reaction is concentration dependent for one material. Brock suggests that the heat effect is on a large single structure such as membranes and not on heat sensitive enzymes or ribosomes of which there are many copies in the cell.

Mohr and Stein (1969) studied the effect of various freeze-thaw regimes on the ultrastructure of tomato fruit cells using electron microscopy. They found that rupture and disorganization of membranous components was a common denominator throughout. They found that the plasmalemma was more resistant than the tonoplast and several other membranous components. The endoplasmic reticulum, ribosome, golgi bodies, osmiophilic globules and mitochondria were absent or unrecognizable following any of the freeze-thaw combinations they tried, except where special prefreezing plasmolysis treatment was used.

Studies of senescing bean leaves (Barton, 1966, cited by Mohr and Stein, 1969) and senescing wheat leaves (Shaw and Manocha, 1965) also revealed that the plasmalemma was nearly always the last structure of the cytoplasm to disappear, the tonoplast having ruptured at a relatively

early stage. However, Chambers and Hale (1932, cited by Mohr and Stein, 1969) found the tonoplast of onion epidermal cells more resistant than the "protoplast".

Pomeroy and Siminovitch (1971) studied cytological changes of black locust phloem parenchyma cells with the electron microscope. They found that total protoplasm, including mitochondria, lipid bodies and membrane-bound vesicles derived from invaginations and folding of the plasmalemma increased with increasing frost resistance. The endoplasmic reticulum also changed from long cisternae-like units in the summer to a vesicular form in the winter. Using chemical techniques, Siminovitch, et al. (1968) found an increase in lipid phosphorus and lipoprotein during hardening of black locust bark. This agrees with the cytological evidence that total membranes increase during hardening. There may also be a qualitative change of membranes since total lipid phosphorus also increased in starved tissue where total protoplasmic augmentation did not occur.

Heber (1967) showed that freezing chloroplast membranes uncouples photophosphorylation from electron transport and inactivates proton uptake and light-dependent shrinkage of chloroplast lamellae. Mitchell's theory of phosphorylation requires a pH gradient across the vesicle membrane. Heber proposes that frost dehydration alters the membrane permeability, thus preventing a pH gradient. Sucrose can protect the chloroplast from damage and salt interferes with this protection. Chloroplast membrane integrity may be maintained by macromolecules such as the one isolated by Heber (1968, cited by Mazur, 1970).

Possible functions of membranes in frost resistance and the susceptibility of membranes to frost damage have been discussed in the first sec-

tion of the literature review.

Electrical resistance of plant tissue in the frozen state can be measured using an applied low frequency or direct current. The values obtained appear to be related to the intactness of cell membranes (Greenham, 1966). Using this principle and studying four cultivars of alfalfa, Greenham showed that injury can occur either during freezing, in the frozen state or during thawing.

Electrolytic measurements have been used to measure the amount of injury to plants caused by freezing. They are based on the loss of selective permeability of the plasmalemma. This type of test along with the water soaked appearance of plants killed by frost suggest that injury is to the membranes (Weiser, 1970).

However, Weiser (1970) presents three arguments against injury being to the plasma membrane. First Evert (1970, cited by Weiser, 1970) found that stem sections killed by freezing showed no immediate change in admittance (current passing through a sample divided by applied voltage) to low frequency current upon thawing. Secondly, evidence that lysosomes are injured suggests damage may be to membranes of cell constituents. Thirdly, Gusta (1970, cited by Weiser, 1970) found a rapid destruction of nucleic acid components by ribonucleases after freezing and during freeze-drying.

Lipids

Two important functions of lipids in plants are as a carbon and energy storage form and as a structural component of membranes. An example of a typical composition of plant lipids is provided by Tremolieres and Lepage (1971). They found that neutral lipids of young pea leaves

compose not more than ten percent of the total lipid. The free fatty acids of the neutral lipids are much more saturated than the triglycerides or diglycerides. Phosphatidyl choline is the most abundant phosopholipid in the young pea leaves. Phosphatidyl ethanolamine is about half as abundant. Other phospholipids detected in pea leaves were phosphatidyl-inositol, -glycerol, diphosphatidyl glycerol and phosphatidic acid. Linoleic acid is the most abundant fatty acid of phospholipids. They also point out that, "although the lipid composition of green algae and higher plants have common characteristics such as high levels of galactolipids with polyunsaturated fatty acids and the presence of phosphatidyl-glycerol with trans-3-hexadecenoic acid (James and Nichols, 1966), appreciable differences are encountered between them: (a) in algae, the galactolipid polyunsaturated fatty acid consists of 16 and 18 carbon chain fatty acid, whereas in higher plants - linolenic acid alone accounts for eighty percent of the galactolipid fatty acid (James and Nichols, 1966); (b) in higher plants, the turnover of the fatty acid portion of galactolipids is very low while the $^{14}CO_2$ incorporation into the galactose portion is very rapid (Tremolieres, 1970)". A ratio of monogalactolipid to digalactolipid of two, is characteristic of mature chloroplasts (Tremolieres and Lepage, 1971).

Chloroplast membranes are characterized by a large amount of galactolipid and extrachloroplastic membranes are rich in phospholipid (Tremolieres and Lepage, 1971). Chloroplasts of tobacco leaves, for example, contain 83 percent of the total cellular monogalactosyl diglyceride, 88 percent of the digalactosyl diglyceride, 76 percent of the sulfolipid and 74 percent of the phosphotidyl-glycerol (Ongun, et al., 1968).

The fatty acid content of different tissues may differ. For example, after seven days of germination in the dark, pea cotyledons contained 5.0 mg of fatty acid per gram fresh weight, stems contained 0.7 mg per gram fresh weight and leaves contained 2.7 mg per gram fresh weight (Tremolieres and Lepage, 1971).

Many experimentors have observed changes in lipids during cold acclimation. For example, lipids and tannins of fruit trees have been observed to increase as starch decreases in the winter and to decrease as starch accumulates in the spring (Genkel and Oknina, 1966, cited by Alden and Hermann, 1971). Pomeroy and Siminovitch (1971) using electron microscopy found that lipid bodies increased in the bark cells of black locust during hardening in the fall. Experimentors have also correlated lipid content to frost hardiness. For example, both Durmanov (1957) and Eljseev (1964) found that buds and shoots of hardy apple cultivars accumulated more fat and lipid on hardening than less resistant cultivars. However no evidence of fat and lipid accumulation during hardening was found in wheat (Chien and Wu, 1965, cited by Alden and Hermann, 1971).

Changes in lipids at elevated and reduced temperatures have been studied in animals, microorganisms and plants. Changes in lipids of poikilothermic animals, microorganisms and chilling sensitive plants presumably allow the organism to function at a reduced temperature above the freezing point. Changes in homeothermic animals are thought to be related to the animals' ability to maintain at least a minimum body temperature. In frost hardy plants, the changes must function, by aiding the plant to withstand intercellular freezing and/or avoid intracellular freezing.

Simon (1969) grew native arctic mice at 18°C (control) and 5°C for

eight to ten weeks. He then exposed those grown at 5°C to -40°C for two hours. Animals that could maintain an annal temperature of 36°C or greater were considered cold resistant (CR) and those that could not, cold sensitive (CS). Animals were sacrificed and analysed twelve hours and fourteen days after stressing. Two species, Microtus oeconomus and Clethrinomus rutilus were used. The control and CR animals of both species had equal quantities of lipid expressed on a dry weight basis, whereas the CS animals had a lower quantity. Most of the decrease appears to be due to a decrease in the neutral lipid fraction. M. oeconomus, CR animals also showed a decrease of neutral lipid, but it was offset by a corresponding rise in phospholipid. With the exception of diphosphoglycerol ratios in C. rutilus there are no differences between the phospholipid classes expressed on a ratio basis twelve hours after the stress in either species.

Animals sacrificed fourteen days after stress show that in CS of C. rutilus the percent total lipid of mouse dry weight returns to normal but the percent phospholipid of mouse dry weight remains at a lower level. Lipid content of M. oeconomus does not return to normal. The phospholipid ratios (weight of individual phospholipid over total phospholipid) of stressed M. oeconomus were not different from the control fourteen days after stressing. However C. rutilus did modify its phospholipid ratios as a result of stress.

The one consistent change in both species was a two to three fold increase in diphosphoglycerol expressed on a dry weight basis of the CR animals over the CS animals, twelve hours after the stress. Phospholipids are essentially a membrane component with the exception of blood. Diphosphoglycerol is located only in mitochondria of most tissues. Thus Simon

predicted that the increase in diphosphoglycerol represented an increase in mitochondria. Cytochrome oxidase activity, which is a measure of the quantity of mitochondria, should follow the same pattern as the diphosphoglycerol ratios. That is, the CR should have the highest values, the CS the lowest, and the controls an intermediate value. Simon found that this in fact occurred. He also measured the area of mitochondria on electron micrographs and found that it followed the same trend. He concluded that the ability to withstand acute cold stress is an inherited characteristic and is a function of the quantity of the muscle that is mitochondria.

Eybel and Simon (1970) studied three species of arctic mice and classified them into CS and CR as above. They analysed the fatty acid composition of the phospholipid and neutral lipid fractions. The only consistent result in all three species was a marked elevation in 14:0 of the phosphatidic acid fraction of the CS mice.

Climatic temperature is one of the main factors determining the relative saturation of seed fats (Hilditch and Williams, 1964). Production of fats solid at the prevailing temperature is unlikely to occur. This does not mean that fat produced in the tropics is more saturated than fat produced in temperate regions. Many of the most unsaturated fats are synthesized in the fruit of tropical species. However, fat of plants growing in one climatic region will be more unsaturated under cooler conditions.

Patterson (1970) studied the fatty acid composition of Chlorella sorokiniana at six growing temperatures between 14 and 38°C. He found that the total lipid remained constant at ten percent of tissue dry weight at all temperatures. The fatty acids were at a minimum concentration (13% of total lipid) at 26°C and increased towards both extremes (50-60%

of total lipid). The maximum degree of unsaturation of fatty acid was attained at 22°C with less unsaturation of fatty acid at lower or higher temperatures. However, the proportion of total unsaturated fatty acid was always increased at lower temperatures. Chain length of fatty acids always decreased with increasing temperature.

The lipid composition of the algae Cyanidium coldarium was studied at two temperatures, 20°C and 55°C (Klienschmidt and McMahon, 1970a). It was found that total lipid in terms of mg/g dry weight was greater in cells grown at 20°C. The total lipid was then separated into five fractions. Expressed as a percent of total lipid, these fractions showed no gross difference between the lipid of cells grown at 20°C or 55°C. Thus it is believed that a nonlipid constituent increases at 55°C. They have unpublished data that the cell wall increases. The ratio of unsaturated to saturated fatty acid in the total, as well as in each of the five fractions, decreased at the higher temperature. Linolenic acid composed 30 percent of the fatty acid at 20°C but was not detectable at 55°C.

Klienschmidt and McMahon (1970b) then analysed the fractions that contained the glycolipids and phospholipids. It was found that mono- and di-galactosyl diglyceride and sulfolipid are higher in cells grown at 20°C and lower in cells grown at 55°C, while an unidentified glycolipid is very low in cells grown at 20°C but much higher in cells grown at 55°C. Glycolipids and sulfolipids are associated with the photosynthetic apparatus (Singh and Privett, 1970; Ongun, et al., 1968). Mono- and di-galactosyl diglyceride of cells grown at 20°C contain no detectable amounts of stearic acid and the diglycerides of cells grown at 55°C contain no detectable amounts of linolenic acid. The fatty acid composition of mono- and di-galactosyl diglyceride were essentially the same in cells grown at the

same temperature. Phosphotidyl choline and ethanolamine comprise about 80 percent of the total phospholipid of cells grown at both temperatures. Phosphotidyl choline is not greatly affected by temperature but phosphotidyl ethanolamine is markedly decreased in cells grown at the higher temperature. No linolenic acid was detectable in phosphotides of cells grown at 55 C. Oleic acid composed 50 percent of the fatty acid of phosphatidyl ethanolamine at both temperatures. Thermostability of the cell membranes may be related to fatty acid saturation since cells grown at 55°C were thermostable at a temperature 15°C higher than cells grown at 20°C. Klienschmidt and McMahon postulate that the decrease in glycolipids and phospholipids in cells grown at higher temperatures may be due to a decrease in carbon dioxide solubility causing normal autotropic cells to grow heterotropically.

Okuyamo (1969) studied phospholipid metabolism in E. coli during a five hour lag period of growth, which results when the temperature is changed from 37°C to 10°C. The phospholipid content increased 20 percent. This information, along with data from ^{32}P incorporation experiments, indicated that 33 percent of the existing phosphodiester moiety of phospholipids was degraded to water soluble products and about 54 percent more phosphodiester moiety was synthesized from inorganic phosphorus derived from the medium and phosphorus containing components of the cell. The fatty acid moiety of lipids was not degraded but was maintained as fatty acids or fatty acyl esters. It was also shown that the fatty acyl moiety of the neutral lipid fraction, including free fatty acids, was actively reutilized for phospholipid synthesis. There was turnover of phosphatidyl ethanolamine and phosphatidyl glycerol which are known to be very stable

in normally growing cells of E. coli. The amount of vaccenic acid increased two-fold while the amount of other fatty acids in the phospholipid fraction changed relatively little.

The ability of mitochondrial membranes to swell in hypotonic solutions is correlated to the unsaturation of fatty acids (Richardson and Tappel, 1962, cited by Lyons, et al. 1964). Richardson and Tappel showed a difference in flexibility of mitochondrial membranes from warm blooded (rat liver) and cold blooded (fish liver) animals. Lyons, et al. (1964) demonstrated that mitochondria from chilling sensitive sweet potato roots and tomato fruit showed little ability to swell. Chilling resistant pea seedlings, turnip root and cauliflower buds had mitochondria with a striking ability to swell. Mitochondria from chilling sensitive corn and bean seedlings could swell to the same extent as those from chilling resistant plants. However, analysis of the shape of the swelling curves of corn and bean seedlings showed that the shapes were different than those of chilling resistant species, suggesting that they may have different properties related to their chilling classification. The double bond index (a measure of unsaturation) of the fatty acids was highest in cauliflower buds and turnip roots, intermediate in pea seedlings, bean shoots and tomato fruit and lowest in sweet potato root and corn seedlings. Swelling rate of plant mitochondria is essentially temperature independent, whereas in animals it is affected by temperature, suggesting a metabolic control of swelling in animals.

Lyons and Asmundson (1965) measured the freezing point of a range of mixtures containing different concentrations of linoleic, linolenic and oleic acid with palmitic acid. They found that a small increment in

mole percent unsaturated fatty acid between 60-80 percent resulted in a relatively large drop in the freezing point. Linoleic and linolenic acid with palmitic acid behaved identically down to the eutectic point. For the mixture of oleic and palmitic acid there was less of an effect on the freezing point for a given change in mole percent unsaturated fatty acid. Several species of plants were analysed and found to have a mole percent unsaturation in the critical range of 60-80 mole percent. The chilling resistant plants tended to have a higher mole percent unsaturation, but there was considerable overlap. Because linolenic and linoleic acid have similar effects on the freezing point, Lyons and Asmundson believe that the mole percent unsaturated fatty acid is more meaningful than the double bond index for plants because these two fatty acids make up approximately 90 percent of the unsaturated fatty acid of plants. The double bond index may be more meaningful for animals which have a more complex fatty acid composition.

Cotton plants are chilling sensitive (injured at $5^{o}C$) but can be hardened by exposing to $15^{o}C$ days and $10^{o}C$ nights (Guinn, 1971). Lipid soluble phosphorus decreases during this hardening process.

Gerloff, et al. (1966) studied the fatty acid composition of alfalfa roots of a frost hardy cultivar (Vernal) and a less hardy cultivar (Caliverde). Total fatty acid content increased during hardening in both cultivars. The total saturated fatty acid content of the root tissue increased with hardening all three years in the nonhardy cultivar, but decreased two out of three years in the hardy cultivar. Unsaturated fatty acid, on the other hand, increased during hardening in both cultivars all three years primarily due to increases in 18:2 and 18:3. The most consistent cultivar differences were that monounsaturated fatty acid increased

during hardening two out of three years in the nonhardy cultivar while the hardy cultivar showed decreases all three years. The hardy cultivar accumulated more polyunsaturated fatty acid than the less hardy cultivar two out of three years. It was suggested that since both cultivars can harden to some extent, the hardening treatment may not have been severe enough to differentiate between the hardy and nonhardy cultivars.

Kuiper (1969) found that two frost susceptible citrus cultivars, March grapefruit and Eureka lemon, had a higher neutral lipid and a lower phospholipid composition than the more resistant Dancy and Satsuma tangerine citrus cultivars. There was no consistent difference in the glycolipid fraction. He also found that decenylsuccinic acid protected flowers of peach, pear and apple during a frost of -6.0°C for two hours and increased cell permeability. He suggested that charged lipids occurring in the plasmamembrane may contribute in the same way to the permeability characteristics and increase frost hardiness.

Green, et al. (1970) found that decenylsuccinic acid (DSA) did not increase the hardiness of winter wheat or barley in a nonhardening environment. They found that it failed to increase ^{32}P uptake but instead caused leakage to ^{32}P previously taken up. They concluded that the increased permeability results from injury to the roots.

Kuiper (1970) studied the lipid composition of two alfalfa cultivars (Vernal and Caliverde) grown at 15°C, 20°C and 30°C. The content of acidic phospholipids phosphatidyl-glycerol and -inositol and sulfolipid was directly related to the growing temperature, while an inverse relation was observed for the less acidic phospholipids, phosphatidyl-choline and-ethanolamine and the neutral glycolipids mono- and di-galactosyl diglyceride. There was

also a difference between the hardy and nonhardy cultivar. At corresponding growth temperature the hardy Vernal plants showed a higher percentage of mono- and di-galactosyl diglyceride and of phosphatidyl-choline and-ethanolamine than the Caliverde plants, while the opposite was true for the sulfolipid and phosphatidyl-glycerol and-inositol. Fatty acid composition of the lipid classes was quite constant. For example, monogalactosyl diglyceride was predominantly esterified with 18:3 fatty acid. This was also true for phosphatidyl ethanolamine (76% 18:3). The other lipids characteristic of hardy plants, digalactosyl diglyceride and phosphatidyl ethanolamine showed smaller amounts of linolenic (34 and 24 percent respectively) but in addition contained 30 and 28 percent linoleic acid. Thus, lipids related to hardiness had predominantly polyunsaturated fatty acids. Phosphatidyl inositol characteristic of cold sensitive plants showed a large percent of saturated fatty acid and long chain saturated fatty acids such as arachidic acid (20:0) and behenic (22:0), which were only observed in this lipid fraction. Only two lipid fractions, monogalactosyl diglyceride and phosphatidyl choline showed a minor but significant relation between cold hardiness and the content of polyunsaturated fatty acid.

Siminovitch, et al. (1968) found a greater amount of lipid phosphorus and lipoprotein in winter cells of black locust bark compared to its summer cells. They found that the nonpolar lipids decreased as the polar lipids increased. The phospholipids of black locust were composed mainly of phosphatidyl-inositol, -choline and-ethanolamine. Each of these increased during hardening. The non-phosphorus polar lipids also increased during hardening. Hardening of black locust bark cells usually involves

total protoplasmic augmentation along with the increase in membranes indicated by the increase in phospholipid and lipoprotein. However when Siminovitch, et al. (1968) starved the cells by using the double girdling technique, they found that lipid phosphorus still increased as did hardiness, but without total protoplasmic augmentation. Thus they suggest that qualitative as well as quantitative changes in the membrane may be involved.

Gerloff (1966) found that crude lipid of alfalfa roots increased during hardening in all cultivars. He also found a ninhydrin-positive, unsaturated, U.V. absorbing phospholipid that was only present in fully hardened roots of all cultivars.

In the frost resistant grape cultivar, Concord, the quantity of triglycerides and phospholipids remains constant during the critical winter period (Marutyan and Petrosyan, 1968). In the non-resistant cultivar Spitak arakensi these compounds were hydrolyzed and the amount of free fatty acid increased. Marutyan and Petrozan postulate that the ability to synthesize triglycerides may be related to frost hardiness.

Lyons and Asmundson (1965) hypothesize that chilling injury is due to physical changes in membrane lipids. It has been indicated that the physical condition of a lipoprotein complex such as a cell membrane is on a borderline of a phase transition from a liquid-crystalline structure to a coagel (Luzzots and Husson 1962, cited by Lyons and Asmundson, 1965). Lyons and Asmundson propose that when the temperature is lowered the hydrocarbon chains crystallize, thus blocking some physiological activity of the membrane. For example, cytoplasmic streaming stops when the temperature falls below $10^{o}C$ in chilling sensitive plant cells, but continues in

resistant plant cells (Lyons, et al. 1964). There is also a close correlation between oxidative phosphorylation and mitochondrial swelling and contraction (Lyons, et al. 1964). The other hypothesis of Lyons and Asmundson (1965) is that chilling injury involves the thermostability of lipid protein complexes as a function of both complexing substances.

Lovelock (1957) provides evidence that lipoproteins are easily damaged by conditions that are believed to exist in cells dehydrated by freezing. He proposes that these molecules are especially susceptible because of the weak forces binding them together. He found that human red blood cell membranes are increasingly damaged (measured by the release of phospholipid) as the ionic concentration (he used NaCl) of the suspension medium is increased. Next he showed that lipovitellin, an egg yolk lipoprotein, is not damaged (measured by loss of solubility in physiological saline) by high ionic strength, but is damaged if the pH falls below 5.2. Lastly, he studied β lipoprotein of human plasma. He found that ionic or pH changes were not sufficient to account for the damage (measured by loss of solubility in physiological saline) when it is frozen. By using different salts with different eutectic points he was able to show that β lipoprotein is damaged when all of the water is removed. He concludes that damage during freeze-thawing will be caused by the factor which the particular lipoprotein is most susceptible to.

Ingmar and Bertil (1969) measured lipid changes in the muscle of Baltic herring stored at -15°C for twelve weeks. They found that free fatty acids increased in both dark and white muscle. The free fatty acids increased due to the hydrolysis of the phospholipids lecithin and

cephalin and triglycerides. In dark muscle 45 percent of the hydrolysed fatty acids came from phospholipid and in white muscle 75 percent came from phospholipid. The enzymic hydrolysis of the phospholipids was not fatty acid specific but lecithin was hydrolyzed faster than cephalin.

Harris and James (1969) provide the following scheme for fatty acid synthesis:

$$\text{acetate} \xrightarrow{A_1} \underset{\text{Lipid}}{C_{16:0}} \xrightarrow{A_2} \underset{\text{Lipid}}{C_{18:0}} \xrightarrow{A_3} \underset{\text{Lipid}}{C_{18:1}} \xrightarrow{A_4} \underset{\text{Lipid}}{C_{18:2}}$$

They say that any restraint in A_3 or A_4 will result in a change of fatty acid composition, provided there is no feedback inhibition, resulting in an increase of stearic acid (18:0). They define the following terms:

a desaturation = $\dfrac{^{14}C_{18:1 \text{ and } 18:2}}{^{14}C_{18:0}}$

b relative desaturation = $\dfrac{\text{desaturation of treatment } (t^{\circ}C)}{\text{desaturation of the control } (30^{\circ}C)}$

It is apparent that if the relative desaturation is greater than one, t is synthesizing unsaturated fatty acids more rapidly than the control. Thus its lipids will be more unsaturated. When they incubated non photosynthetic bulk tissue (narcissus bulbs) for five hours, the relative desaturation decreased with increasing temperature. However, if they artificially increased the oxygen concentration this was reversed. Thus it appears that oxygen is the rate limiting factor of desaturation of fatty acids. In photosynthetic tissue such as Chlorella oxygen was not found to be limiting, but in the leaf tissue of spinach it appeared to be slightly limiting below 25°C because the relative desaturation below 25°C was greater than one. The control samples were incubated at 30°C. The

reason oxygen is not as limiting in photosynthetic tissue as bulk tissue, is that photosynthetic tissue produces oxygen during photosynthesis. Because the solubility of oxygen increases in an aqueous phase as temperature decreases, they suggest this is a simple and self-regulating control of desaturation. Thus when the plant requires more unsaturated fatty acids at lower temperatures, the availability of oxygen is increased due to increased solubility which increases desaturation providing the unsaturated fatty acids.

A higher content of chlorophyll was characteristic of hardened cucumber plants and frost-resistant cultivars of winter wheat (Shapovalov, 1965, cited by Alden and Hermann, 1971). The chlorophyll content of both frost-resistant and non-resistant cultivars of winter wheat declined during the autumn, increased slightly during early winter and declined again in late winter on exposure to subfreezing temperatures. Godnev, et al. (1969, cited by Alden and Hermann, 1971) found that the chlorophyll and carotenoid content in needles of Picea pungens and Picea excelsa were retained during the winter. McGregor and Kramer (1963) found that chlorophyll increased to a maximum in September and then declined slightly during the winter in both white pine and loblolly pine. The bonds between chlorophyll and the chloroplast-lipid-protein complex were more stable for frost resistant cultivars of wheat (Shmat'ko and Ostaplysuk, 1964) and frost resistant cultivars of apple (Protsenko and Chikalenko, 1962) than for less resistant cultivars.

Chloroplasts of cucumber leaves at 2°C lost their photosynthetic and fluorescence abilities, swelled and became round and the swollen stroma became transparent (Kislyuk, 1967, cited by Alden and Hermann, 1971). These effects were reversible until after 25 hours. Light is

particularly damaging during cooling, probably related to the sensitive photo-oxidative constituents of the chloroplasts.

Juvenile Tissue

Plants are exceptionally susceptible to frost damage during rapid growth in the spring. For example, maximum resistance to cold can be induced in red-osier dogwood in seven weeks at any stage during its annual cycle of development except for one to two months after growth resumes in the spring (Van Huystee, et al., 1967). Clauson (1964, cited, by Alden and Hermann, 1971) examined 50 species of hepatica (liverworts) and found that freezing to -10°C for four days destroyed undeveloped cells in young shoots but fully developed cells survived the same treatment.

It is well established that cold hardiness in woody plants is inversely related to growth rate (Levitt, 1966). On the other hand, Cox and Levitt (1969) reported that the leaves of cabbage hardened most when growth was rapid, presumably because proteins that promote both hardiness and growth were synthesized at low temperatures. However, the very youngest leaves were exceptions to this.

Dantuma and Andrews (1960) found that seedlings of winter wheat and barley germinated and grown at 1.5°C developed maximum hardiness at two stages. The first stage was during the first week after germination and the second stage was around the sixth week after germination. Spring cultivars and some less hardy West European wheats showed the first maximum in hardiness but not the second. An additional hardening for one week

at -4°C produced a significant increase in hardiness. Barley responded to the additional hardening during all stages. Wheat, on the other hand, showed the largest response to the additional hardening only during the youngest stages of germination. For example, after five weeks at 1.5°C only the less hardy wheat cultivars showed much response to hardening at -4°C. The spring cultivars of both wheat and barley responded to the additional hardening at -4°C. Andrews (1960) found similar results using winter rye. Maximum hardiness occurred after germination when the coleoptile was one millimeter or less and the roots less than five to ten millimeters in length, and again after five to six weeks of growth at 1.5°C when the first leaf was breaking through the coleoptile. Additional hardening at -4°C for one week was only effective for very young seedlings, but did not affect seedlings that had reached maximum hardiness at 1.5°C (approximately five weeks old).

Winter wheat has maximum resistance to temperatures between -10°C and -15°C at two stages of development (Robertson, 1970). The first stage is the dry or freshly moistened seed and the second stage is during the fourth and fifth leaf stage. Robertson gives the following critical low temperature for development and suggests that there is a common genetic reason for the peak in cold hardiness at the four and five leaf stage and the low threshold temperature of development between emergence and jointing:

planting to emergence	6.9°C
emergence to jointing	-4.7°C
jointing to heading	5.9°C
heading to soft dough	5.7°C
soft dough to ripe	3.2°C

Evidence that frost hardiness is controlled genetically is provided by Muehlbauer, et al. (1970) who found significant differences in the F_3 population of reciprocal crosses in oats. They also found that spring oats are a good source of genetic diversity for winter hardiness.

Winter Rape

Torssall (1959) reviewed the winter hardiness studies of rape (Brassica napus L.) and turnip rape (Brassica campestris L.). Rape has a killing temperature of around -9°C. Rape is more cold hardy than turnip rape as a species. The terminal bud is more hardy than the epicotyl which is more hardy than the root. The vascular tissue is more hardy than the pith. These morphological and anatomical relationships are more pronounced in rape than in turnip rape.

The field hardiness was greater for turnip rape than rape when the stand was unprotected by snow or by a snow cover of only 10 centimeters (Torssall, 1959). This was because turnip rape developed an entirely subterranean hypocotyl and an epicotyl situated at, or below, the ground surface,with very short internodes. Rape, on the other hand, developed a hypocotyl the upper part of which, as a rule, reached above the ground surface causing the epicotyl to be entirely above the ground surface and the internodes of the epicotyl were more or less elongated. At higher snow levels, the species relationships to winter hardiness could be changed due to thermal properties and depth of snow.

In turnip rape and rape, starch content decreased and total sugars increased in all cases during hardening (Hellstrom, 1954). There was

evidence that starch was not converted quantitatively into a simple carbohydrate. Hellstrom believes that certain cell wall constituents such as pectin, cellulose and hemicellulose are also involved. Torssall (1959) also stated that changes in frost hardiness was parallel with changes in total sugar content and lignification of the secondary xylem.

Starzychi, et al. (1964) used the ratio of ^{32}P uptake $\frac{1\text{-}2^{o}C}{19\text{-}20^{o}C}$ as an an index of frost hardiness. Resistant cultivars of winter rape had a higher ratio than less resistant cultivars.

Evaluation of Frost Hardiness

General.

Frost hardiness is generally measured by freezing the plants or plant parts to a test temperature and then using some method to estimate the damage or lack of damage. Some tests have been used which do not involve actual freezing.

When actual freezing is done the freezing rate, time frozen, thawing rate, and post thawing conditions must all be controlled (Levitt, 1956). The amount of damage is then estimated. A visual estimate of damage was found satisfactory for red-osier dogwood stem sections (Fuchigami, et al. 1971). However, quantitative chemical estimates are generally considered less biased. The refined triphenyl tetrazolium chloride (TTC) method of measuring cold injury is based on the quantity of cells that survive freezing and thus are able to reduce TTC producing a red color which can be measured colorimetrically (Stephonkus and Lanphear, 1967). Some methods measure the death of cells. These methods are generally based on the loss of selective permeability of the membranes. For example, Wilner 1960 used the release of electrolytes from the cell as a measure of death and

Siminovitch, et al. (1964) used the release of amino acids and other ninhydrin-reacting substances as a measure of death after thawing. Greenham (1966) believes that freezing injury is to the plasmamembrane. He believes that a decrease of electrical resistance to low frequency current measures damage to the plasmamembrane and he used this principle to identify the time of injury during freezing, to alfalfa.

Some methods of estimating frost hardiness have been used or proposed which do not require actual freezing of the tissue. A correlation of peroxidase in young tissue of carnation and willow and hardiness has led to the suggestion that peroxidase be measured to estimate hardiness (McCown, 1969). As already mentioned, the ratio of ^{32}P uptake $\frac{1\text{-}2^{o}C}{19\text{-}20^{o}C}$ was used as an index of frost hardiness of winter rape (Starzychi, et al. 1964).

Exotherms.

Plots of tissue temperature during cooling vs. time produce curves which are deflected by heat of water crystallization at points where plant tissue freezes (McLeester, et al. 1969). The lowest temperature reached before the deflection is considered the supercooling point and the highest temperature reached after a deflection is considered to be a freezing point (Appendix page 154). Living plants exhibit two or more deflections while dead plants exhibit only one (McLeester, et al. 1969). The third exotherm occurs at the moment of death (Weiser, 1970). The hypothesis involving the third exotherm has been stated in the general section. Most workers agree that the first exotherm is due to freezing of extracellular water. Luyet and Gehenio (1937, cited by McLeester, et al. 1969) suggested that the second exotherm was due either to freezing of intracellular fluid after its extraction from the cell by rapid osmosis or freezing of vascular sap. Hudson and Idle (1962, cited by McLeester, et al. 1969) suggested that the second

freezing point was caused by a sudden leakage of inorganic solutes from the cells after the first freezing point had been reached. They postulate that the released solutes cause extracellular thawing by lowering the freezing point and that subsequent freezing points result from refreezing of thawed extracellular water. Block, et al. (1963, cited by McLeester, et al. 1969) suggested that the second freezing point represents a depressed freezing point of water structurally bound in a gel network. Their work was based on studies with non living model systems, which also show double freezing points. The first freezing cycle of these gels destroys their ability to bind water and eliminates the second freezing point in subsequent freezing cycles. Salt and Kaku (1967) after studying freezing curves of spruce needles suggested that the two freezing points represented ice crystallization in two different major tissues such as stele and spongy mesophyll. Salt and Kaku believed the deflections should be called rebounds because they say that the deflections do not represent the true freezing points of the tissue. Red-osier dogwood twigs with all bark removed and homogeneous tissue from storage organs show two distinct exotherms (McLeester, et al. 1969).

McLeester, et al. (1969) developed a viability test of stem sections of several genera of plants, based on the fact that dead tissue does not have a second exotherm. They found that the results which were immediately available, were identical to those of regrowth tests which took up to twenty days.

McLeester, et al. (1969) found that dormant twigs allowed to imbibe water showed an increase in length of the first freezing point plateau, which was correlated with the increase in moisture content. When the water

content reached 70 percent, the first freezing point plateau masked the second freezing point. They observed that if the stem sections were not wrapped in saran, the stem sections lost moisture during the first freezing cycle and on a second freezing cycle, the second freezing point was more distinct due to the loss of moisture. They also found that twig length had little effect on the freezing curves but that twig diameter did. Samples of smaller diameter twigs showed a depression of the freezing point, due to excessive heat loss in relation to the latent heat produced. Placement of the thermocouple in the pith gave better resolution of freezing point two, than if it was pressed tightly against the bark. Slow rates of cooling gave the best resolution of the second freezing point for samples with low moisture content, while rapid freezing was best for more hydrated samples. Lastly, they found a wide and unpredictable variation in the supercooling of uniform samples frozen under the same conditions. Samples with only slight supercooling had freezing point one plateaus, which were proportionately longer than those for samples which supercooled more.

Salt and Kaku (1967) found that needles of Colorado blue spruce have a freezing curve with a single rebound when the needles have sufficient water and a double rebound when water is deficient. The first rebound is small representing a small amount of water freezing. Visual observations under the microscope lead them to believe, that ice first freezes in the stele and then in the bulky mesophyll tissue. Because the second rebound occurs even if the temperature is not lowered beyond the temperature that causes the first rebound, they believe that it is caused by impeded propagation of ice and not a second nucleation. They also found that freezing appears to occur in the intrawall area with interior ice accretion and no intraprotoplasmic freezing.

Young (1966) measured the freezing point of citrus leaves by recording the leaf temperature every 20 seconds when they were placed in a growth cabinet at a given constant temperature. Freezing points of leaves attached to the plant did not differ significantly from those of detached leaves when exposed to a chamber temperature of 19°F. However, the attached leaves generally froze sooner. Freezing points of plants frozen at faster rates (lower chamber temperature) were depressed because a lower minimum temperature is reached before freezing occurs and thus more heat is removed during the cooling process. Thus, on freezing, the heat released from ice formation is not sufficient to offset the heat removed during the extra cooling and so the freezing point is depressed. He found that the LT 50 of citrus plants is lower than the freezing points observed. He states that if the true freezing point is obtained by prolonged exposure to moderate freezing, the LT 50 and the freezing point should approach each other but the freezing point will always be slightly higher. He found that as the time the plants were exposed to the low temperature increased from two to 24 hours, the LT 50 increased.

Supercooling and frost hardiness.

Supercooled water is water at a temperature below that required for ice formation but still in the liquid state. It offers the plant a method of avoiding frost. However, it is a very unstable state. Many insects survive winter in the supercooled state, but even in them it is not stable and freezing occurs at irregular intervals over long periods of time (Levitt, 1966). Supercooling is probably only important to plants exposed to moderate freezes of short duration (Levitt, 1966).

A rapid decline in temperature increases the chances for supercooling

and greater supercooling increases the probability of intracellular freezing (Mazur, 1970).

Salt (1966) lists temperature, time and chance as factors influencing nucleation in supercooled insects. A study of supercooling of Cephus cinctus Nort. showed that the time for intracellular ice formation doubled with every 0.53°C rise in temperature. For example, freezing time increased from 1.2 seconds at -30°C to more than a year at -17°C. No substance has been identified as a primary nucleating agent, but a variety of tissue substances and structures are believed to possess nucleating ability. Nucleating agents were associated with the cell walls of blue spruce and not the extracellular water (Kaku and Salt, 1968) because nucleation was a function of the length of the stele and not of weight of tissue or water. "Consistent supercooling after repeated freezing and thawing is attributed to the action of the same nucleating agents. Erratic supercooling, however, appears to involve inhibition, destruction, substitution and reactivation of nucleating agents, although the nature of these actions is unknown" (Alden and Hermann, 1971).

Internal and external moisture deficiencies more than any other factors favored the supercooling of tender fruit blossoms and leaves (Modlibowska, 1962).

The increase in killing temperature when frost was present on leaves of grapefruit seedlings, indicated that supercooling was a factor in resistance to freezing injury in citrus species (Young, 1969). Leaves of citrus species that are not cold resistant do not recover from ice formation in their tissues (Young, 1968).

Cells of immature leaves, in contrast to cells of mature leaves which have a larger volume of intercellular space, froze rapidly and almost all were killed before freezing was complete (Kaku, 1964, cited by Alden and Hermann, 1971). Saturation of intercellular spaces with water caused a decrease in supercooling ability of mature leaf tissue and less difference in supercooling ability between mature and immature leaves. Thus Kaku concluded that supercooling ability is not an index of frost tolerance because it depends on intercellular water content and leaf maturity, which vary within the same plant and between different plants. Yelenosky and Horanic (1969, cited by Alden and Hermann, 1971) stated that the ability of unhardened citrus to supercool was not a satisfactory index of cold hardiness.

Increased osmotic values and decreased water contents with maturation did not affect supercooling points (Kaku 1966, cited by Alden and Hermann, 1971). The hypothesis is that rapid cooling rate lowers the supercooled point below which spontaneous freezing occurs, because the probability of ice nucleation increases as the duration of undercooling is lengthened (Alden and Hermann, 1971).

METHODS AND MATERIALS

Environmental Growing Conditions

Greenhouse.

The minimum night temperature was 18°C, during both summer and winter. Three hundred foot candles of supplemental light at plant level was supplied by fluorescent and incandescent lights during both the summer and winter. Experiments performed during the winter had a 16 hour photoperiod and those during the summer an 18 hour photoperiod.

Cold room.

The temperature was maintained at 4.4 ± 1.0°C. Three hundred foot candles of light at plant level was supplied by fluorescent light bulbs. The photoperiod was nine hours.

Growing of Plants and Experimental Design

Exotherm experiments.

Hardening experiments. Four flats (1 x 1.5 ft.) containing a loam soil were broadcast seeded with Target rape seed and placed in the greenhouse. Germination occurred five days after planting.

Five days after germination, exotherm measurements were begun on the cotyledons. Two flats selected randomly were transferred to the cold room. Weekly exotherm measurements were made on the cotyledons, throughout a five week hardening treatment.

Nineteen days after germination, greenhouse grown plants were in the rosette stage of growth with fully expanded leaves. At this time exotherm measurements of the leaves were begun and two flats were transferred to the cold room. Weekly exotherm measurements of the true leaves were performed during a five week hardening treatment. The plants remained in the rosette

form throughout this period.

Life cycle experiments. Four flats (1 x 1.5 ft.) containing a loam soil were broadcast seeded with Target rape seed. Two of these flats were placed in the greenhouse and two in the cold room.

Seeds in the greenhouse germinated in five days. Five days after germination, exotherm measurements were begun on the cotyledons. The next week exotherm measurements were performed on the true leaves. Weekly exotherm measurements were continued on the true leaves throughout the life cycle of the plants.

Seeds in the cold room germinated 21 days after planting and after a further seven days were moved to the greenhouse. At this time the seedlings were approximately three inches high and their cotyledons were small and yellow. Then after seven days in the greenhouse exotherm measurements were begun. The cotyledons were analyzed the first week. At this time they were green but smaller than the cotyledons from plants germinated in the greenhouse. Weekly exotherm measurements were then performed on the true leaves throughout the life cycle of the plants.

Cultivar comparison experiment. Two small flats (0.5 x 1 ft.) containing a loam soil were broadcast seeded with Nugget rape seed and two with Target rape seed and placed in the greenhouse.

The Nugget seed germinated in seven days. The cotyledons were analyzed 11 and 16 days after germination.

The Target seed germinated in four days. One flat of Target was placed in the cold room eight days after germination. It was analyzed 12 days after

germination. The flat of Target in the greenhouse was analyzed five and twelve days after germination.

General. The above experiments were performed in the summer. The plants were fertilized with 8:16:0 every seven to ten days. Watering was carried out when required and the day proceeding exotherm analysis. Each exotherm analysis consisted of measuring one exotherm curve of one leaf of seventeen different plants, chosen randomly from the two flats. Moisture determinations were carried out each time exotherm measurements were made. The Target seed used was produced in 1965 and the Nugget seed in 1961.

Lipid experiments.

Lipid composition of seedlings of two rape cultivars before and after a short hardening treatment. Nine flats (1 x 1.5 ft.) containing a loam soil were broadcast seeded with Target rape seed and twelve with Nugget rape seed. The flats were then placed in the greenhouse. The Target seed germinated three days after planting and the Nugget seed four days after planting.

Two days after germination three flats of Target seedlings chosen randomly were transferred to the cold room and three were harvested. Five days after germination the three flats of Target seedlings in the cold room were harvested. On the third day after germination the three flats of Target seedlings still in the greenhouse were harvested. Each flat was harvested as one sample. The total shoot of the seedling was harvested. Each sample was then immediately frozen.

Five days after the Nugget seeds germinated, six flats chosen randomly

were transferred to the cold room and six were harvested. Eight days after germination the six flats in the cold room were harvested. Two flats were harvested as one sample because of the lower percent germination of Nugget. The samples were immediately frozen. This experiment was performed in the fall.

Lipid composition of leaves from the rosette growth stage before and after a 35 day hardening treatment and of leaves from the nonhardened bolted growth stage. Nine flats containing a loam soil were broadcast seeded with Target rape seed and put into the greenhouse. Germination occurred in eight days.

Twenty-eight days after germination, the plants were in the rosette stage of growth and the leaves were fully expanded. The leaves of all the plants from three flats, chosen randomly, were harvested as one sample and immediately frozen. A bulk sample was made to reduce variation in the lipid values due to microenvironmental influences. For lipid analysis, the leaves were divided into four samples after freeze drying. When possible the data for the three samples showing closest agreement were used to decrease the experimental error of each estimate.

At the same time (28 days after germination) three flats chosen randomly were transferred to the cold room. After thirty-five days of hardening, the leaves of these plants were harvested and treated as above. The hardened plants were still in the rosette stage of growth.

Forty-nine days after germination, the leaves of the plants still in the greenhouse were harvested and treated as above. These plants had bolted

and were flowering. This experiment was done in the winter.

General. These plants were fertilized and watered in the same manner as those plants grown for the exotherm experiments. The seed source was also the same.

Method of Measuring Exotherms

The temperature of a leaf disc (11 mm in diameter) during cooling, was measured and recorded with a Honeywell dual pen recorder (Electronic 194). The thermocouple was copper constantan (gauge No. 30) with welded junction (Campbell, et al. 1968). A reference thermocouple at 0.6°C (ice water bath) was used. The leaf disc, covered by a piece of synthetic sponge (80 x 25 x 15 mm) was folded over the thermocouple junction. This was then placed into a weighing bottle (60 x 40 mm), the sides of which prevented the sponge from unfolding and forced the sponge to hold the leaf disc firmly against the thermocouple junction. The bottle was then covered with a rubber stopper, which had a hole in the center to allow the thermocouple wires to pass through. The weighing bottle was then placed to a specific depth into a methanol bath (one litre) cooled to some temperature below -35°C by a bath cooler (PBC - 4 Neslab Instruments Inc.). This resulted in a leaf disc cooling rate of approximately 1200°C/minute.

The recorder span was one milli volt, which gave a full scale reading of approximately 30°C. The chart paper was scaled from 0 to 100. Zero degrees centigrade was set at a chart reading of 50. A standard curve was prepared and temperatures read from it. A chart speed of one inch per minute was used.

Method of Measuring Moisture Content

Leaves were removed from the plants and thirty leaf discs were immediately taken, placed into tarred tins and weighed. They were then dried at 105°C for 18 hours. Tins were then removed from the oven, covered and allowed to cool for ten minutes before weighing. For cotyledons the whole cotyledon was used and not discs. Two replications were used for each determination. These moisture determinations were carried out at the same time that the exotherm measurements were being made.

Conductivity Measurements

Detached leaves were washed with distilled water, dried with paper toweling, rolled into a cylindrical shape and held like this with a small piece of masking tape. Care was taken not to roll the leaves so tightly that injury occurred. The leaves were then put into a test tube (25 x 150 mm). If exotherm measurements were to be carried out, unwashed leaves were used and the thermocouple was placed inside the leaf cylinder. The test tube was then put into a stoppered thermos bottle which was then placed in the freezer for the cold treatment. After the cold treatment, the thermos was removed from the freezer and allowed to warm to room temperature. Twenty-five ml of dionized water was added. This was sufficient to completely cover the leaf. The leaf was left in the water for 24 hours, at which time the conductivity was measured with a Radiometer conductivity meter (Type CDM 2d). The leaves were then killed after pouring the water into a 50 ml beaker, by placing the test tube containing the leaf into liquid nitrogen for 1.5 minutes. The water was then poured back into the test tube. It was allowed to come to equilibrium for another 24 hours before again measuring the conductivity. The conductivity change after the cold treatment as a percentage of the total conductivity change caused by

killing with liquid nitrogen was then used as an estimate of the amount of damage caused by the cold treatment.

Method of Determining the Weight of Plant Material and Lipid

Immediately after harvesting, the plant material was frozen at -20°C, then lyophilized in a Virtis model 10-MR-TR freeze drier and stored at -20°C. Before analysis each sample was placed into tarred tins and brought to constant weight in a vacuum desiccator (containing drierite and KOH pellets). The tins were removed from the vacuum desiccator, immediately covered and weighed to one ten thousandth of a gram with a Mettler gramatic balance. The sample was then extracted for lipids (see below).

The lipid extract was transferred to tarred 50 ml beakers with 40 ml of chloroform. The chloroform was evaporated under a stream of nitrogen. The beakers were placed in a vacuum desiccator containing drierite, KOH pellets and paraffin wax for 24 hours and dried to constant weight. The beakers were removed from the vacuum desiccator and immediately weighed. After extracting the lipid from the beaker with a total of 15 ml of chloroform in three aliquots, the beakers were put back into the vacuum desiccator for eight hours. The beaker was again weighed to allow for any non lipid material.

Weight of the lipid fractions and fatty acids were determined in a similar manner using the appropriate solvents.

Method of Lipid Extraction and Purification

Lipid extraction was carried out according to Bligh and Dyer (1959),

on one to three gram samples of dried plant material. The solvent volumes used for all samples, were those recommended by Bligh and Dyer for samples of 25 grams fresh weight at 80% moisture content. Blending was carried out in a homogenizer (model MIMI manufactured by Lourdes Inst. Corp.) for periods of time twice those recommended. NaCl in water (0.9% W/V) was used instead of distilled water to prevent emulsion formation during partitioning (Kuiper, 1968). Partitioning of the chloroform phase from the methanol water phase was carried out in a 500 ml sepratory funnel. The methanol water phase was re-extracted twice with chloroform to prevent loss of galactosyl diglycerides (Kuiper, 1970).

The combined chloroform extracts were purified of water and nonlipid components according to Williams and Merrilees (1970). Two grams of sephadex G-25 and 5 ml of distilled water were added to each sample. The mixture was poured into a glass column (1.0 cm I.D.) and the chloroform solubles were eluted by the addition of 200 ml of chloroform. The chloroform was removed by vacuum evaporation at 40°C. The lipid was then weighed.

Method of Fractionating the Lipid

The total lipid extract was separated into neutral and complex lipids by the method of Dittmer and Wells (1969). Silicic acid was suspended several times in distilled water and the fines were decanted. It was then washed with acetone to remove the water, air dried overnight and activated at 110°C for one half hour. This gave a solvent flow rate of one to 1.5 ml per minute. The column was prepared by suspending the silicic acid in chloroform and pouring it into a glass column (1.5 cm I.D.) plugged with

absorbent cotton. The column was washed with 150 ml of chloroform, then the lipid sample was transferred to the column as three 10 ml aliquots. The neutral lipids were eluted with 200 ml of chloroform and the complex lipids with 200 ml of methanol. After fractionation, the solvents were removed by vacuum evaporation and the simple and complex lipids weighed.

Method of Saponification of Lipids and Methylation of Fatty Acids

The lipid fractions were diluted to 25 ml with chloroform. Ten ml samples were used for fatty acid analysis. Saponification was carried out by a modification of the method of Gerloff, et al. (1966) using ethanolic KOH. Skelly F (10 ml) was added to the ethanolic KOH to increase lipid solubility. The combined skelly F extracts, containing the fatty acids were washed twice with distilled water to remove nonlipid material. Water was removed from the combined skelly F extracts with Na_2SO_4. The skelly F was removed by vacuum evaporation at 25°C and the weight of the fatty acids was determined.

The fatty acids were methylated by refluxing under nitrogen in 60 ml of methanolic 2N HCI (methanol-concentrated HCl, 5:1, V/V) for three hours (Kates, 1964). Ten ml of skelly F was used to increase the lipid solubility during refluxing. After refluxing, 40 ml of distilled water was added and the fatty acid esters were extracted four times with 40 ml of skelly F. The combined skelly F extracts were washed twice with 40 ml of water and dried with Na_2SO_4. The skelly F was removed by vacuum evaporation at 25°C. The methyl esters dissolved in skelly F were then transferred to small screw cap vials and stored at -20°C until analysed. The screw caps

were lined with aluminum foil.

Method of Fatty Acid Analysis

The fatty acids were analysed on a Varian aerograph series 1200 gas chromatograph. The column used was 1/8 in. x 8 ft copper tubing packed with 10% diethylene glycol succinate on a stationary phase of chromosorb W (60-80 mesh). The injector temperature was 240°C and the detector temperature 255°C. The hydrogen flame detector was supplied with 200 ml/minute of air and 20 ml/minute of hydrogen. The carrier gas was nitrogen at a flow rate of 12 ml/minute room temperature. Temperature programing was used between 80 and 180°C at 4°C/minute. Sensitivity as measured by the product of attenuation and range was generally between 16 and 64. The area of each peak was measured by triangulation (height x width at one half height). Peak area was presented as a percent of total. The mean percent from three injections was used for each sample. Fatty acids were identified by co-injection of sample plus a standard mixture of fatty acid esters. The 16:0, 18:0, 18:1, 18:2, 18:3 and 20:0 fatty acids were identified this way. The 16:1 fatty acid was identified by co-injection of sample and a 16:1 fatty acid standard. The samples were also hydrogenated (Eybel and Simon, 1970) to identify the unsaturated fatty acids, 16:2 and 16:3.

Method of Phosphorus Determination

Total phosphorus of the complex lipid was determined in triplicate by the modified Fiske and Subba Row method given by Dittmer and Wells (1969). Heating during wet ashing was carried out in a sand bath. Optical density was measured with a Zeiss spectrophotometer (PMQ11) as were the optical

densities for sugar and chlorophyll determinations.

Method of Sugar Determination

The complex lipid was hydrolyzed with five ml of 3N H_2SO_4 in a 150 x 10 mm test tube (covered with a marble) for 2.5 hours, in a boiling water bath. It was then extracted four times with five ml of skelly F to remove the lipid material, heated to drive off any remaining skelly F and made up to 10 ml with water. One ml of this was analyzed quantitatively for sugar. This hydrolysis procedure is a modification of that used by Dittmer and Wells (1969).

The quantity of sugar was measured by the phenol method given by Dubois, et al. (1956). It was assumed that galactose was the only sugar present. A qualitative analysis of the sugar from one lipid sample, by ion exchange chromatography (Kesler, 1967) gave only one peak with an R_f similar to galactose. Three hydrolysis were performed for each sample and three color developments were performed for each hydrolysis. Thus the value for each sample was the mean of these nine values.

Method of Chlorophyll Determination

The quantity of chlorophyll was determined according to the method of Mackinney (1941). It was determined after the lipid extract was purified with sephadex G-25. Three ml of the 200 ml was used for analysis, one ml for each replication.

Chemicals

Organic solvents were technical grade, supplied by Baker Chemical Co.

The chloroform and methanol were redistilled before use.

The silicic acid was 100 mesh analytical reagent manufactured by Mallinckrodt Chemical Works.

The sephadex G25-80 had a particle size of 20-80μ and was supplied by Sigma Chemical Works.

Chromosorb W was acid washed, DMCS treated, 60-80 mesh and produced by Johns-Manville Products Corp., Celite Division.

Diethylene glycol succinate type LAC-728 was supplied by Varian aerograph.

The air, nitrogen and hydrogen were supplied by Union Carbide Canada Ltd.

The mixture of fatty acid standards and the 16:1 standard were produced by Applied Science Laboratories Inc.

All other chemicals used were reagent grade and supplied by either Baker Chemical Co. or Fisher Scientific Co.

Statistical Analysis

The statistical values (mean, standard deviation and standard error) were calculated for each treatment. The means of similar measurements for the different treatments were tested for significant differences using the Student's t test. Significant differences at the levels $p=0.10$, 0.05 and 0.01 were recorded. I accept the level $p=0.05$ as indicating a significant difference, however because of the small sample size for the lipid work

(three or four) and the large variation in some measurements, I indicate when a significant difference at the level $p=0.10$ exists and accept it as a potential difference. All calculations (statistical values, correlations, Student's t test and percent fatty acid composition) were performed on an Olivetti Underwood programma 101.

RESULTS AND DISCUSSION

Exotherm Studies

Effect of a hardening treatment on exotherm values.

Cotyledons. After a late spring frost in 1969, it was observed that some spring rape seedlings survived while others were killed. This apparent range in frost hardiness of spring rape and the growing economic importance of rape, suggested that frost hardiness studies of spring rape may be fruitful. The first objective was to see if the cotyledons could harden, as a result of the proper environmental stimulus.

Seeds of the cultivar Target were planted and germinated in the greenhouse. When the cotyledons were fully developed, the seedlings were transferred to the cold room. Weekly exotherm measurements, which may be related to frost hardiness, were performed on the cotyledons. The hardening treatment lasted five weeks. The exotherm measurements are defined in the appendix (Page 154).

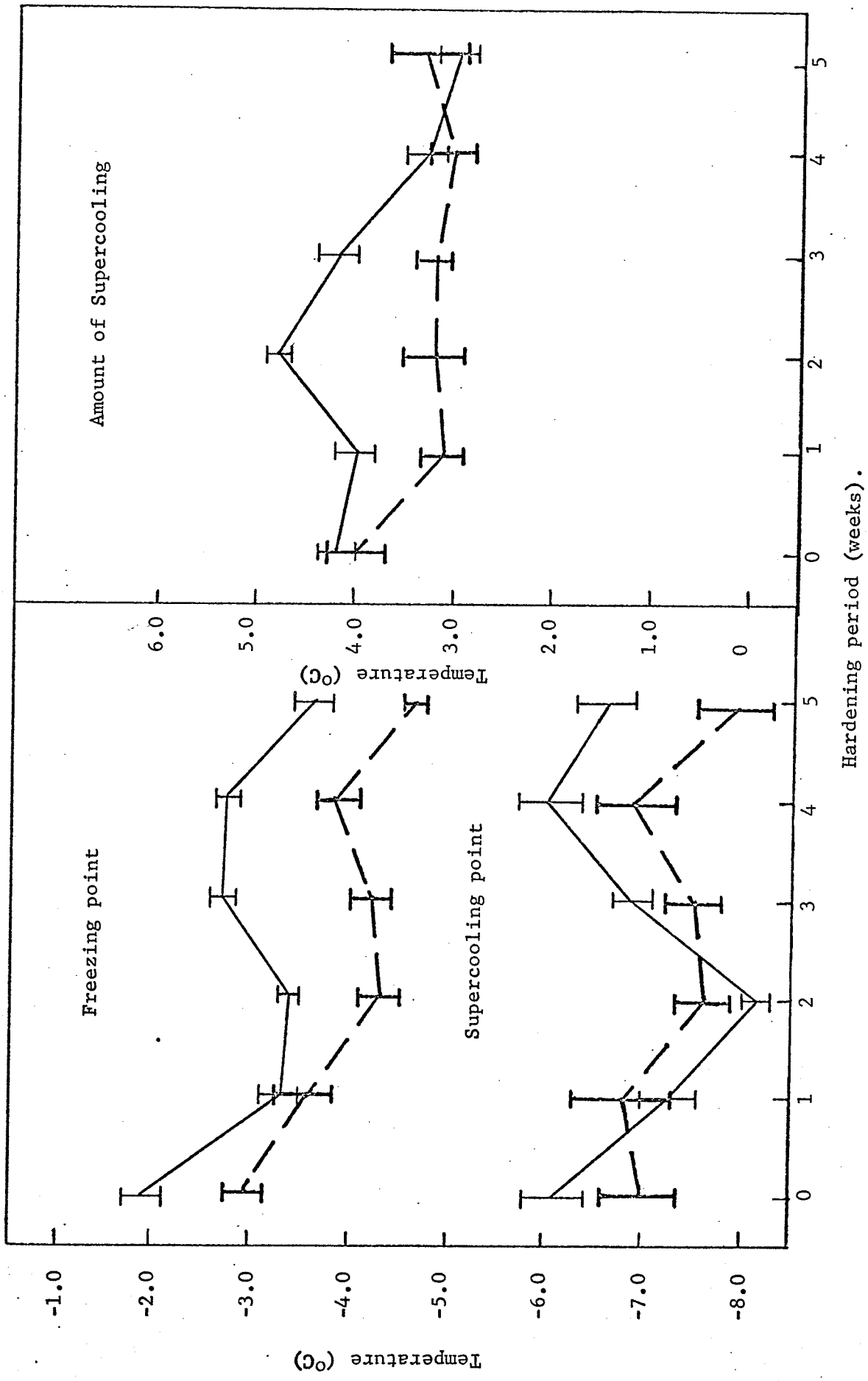
The supercooling point of the cotyledons decreased from $-6.1\pm0.3^{o}C$ (nonhardened) to a minimum value of $-8.2\pm0.1^{o}C$ during the first two weeks of hardening (Figure 1). It then increased to the value of the nonhardened plants (zero week hardening) during the third week of hardening and remained at this level for the remainder of the five week hardening treatment.

The amount of supercooling of the nonhardened cotyledons was $4.2\pm0.2^{o}C$ (Figure 1). It remained constant during the first week of hardening and then increased slightly during the second week. The amount

Figure 1 . The effect of hardening on the exotherm values of Target leaves and cotyledons. Each value is represented by the mean and standard deviation of 17 determinations.

Leaves - - - - - -

Cotyledons ——————


Freezing point
Supercooling point
Amount of Supercooling
Temperature (°C)
Temperature (°C)
Hardening period (weeks).

of supercooling then decreased continuously from the second week high of $4.8 \pm 0.1^{\circ}C$ to a minimum of $3.3 \pm 0.2^{\circ}C$ reached after four weeks of hardening. It remained constant during the fifth week of hardening. The increase in the amount of supercooling during the second week accounted for the decrease in the supercooling point during the second week.

The freezing point decreased from the nonhardened maximum of $-1.9 \pm 0.2^{\circ}C$, to its minimum value of $-3.3 \pm 0.2^{\circ}C$ within one week of hardening (Figure 1). It remained constant during the second week of hardening, then increased to $-2.7 \pm 0.1^{\circ}C$ during the third week. The freezing point remained at this value during the fourth week, then during the fifth week it again decreased to a value not significantly different from that reached after one week of hardening. The decrease of the freezing point during the first week of hardening accounted for the decrease of the supercooling point during that week. It should also be noted that during the hardening treatment the freezing point was always significantly lower than for nonhardened cotyledons (zero week hardening).

The observed development of a minimum freezing point during the first and fifth week of hardening is similar to results obtained from seedlings of winter wheat and barley (Dantuma and Andrews, 1960). With these species, it was found that maximum frost hardiness occurred during the first and sixth week after germination, when the plants were germinated and grown at $1.5^{\circ}C$. These species had minimum frost hardiness at two to three weeks after germination. The increase in the freezing point of the rape cotyledons during the third week corresponds with this. These relationships are unexpected because Dantuma and Andrews (1960) found that spring cultivars and winter wheats with low winter hardiness did not show

the second maximum hardiness at six weeks after germination.

The moisture content of the cotyledons was measured each week to see if it was related to any of the exotherm values. The percent moisture remained almost constant during the hardening treatment. Thus no correlation between percent moisture and exotherm values existed (Figure 2).

True leaves. The next objective after studying the effect of hardening on cotyledons was to study the effect of hardening on true leaves. Because the true leaves are structurally more complicated than cotyledons, they may respond to a hardening treatment differently.

Seeds of the cultivar Target were planted and grown in the greenhouse. Plants with fully expanded leaves in the rosette stage were transferred to the cold room. Weekly exotherm measurements were performed during a five week hardening treatment.

The supercooling point of the true leaves did not change significantly during the entire five week hardening period (Figure 1). It varied from $-6.8\pm0.5^{\circ}C$ to $-7.9\pm0.4^{\circ}C$. This represents a maximum range of $2^{\circ}C$. Significant differences could not be detected within this range due to the large variation of each estimate. The maximum coefficient of variation for an estimate was 25 percent. This was not unexpected because of the involvement of chance in supercooling.

It can be concluded from Figure 1, that the numerical value of the supercooling point of the leaves and cotyledons are not significantly different during the first four weeks of hardening. However during the fifth week the cotyledons have a higher value. The qualitative response

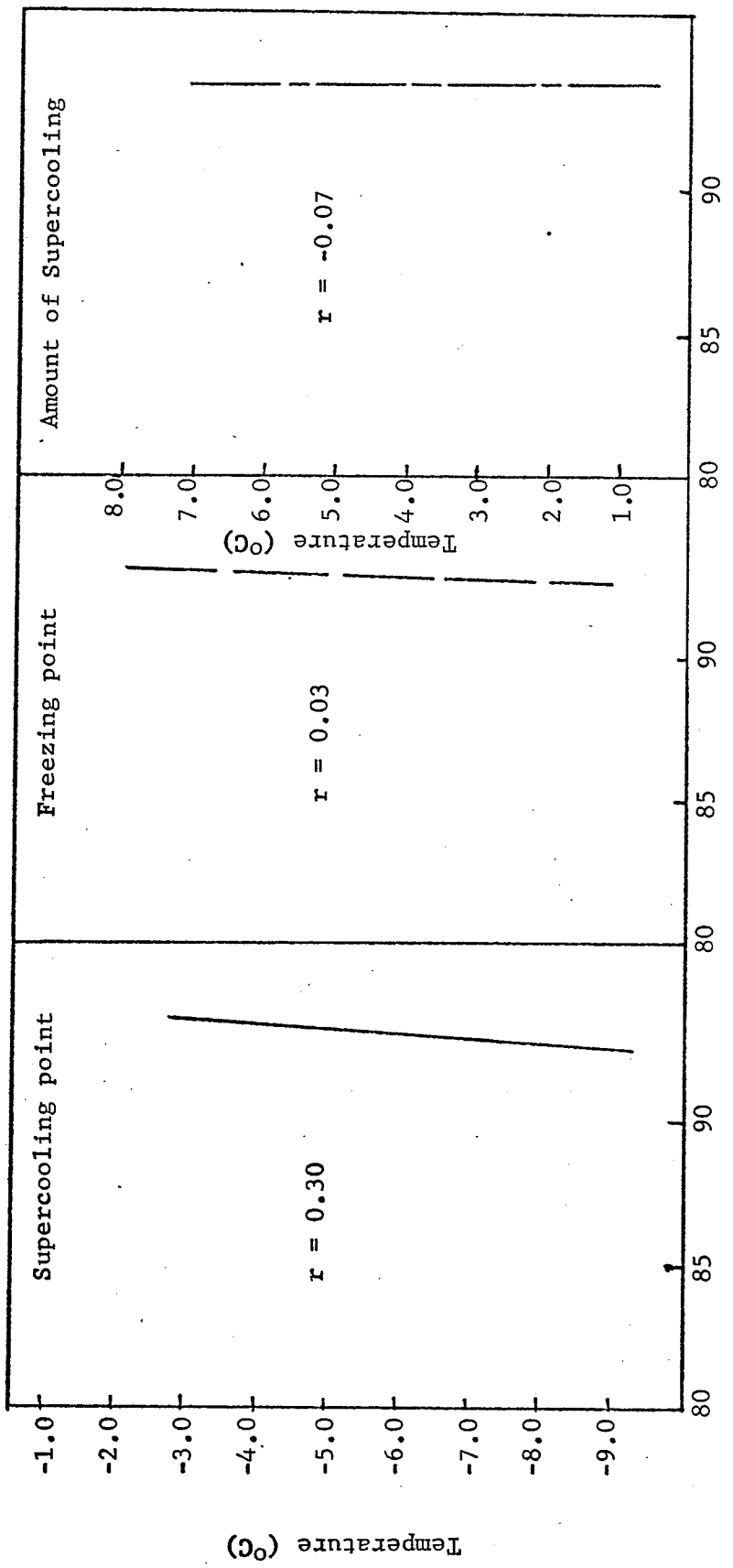


Figure 2. Regression lines of exotherm values vs. the percent moisture of hardened cotyledons.

*, ** and *** indicate a significant correlation at P = 0.10, 0.05 and 0.01, respectively.

to the hardening treatment was different. During the hardening treatment, the supercooling point of the cotyledons changed significantly whereas it did not for the true leaves. Figure 1 also shows that the standard errors for measurements of cotyledons are less than those for the leaves. It is interesting to speculate if there is a cause for this. For example, could the nucleating agents of the cotyledons induce more consistent freezing?

The amount of supercooling for the nonhardened leaves was $4.1 \pm 0.3^{o}C$ (Figure 1). After one week of hardening it decreased to $3.2 \pm 0.2^{o}C$ and then remained constant for the duration of the hardening treatment.

When the amount of supercooling of the nonhardened leaves (zero week hardening) was compared to the nonhardened cotyledons (zero week hardening) no significant difference existed. However during the first three weeks of hardening the amount of supercooling of the cotyledons was significantly greater than for the leaves. During the fourth and fifth week of hardening no significant difference existed.

The freezing point of nonhardened leaves (zero week hardening) was $-2.9 \pm 0.2^{o}C$ (Figure 1). During the first two weeks of hardening it decreased to a minimum of $-4.3 \pm 0.2^{o}C$. It then may have increased a small amount during the fourth week of hardening as indicated by the inflection. It then decreased to the original minimum during the fifth week. After two weeks of hardening the freezing point of the hardened leaves always was significantly lower than the freezing point of the nonhardened leaves.

The freezing point of the nonhardened leaves was lower than the freezing point of the nonhardened cotyledons ($-2.9 \pm 0.2^{o}C$ compared to

-1.9±0.2°C). The minimum freezing point of the leaves during hardening was lower than the minimum freezing point of the cotyledons during hardening (-4.6±0.1°C compared to -3.6±0.2°C). The difference between the nonhardened states produced the difference between the leaves and cotyledons, because the freezing point of both decreased approximately 1.7°C as a result of hardening. In fact the freezing point of the hardened cotyledons was not significantly different from the freezing point of the nonhardened leaves. However there was a qualitative difference in response to hardening. The cotyledons reached their minimum freezing point in one week, whereas the leaves required two weeks. The leaves like the cotyledons showed two stages of decreasing freezing point.

The moisture content of the leaves was determined to see if the exotherm measurements were related to it. This was done by weighing leaf discs before and after drying at 105°C. From this one could also calculate dry weight per disc and weight of water per disc. The leaves were fully expanded at the beginning of the experiment. Thus further cell division and expansion were not likely to occur. After cell expansion only cell divisions that increase leaf thickness, could change the number of cells per leaf disc. This is unlikely to occur because the number of mesophyll layers is characteristic of a species (Esau, 1966). Therefore dry weight per disc and weight of water per disc would be directly related to dry weight per cell and weight of water per cell. If a relationship between an exotherm value and percent moisture was found it could be determined if it was a change in the dry matter, the water or the ratio of these (percent moisture) which was important.

Figure 3 showed that percent moisture decreased and dry matter per disc increased throughout the hardening period. No change in water per disc occurred. A similar increase in dry matter per cell was found by Siminovitch, et al. (1968). They found a significant increase in protoplasmic constitutents of a hardened cell as compared to a nonhardened cell.

Correlations between the exotherm measurements and the percent moisture, dry weight per disc and weight of water per disc were calculated (Figures 4 and 5). These correlations must be considered with caution due to the small sample size. However it is considered useful to present them, if only to raise questions.

Figure 4 indicated that the supercooling point and freezing point were positively correlated to the percent moisture. Figure 5 showed that this was due to the change in dry weight and not the weight of water, because both the supercooling point and freezing point were negatively correlated to the dry weight per disc but not to the weight of water per disc. The supercooling point appears to be affected more by factors other than dry weight because the correlation only accounts for 38 percent of the variation. The correlation between freezing point and dry weight accounts for 69 percent of the variation. This suggested that an increase in low molecular weight molecules with colligative properties occurred.

Leaf exotherm values throughout the life cycle of the plant.

Total life cycle in the greenhouse. Spring rape has three distinct morphological forms which reflect changing metabolic function. They are

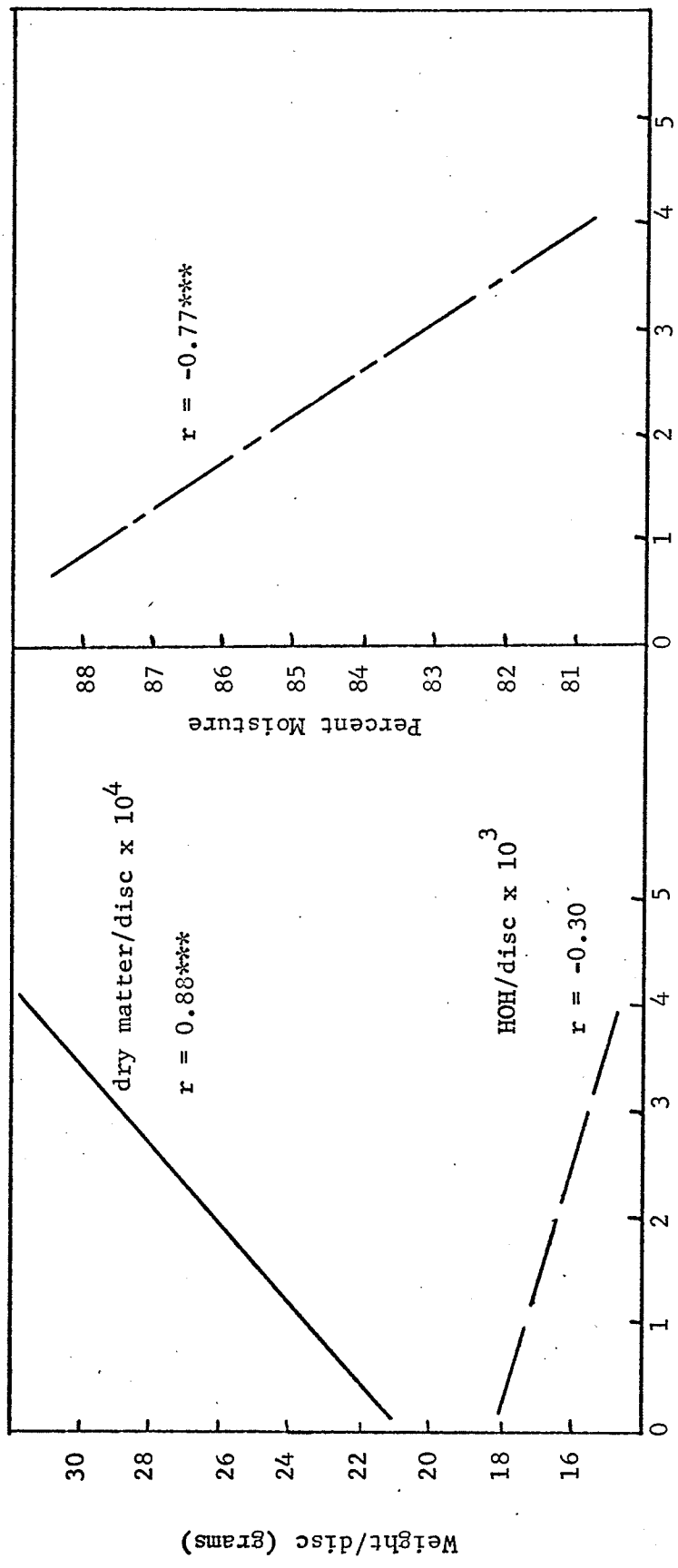


Figure 3. Regression line of percent moisture and weight of dry matter and water per leaf disc vs. length of time of hardening (true leaves).

*, ** and *** indicate a significant correlation at P = 0.10, 0.05 and 0.01, respectively.

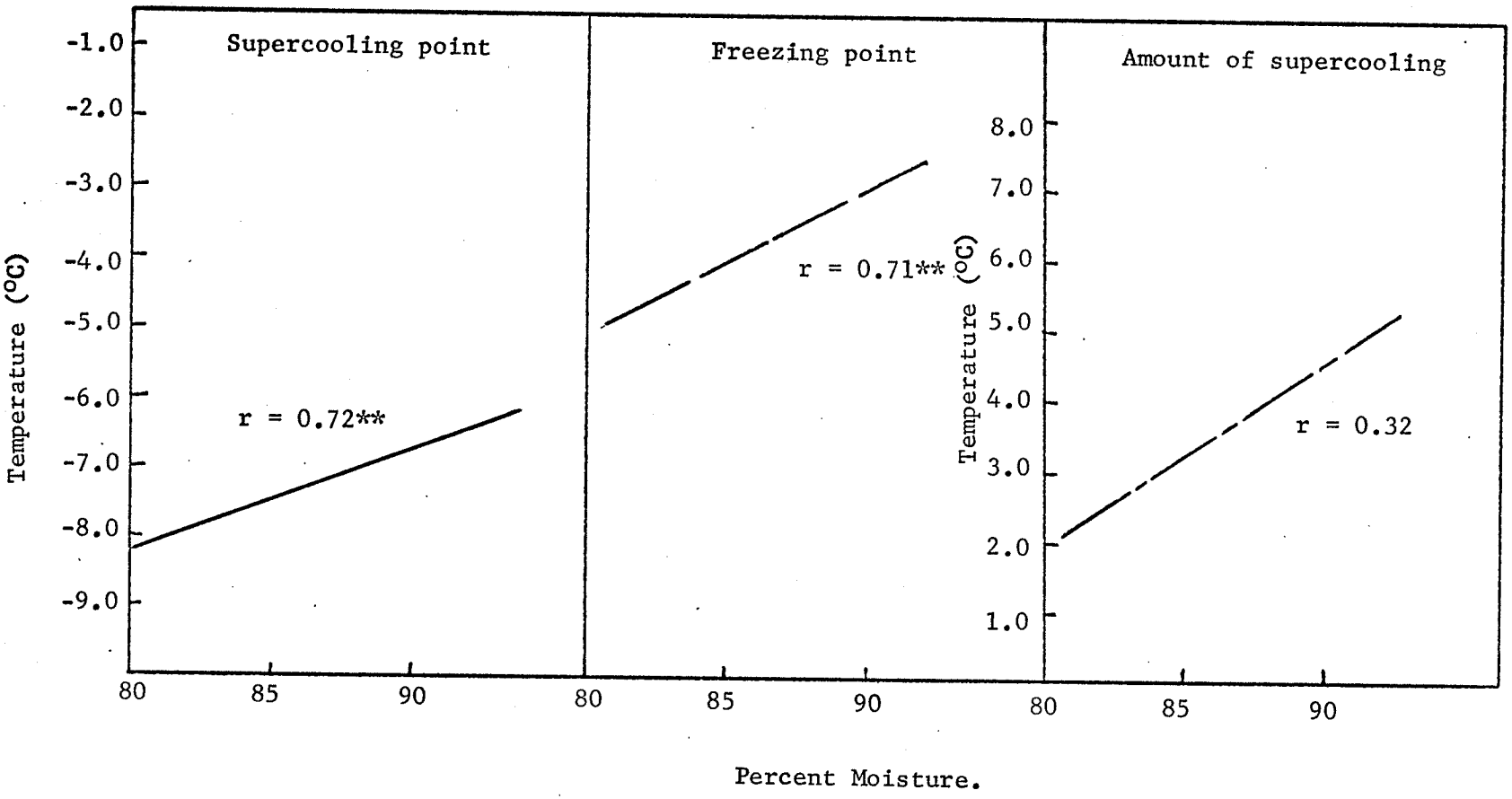


Figure 4 . Regression lines of exotherm values vs. the percent moisture of hardened leaves.

*, ** and *** indicate significance at levels P = 0.10, 0.05 and 0.01, respectively.

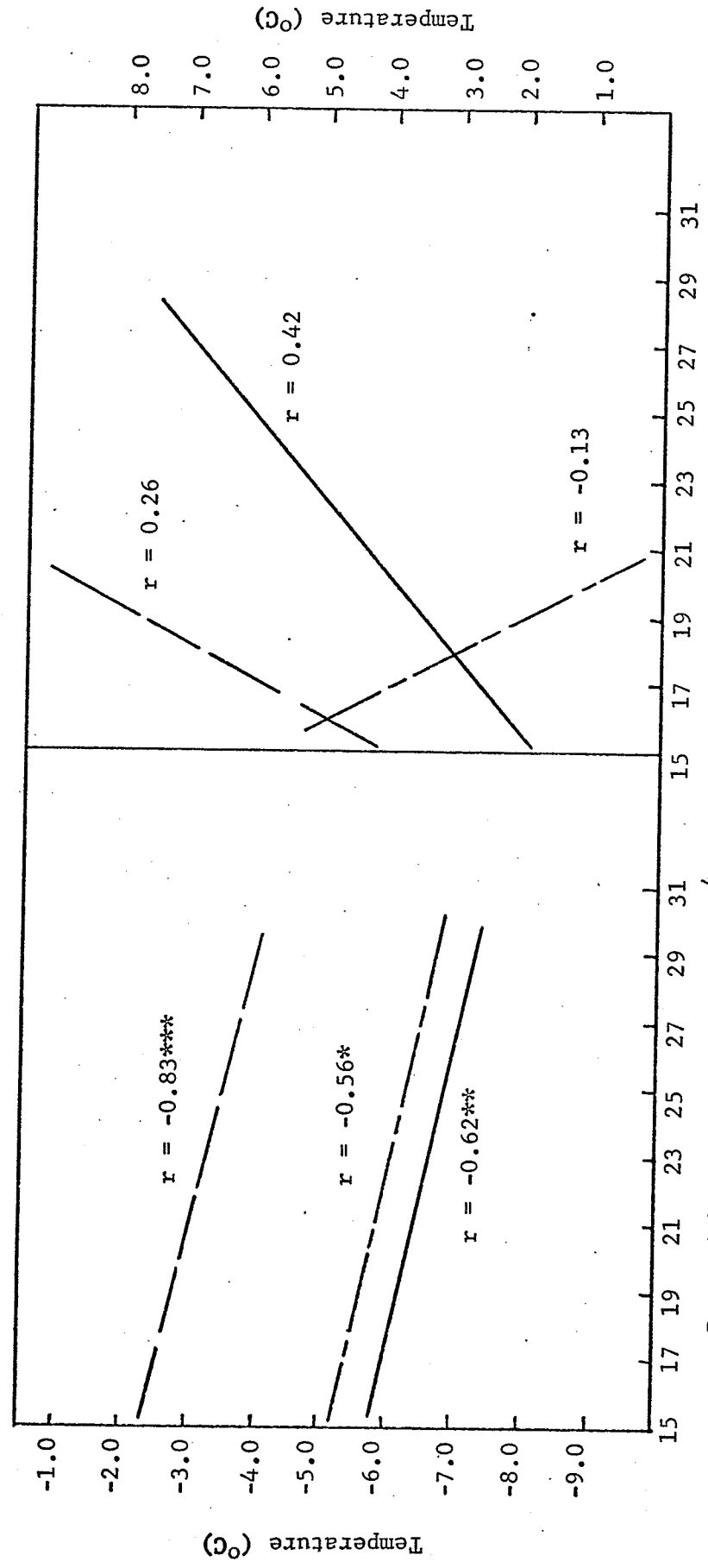


Figure 5 . Regression lines of exotherm values vs. dry weight and weight of water per leaf disc of plants during hardening.

Supercooling point ————; freezing point - - - - - -;
amount of supercooling —— · —— · ——

*, **, and *** indicate significance at P = 0.10, 0.05 and 0.01, respectively.

the seedling, the rosette and the bolted forms. In nature the seedling and bolted form may be exposed to late spring or early fall frosts respectively. A study was undertaken to see if the changing metabolism would result in changing exotherm values and if so, what the exotherm values would be when the plants may be exposed to frost. Weekly exotherm measurements were performed on leaves, throughout the life cycle of the plants.

The supercooling points of the cotyledons and young partially expanded leaves (12 days after germination) were equal (Figure 6). The supercooling point of the leaves decreased from this value as the plants matured to an age of 40 days. It then increased the following week. This increase may have been related to senescence, since the leaves began to lose color during that week.

The freezing point of the cotyledons was higher than the freezing point of the leaves, regardless of leaf maturity (Figure 6). In general, the freezing point of the leaves also decreased as the plants matured. In contrast to the supercooling point the freezing point did not change significantly during the week the leaves were senescing.

Figure 6 showed that the amount of supercooling of the cotyledons was greater than that of the incompletely expanded leaves (12 days after germination). The amount of supercooling of the fully expanded leaves was equivalent to that of the cotyledons until the leaves began to senesce. It then decreased to the value of the unexpanded leaves.

As in the hardening experiments, dry weight per disc, weight of water per disc and percent moisture were measured, to see if they are related to the exotherm measurements. Leaf expansion appeared to be completed

Figure 6 . Change in exotherm values of leaves during the plant's life cycle.

A, cotyledons;

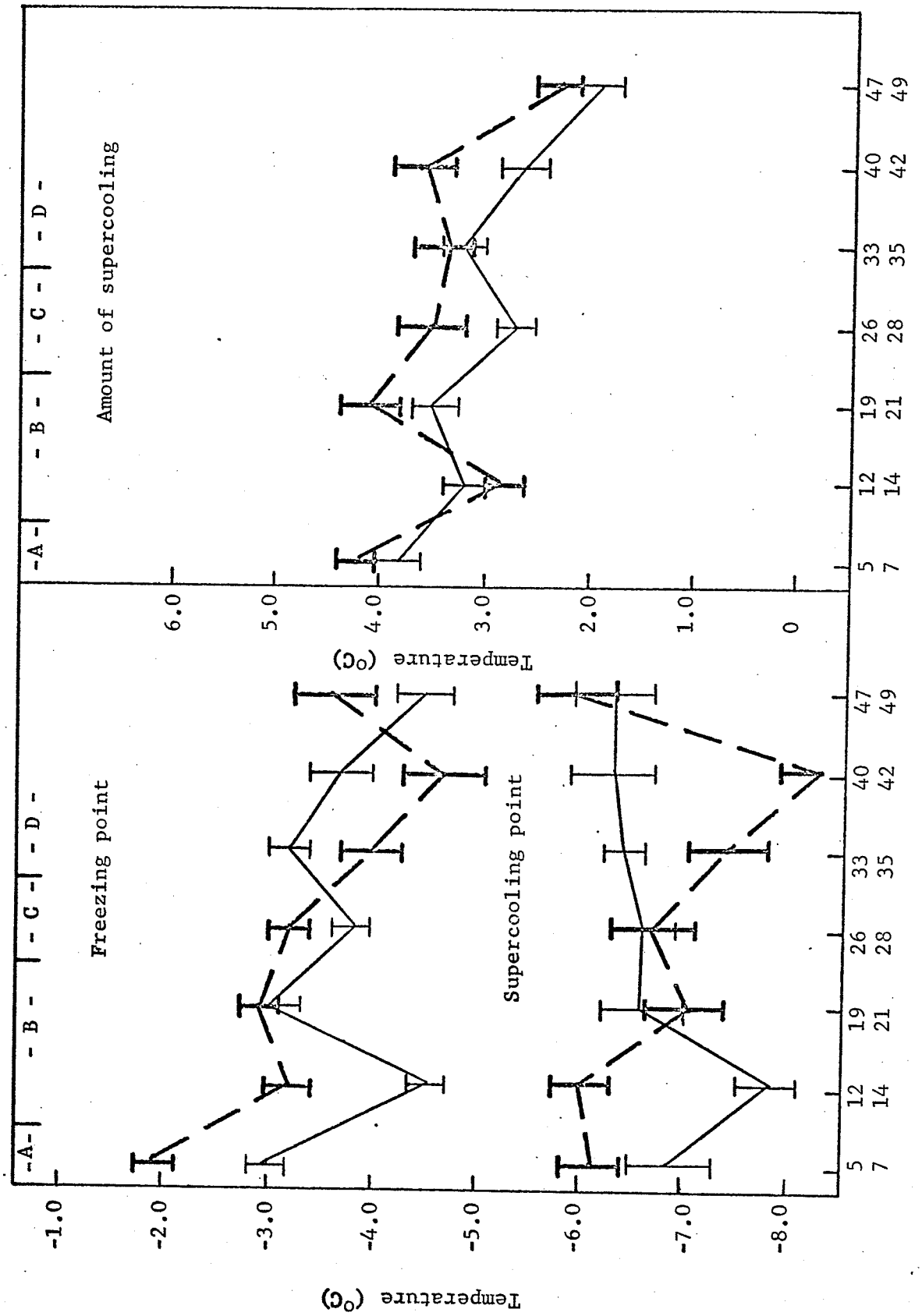
B, rosette stage;

C, bolting stage;

D, bolted stage.

Germination in the greenhouse - - - - -

Germination in the cold room ————


-A-
- B -
- C -
- D -
Freezing point
Supercooling point
Amount of supercooling
Temperature (°C)
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
-7.0
-8.0
6.0
5.0
4.0
3.0
2.0
1.0
0
5 12 19 26 33 40 47
7 14 21 28 35 42 49
Days after germination.
Days after cold treatment.

while the plants are in the rosette stage. Therefore leaf discs from plants at all more advanced stages should have approximately the same number of cells. In this experiment it was not valid to equate weight per disc to weight per cell when comparing the incompletely expanded leaves (12 days after germination) to the completely expanded leaves. After nineteen days from germination the leaves are fully expanded. Preliminary calculations, indicated that the correlation coefficients were not significantly affected by including the datum from the incompletely expanded leaves. Therefore the datum was included to increase the confidence of the correlation.

Figure 7 showed that the percent moisture of the leaves decreased during maturation. The dry weight per disc and weight of water per disc increased with maturation. However, the weight of water increased at a slower rate than the dry weight, thus accounting for the decrease in the percent moisture.

The supercooling point and freezing point of the leaves were positively correlated to the percent moisture at a significance level of $P=0.10$ (Figure 8). This is not in agreement with Kaku's (1966, cited by Alden and Hermann, 1971) results. He found that the supercooling point was not affected by the increasing osmotic values and decreasing water content which accompanied maturation.

The freezing point was negatively correlated to the dry weight per disc (Figure 9). Forty-eight percent of its variation is accounted for by the dry weight per disc. From theory it is expected that if some of the increase in dry weight is due to molecules with coligative properties

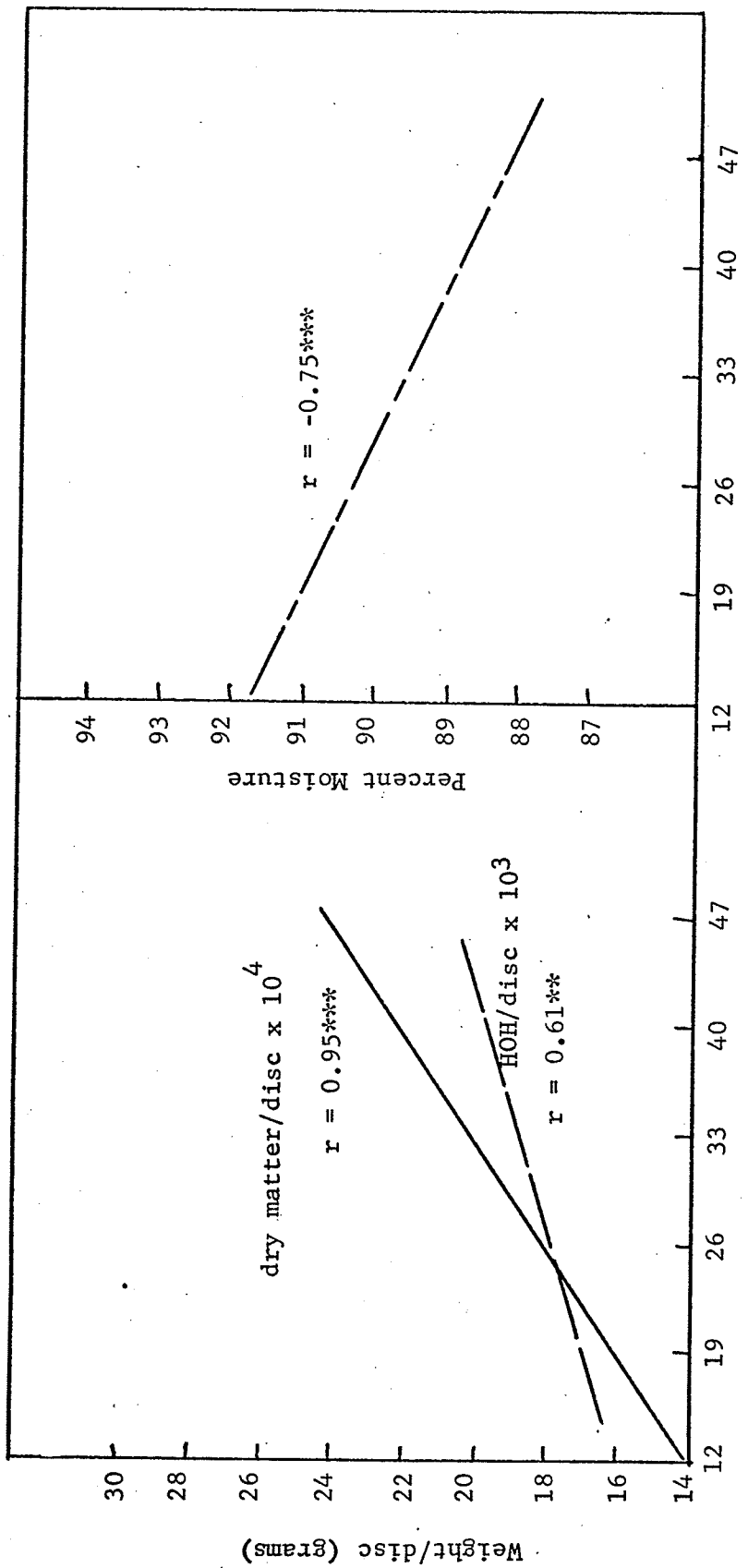


Figure 7. Regression line of percent moisture and weight of dry matter and water per leaf disc vs. age of plant (complete life cycle in greenhouse).

*, ** and *** indicate significance at levels P = 0.10, 0.05 and 0.01, respectively.

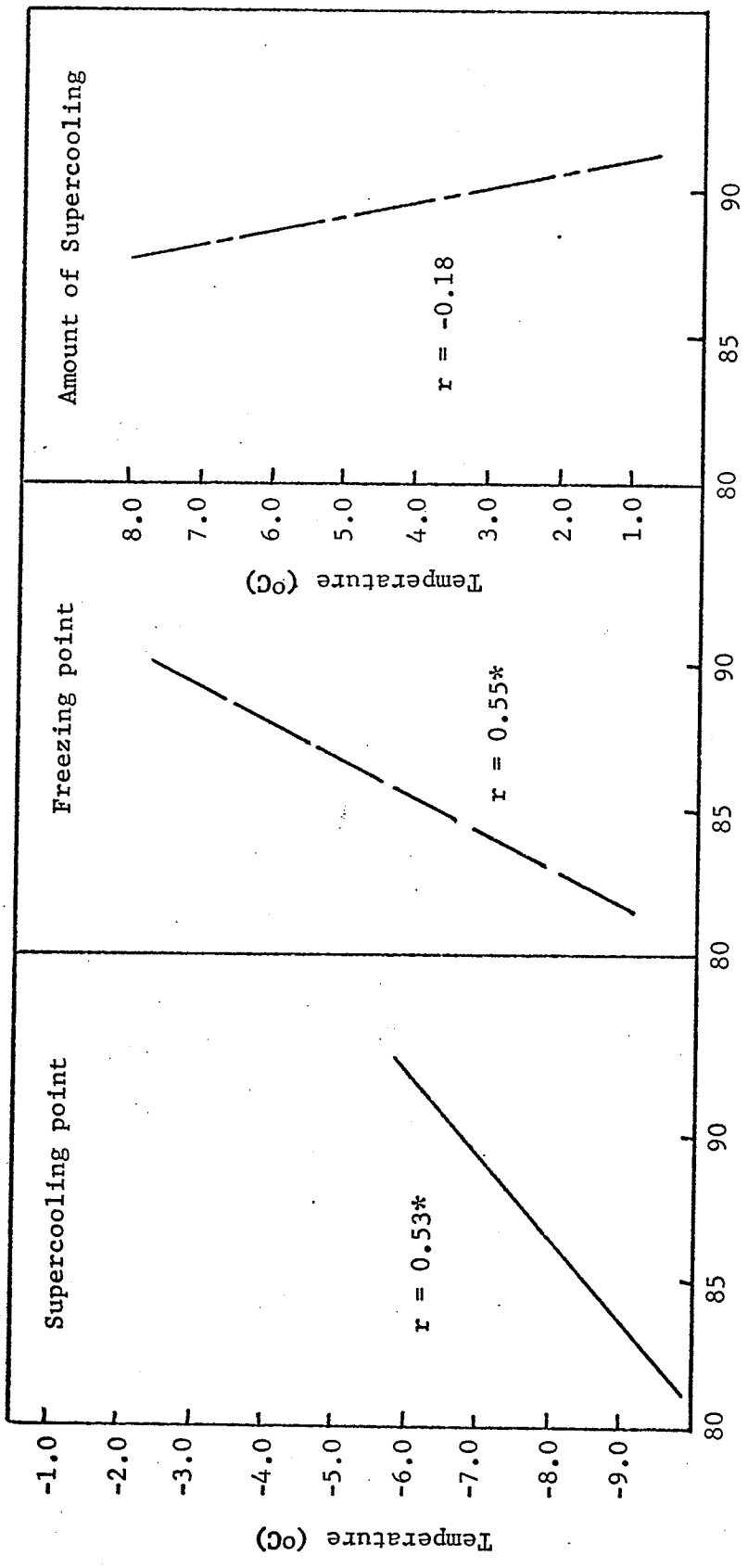


Figure 8 . Regression lines of exotherm values vs. the percent moisture of leaves during the plant's life cycle (germinated in the greenhouse).

*, ** and *** indicate significance at levels P = 0.10, 0.05 and 0.01, respectively.

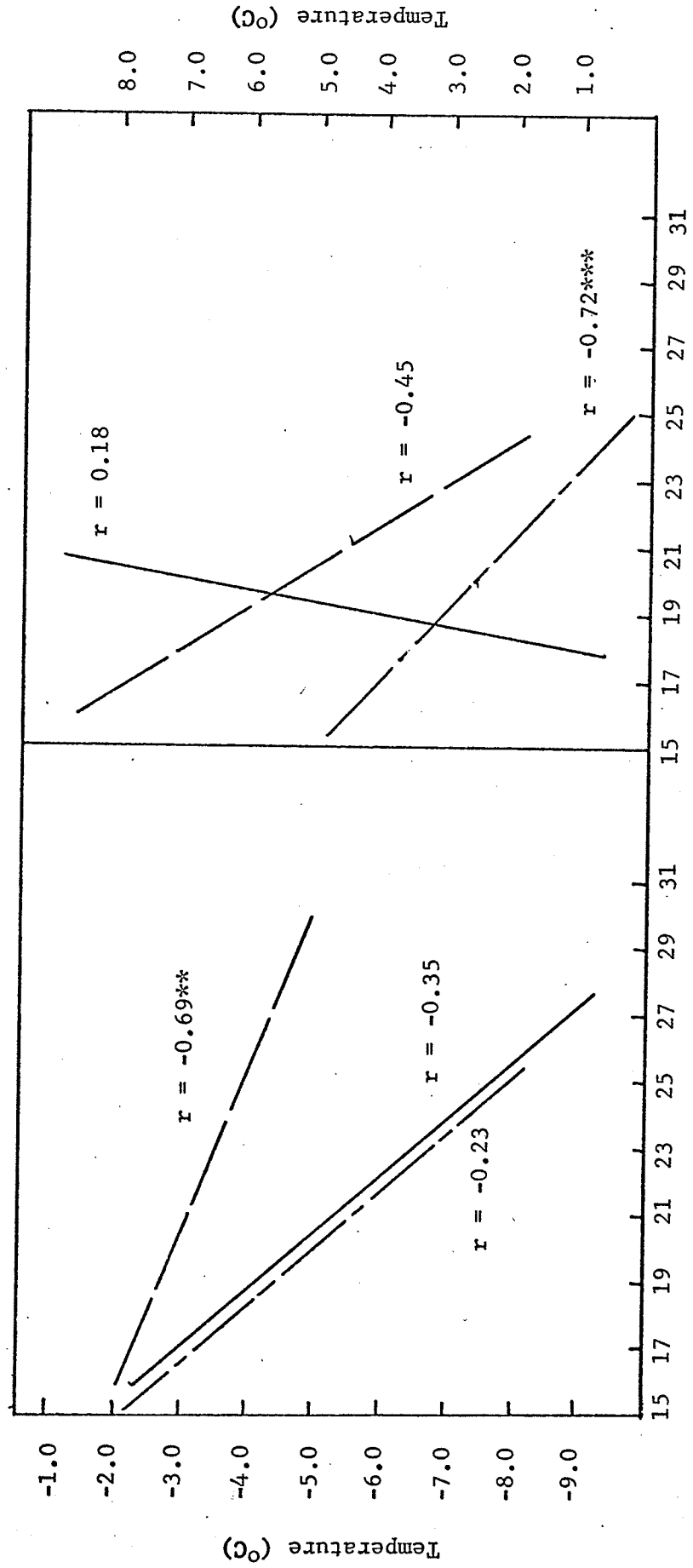


Figure 9 . Regression lines of exotherm values vs. dry weight and weight of water per leaf disc from plants spending their total life cycle in the greenhouse.

Supercooling point ————————; freezing point - - - - - - - - -;

amount of supercooling ——— . ——— . ———

*, ** and *** indicate significance at P = 0.10, 0.05 and 0.01, respectively.

the freezing point will decrease. The decreasing freezing point indicates an increase in such components.

The amount of supercooling was negatively correlated to the weight of water per disc (Figure 9). This agrees with results found by Modlibowski (1962). He found that moisture deficiencies favored supercooling of tender fruit blossoms and leaves.

To compare the average exotherm value for each stage of growth the data were organized into Table 1. The seedling stage was represented by the values for the cotyledons. The rosette stage was represented by the average of the values from 12 and 19 days after germination and the bolted stage by the average of the values from 33 and 40 days after germination. These averages were statistically valid for the supercooling point and freezing point.

Table 1 showed that the supercooling point of the seedling and rosette stages were equal and greater than the supercooling point of the bolted stage. The difference between the rosette and bolted stage was significant only at a significance level of P=0.10.

The freezing point decreased significantly from the seedling to the rosette to the bolted stage (Table 1).

The amount of supercooling was equal for the rosette and bolted stages (Table 1). The amount of supercooling of the seedling stage was greater than for the other two stages.

From these results it was concluded that different morphological stages had different exotherm values. Therefore a chemical analysis

TABLE 1. Effect of stage of growth and temperature treatment on exotherm values of Target rape leaves.

Stage of growth	Treatment (days hardened)	Supercooling Point (°C)	Freezing Point (°C)	Amount of Supercooling (°C)	Percent Moisture
Seedling	0	-6.4±1.7	-2.2±0.4	4.2±1.2	94.2
Rosette	0	-6.5±2.6	-3.0±0.9	3.4±1.7	90.6
Rosette	35	-7.9±1.6	-4.6±0.5	3.4±1.5	80.0
Bolted	0	-7.8±3.4	-4.4±1.6	3.5±1.3	88.2

Values indicate mean and standard deviation. Sample size was 34 for nonhardened plants and 17 for the hardened plants.

TABLE 1a. Comparison of the means in Table 1, using the Student's t test.

Comparison	Supercooling Point	Freezing Point	Amount of Supercooling
Seedling to rosette nonhardened	NS	4.7***	2.2**
Seedling to bolted	NS	5.5***	2.3**
Seedling to rosette hardened	3.0***	15.0***	2.0**
Rosette to bolted	1.7*	4.4***	NS
Rosette nonhardened to hardened	2.0**	6.7***	NS
Bolted to rosette hardened	NS	NS	NS

Values listed are t values.
*, ** and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

of the stages would be justified.

It was also seen that the plants had maximum hardiness in the bolted stage, which may indicate a natural survival mechanism, to withstand late summer frosts. However, the seedling stage had a minimum frost hardiness and is the stage susceptible to late spring frosts. The seedling is small and close to the soil, therefore, it is subject to a lower temperature fluctuation due to conduction of heat from the soil. Thus the seedling may not need as much frost hardiness as more aerial plant parts to survive frosts of short duration.

Life cycle in the greenhouse after germination in the cold room. Seed coating experiments have been underway at the University of Manitoba for several years (Schreiber and LaCroix, 1967). The objective of these experiments is to find a coating, which will prevent the seed of spring crops from imbibing water during the fall when they are planted, but which is destroyed by frost heaving or microorganisms in the spring allowing imbibition and germination. The advantage of this is that growth will begin before the fields are dry enough to support the heavy equipment used for planting. After a late frost in the spring of 1969, it was observed that plants from coated seed planted the previous fall survived, while plants from noncoated seed planted that spring were killed.

We wanted to see if this increased hardiness was due to a cold treatment during germination. To do this, flats of Target seed were planted and put directly into the cold room for treatment. The seed germinated in three weeks as compared to three to four days when in the greenhouse. The seedlings were left in the coldroom for a further nine days and then transferred to the greenhouse. At this time the seedlings were approxi-

mately three inches high and their cotyledons were small and yellow. Weekly exotherm measurements were performed throughout the life cycle of these plants. However in this experiment analysis was terminated before the leaves began to senesce.

The supercooling point of the cotyledons was not significantly different from that of cotyledons of the nontreated plants (Figure 6). The supercooling point of the incompletely expanded true leaves (14 days after transferring to the greenhouse) was significantly lower for the cold treated plants (Figure 6). The completely expanded true leaves of the treated plants had a constant supercooling point regardless of maturity. This was different from the nontreated plants which had a lower supercooling point in the bolted stage. This indicated that the cold treatment during germination had an effect throughout the life cycle of the plant.

The freezing point of the cotyledons of the treated plants was significantly lower than for the nontreated plants (Figure 6). It was equal to the minimum freezing point of cotyledons in the hardening experiment (Figure 1). The incompletely expanded leaves also had a significantly lower freezing point than the corresponding leaves of the nontreated plants (Figure 6). It was also equal to the minimum freezing point of the leaves in the hardening experiment (Figure 1). The decrease in freezing point of fully expanded leaves with maturity, found for plants germinated in the greenhouse was also found for plants germinated in the coldroom (Figure 6).

The pattern of change and magnitude of the amount of supercooling was not significantly different from that found for the plants germinated

in the greenhouse (Figure 6).

Percent moisture, dry weight per disc and weight of water per disc were determined as for the nontreated plants. Correlation coefficients of these to the various exotherm measurements were calculated. The moisture content of cotyledons from treated and nontreated plants was the same. The percent moisture decreased and the dry weight per disc and weight of water per disc increased as was found for the true leaves of the nontreated plants (Figure 10).

The supercooling point of the leaves was negatively correlated to the percent moisture (Figure 11). This was opposite to results from both the untreated plants (germinated in the greenhouse) and the hardening experiment (Table 2).

In the hardening experiment it was concluded that the exotherm values of the cotyledons were independent of the percent moisture. A similar conclusion can be drawn from the data of the life cycle experiments.

The freezing point of the leaves was not correlated to the percent moisture (Figure 11). In both the non-treated plants and the hardening experiment a positive correlation was found (Table 2). Calculation of the correlation coefficient, including values for incompletely expanded leaves gave a positive correlation at a significance level of $P=0.10$. It appeared that the percent moisture had little influence on the freezing point of the incompletely expanded leaves of the treated plants.

The amount of supercooling was positively correlated to the percent moisture (Figure 11). This correlation was not detected in the non-treated plants (germinated in the greenhouse) although the trend of both

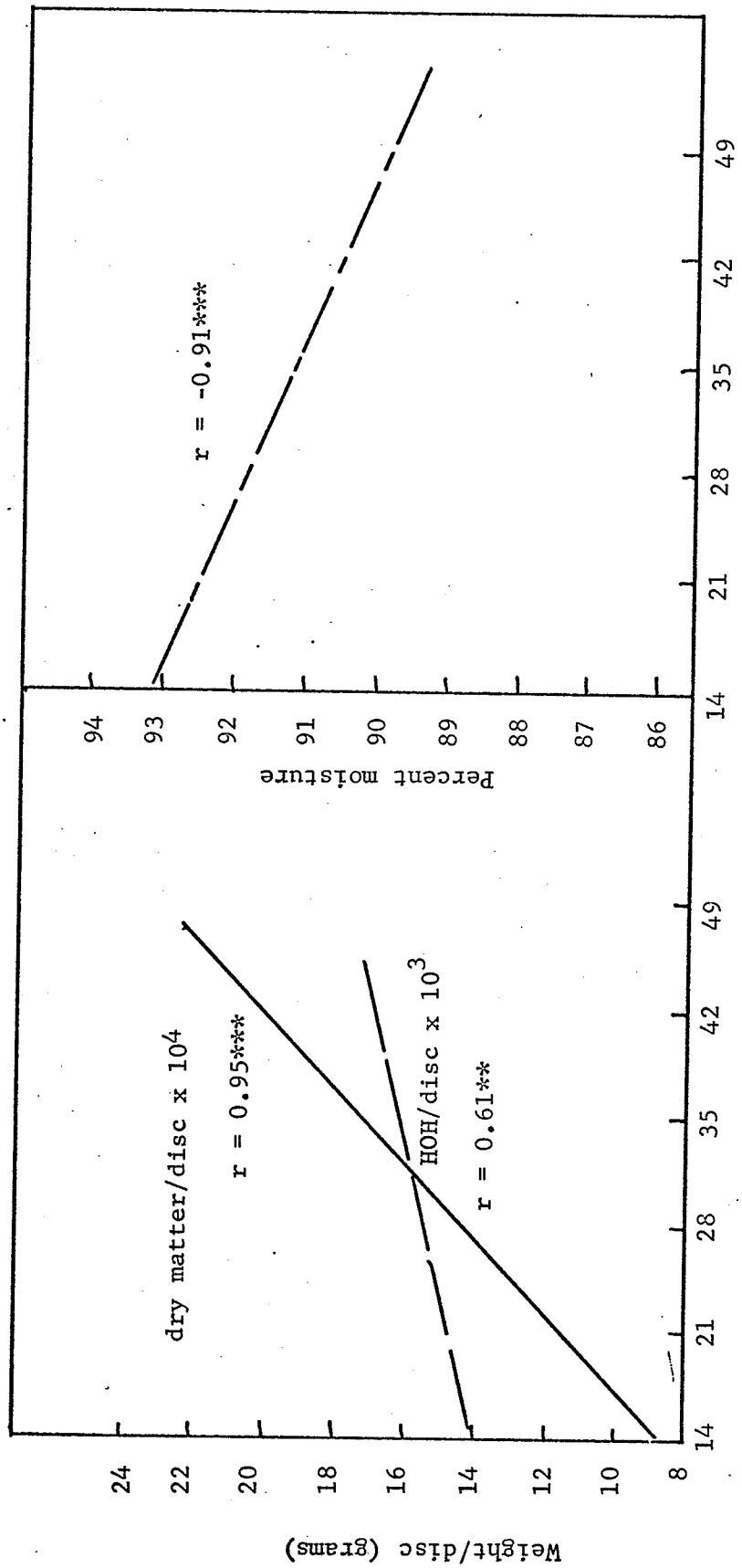


Figure 10. Regression line of percent moisture and weight of dry matter and water per leaf disc vs. age of plant (germinated in the cold room).

*, ** and *** indicate significance at levels P = 0.10, 0.05 and 0.01, respectively.

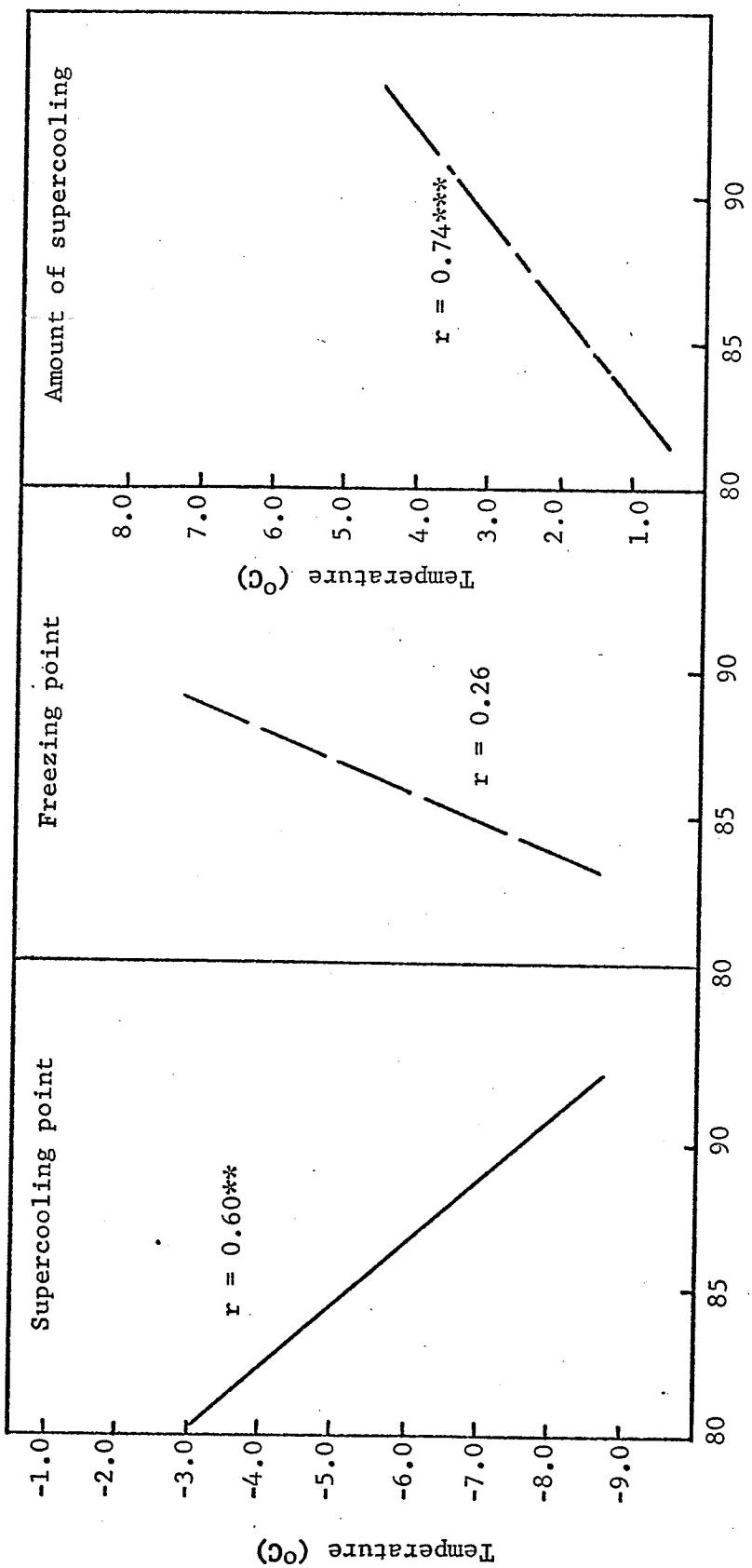


Figure 11. Regression lines of exotherm values vs. the percent moisture of leaves during the plant's life cycle (germinated in cold room).

*, ** and *** indicate significance at levels P = 0.10, 0.05 and 0.01, respectively.

TABLE 2. Comparison of correlation coefficients from all exotherm experiments.

Measurement	Experiment	Measurement		
		Supercooling point	Freezing point	Amount of supercooling
Percent moisture	1a	NS	NS	NS
	1b	+(**)	+(**)	NS
	2a	+(*)	+(*)	NS
	2b	-(**)	NS	+(***)
Dry weight/leaf disc	1b	-(**)	-(***)	-(*)
	2a	NS	-(**)	NS
	2b	+(*)	NS	-(***)
Weight of water/ leaf disc	1b	NS	NS	NS
	2a	NS	NS	-(***)
	2b	NS	NS	-(**)
		Percent moisture	Dry weight/disc	Weight of water/disc
Growth time	1b	-(***)	+(***)	NS
	2a	-(***)	+(***)	+(**)
	2b	-(***)	+(***)	+(**)

Experiment 1a, 1b, 2a and 2b refer to the experiments; hardening of cotyledons, hardening of true leaves, life cycle (germination in the greenhouse) and life cycle (germination in the cold room), respectively.

+ and - indicate a positive or negative correlation.

*, ** and *** indicate significance at levels P = 0.10, 0.05 and 0.01, respectively.

appeared to be similar when one inspects Figure 6. This again indicated that the results from the correlations must be evaluated cautiously.

The supercooling point was positively correlated with dry weight per disc at a significance level of P=0.10 (Figure 12). This correlation did not exist in the nontreated plants (Table 2). In the hardening experiment a negative correlation existed.

The amount of supercooling was negatively correlated to the dry weight per disc (Figure 12). Again this correlation was not found in the non-treated plants (Table 2). However, in the hardening experiment a negative correlation existed.

The amount of supercooling was also negatively correlated with the weight of water per disc (Figure 11). A negative correlation between the amount of supercooling and the weight of water per disc, was also observed for the plants germinated in the greenhouse (Table 2). It was pointed out in the discussion of the plants germinated in the greenhouse, that this negative correlation agrees with the negative correlation reported in the literature.

Genetic influence on the exotherm values of cotyledons of spring rape.

It was desired to see if there is any genetic influence on the exotherm values of seedlings of spring rape. To do this a comparison of the exotherm values for cotyledons of the two cultivars Nugget and Target were made. Nugget germinated more slowly than Target, therefore a chronological comparison may not have been valid. Thus an experiment to observe the effect of chronological age was first performed.

Tables 3 and 3A indicated that for both cultivars, chronological age did not effect the supercooling or freezing points at a significance level

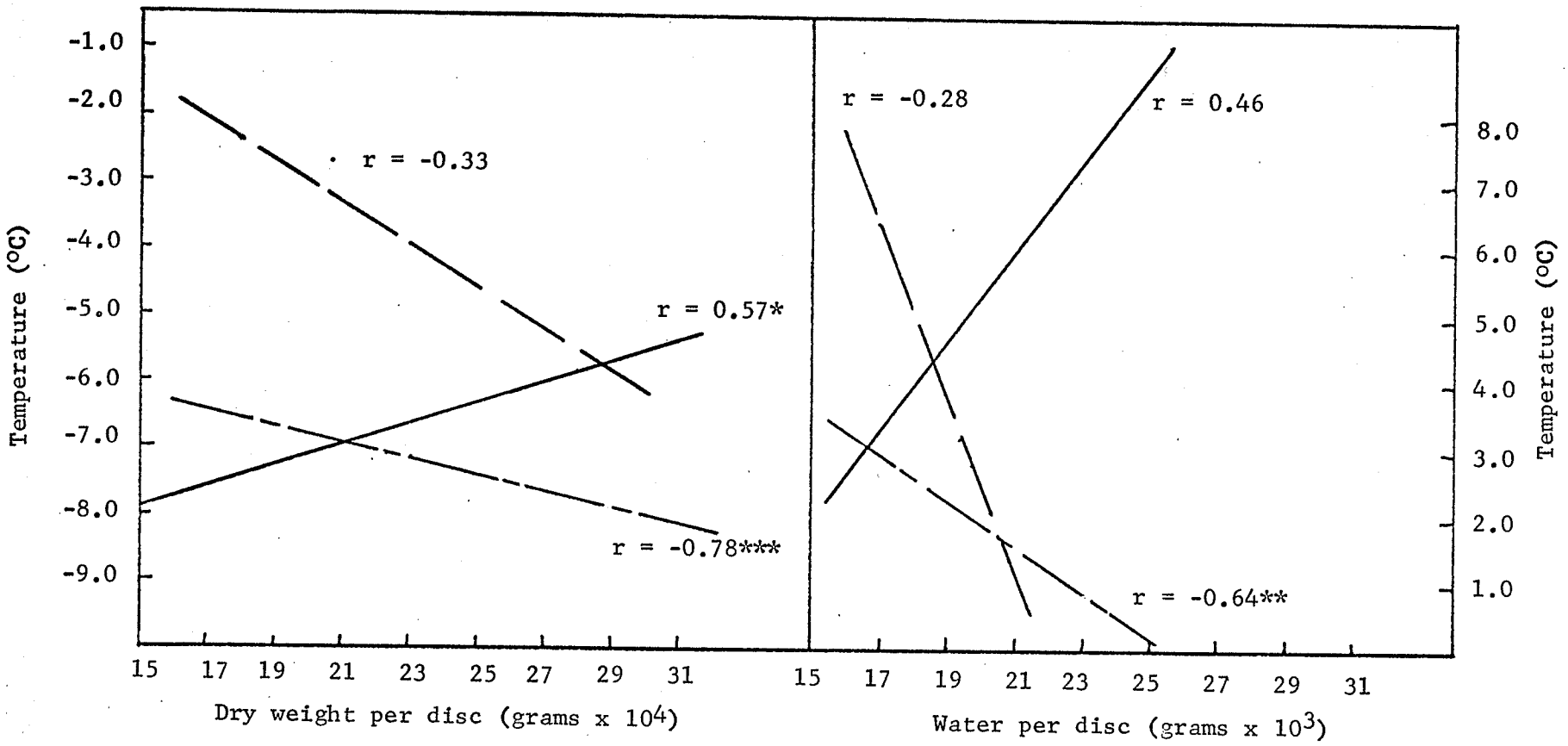


Figure 12. Regression lines of exotherm values vs. dry weight and weight of water per leaf disc from plants germinated in the cold room.

Supercooling point ————————; freezing point - - - - - -;

amount of supercooling ___ . ___ . ___

*, ** and *** indicate significance at P = 0.10, 0.05 and 0.01, respectively.

TABLE 3. Effect of age, cultivar and temperature treatment on exotherm values of rape cotyledons.

Cultivar	Age from Germination (days)	Supercooling Point (°C)	Freezing Point (°C)	Amount of Supercooling (°C)	Percent Moisture	Treatment (Days hardened)
Nugget						
	11	-7.5±1.2	-4.6±1.7	2.9±1.0	92.5	0
	16	-8.2±1.0	-4.0±0.6	4.2±1.0	92.4	0
Average		-7.8±2.1	-4.3±1.8	3.6±1.0	92.4	
Target						
	5	-6.1±1.1	-1.9±0.7	4.1±0.9	94.5	0
	12	-6.8±0.9	-2.4±0.8	4.4±0.7	93.8	0
Average		-6.4±1.7	-2.2±0.4	4.2±1.2	94.2	
	12	-7.2±0.5	-3.2±0.6	4.0±0.5	94.0	4

Values indicate mean and standard deviation. Sample size was 17 for each age and 34 for the averages.

TABLE 3a. Comparison of the means in Table 3 using the Student's t test.

Comparison	Supercooling Point	Freezing Point	Amount of Supercooling
Effect of age of cotyledon			
Nugget	1.8*	NS	3.7***
Target	2.0*	1.9*	NS
Cultivar (averages)	3.9***	6.5***	2.2**
Hardening of Target (to average)	1.9*	6.9***	NS

Values listed are t values. *, **, *** indicate significance at P equal to 0.1, 0.05 and 0.01, respectively.

of P=0.05. Significant differences between cultivars existed at P=0.10, thus putting doubt on conclusive statements. The amount of supercooling in Nugget was significantly different for each chronological age. But because the value of the absolute supercooling point was equal to the sum of the absolute freezing point and the amount of supercooling and because the supercooling point and freezing point were not different, the amount of supercooling should not be different. It was concluded that chronological age did not effect the exotherm values of cotyledons.

The data from both dates were averaged to increase the sample size and thus provide a more accurate estimate for each cultivar. The average values show significant differences between cultivars (Table 3). The supercooling point, freezing point and amount of supercooling were all greater for Target than for Nugget.

Table 3 also showed that a short hardening period of four days was sufficient to decrease the freezing point of Target cotyledons.

Relationship between the exotherm values and frost hardiness.

Ice formation in tender tissue results in death (Olien, 1967). Therefore if one measures the exotherm curve (which indicates ice formation) for a tender plant the lowest temperature reached (the supercooling point) should be related to frost hardiness. This is only true for freezes of short duration since temperature, time and chance influence nucleation in supercooling (Salt, 1966). Thus the greater the amount of supercooling and the longer the exposure time the more likely it is that freezing will occur, causing death. The highest temperature at which ice formation can occur is the freezing point. Supercooling allows the temperature to de-

crease below the freezing point without ice formation. However during long freezes the chance of the plant remaining in the supercooled condition are slight. Therefore the freezing point of tender tissue would indicate its hardiness during a long freeze.

Some preliminary work with a method of evaluating hardiness, similar to the method used by Fuchigami, et al. (1971) was performed. Five detached leaves each in a separate test tube were cooled in a stoppered thermos bottle, in a deep freezer at -25°C. This resulted in a cooling rate of 7.5°C per hour. The temperature within the thermos was measured with a copper constantan thermocouple and recorded with a Honeywell multipoint recorder. The thermos bottles (five per experiment) were removed from the freezer at 2°C intervals, within the temperature range where injury occurred. A slow rewarming rate (6.0°C/hr.) was acquired by letting the thermos bottles warm to room temperature. Twenty five ml of deionized water was added to each test tube containing a single leaf and injury was rated by measuring conductivity before and after killing the leaf with liquid nitrogen.

Injury was related to the ratio, conductivity of leachate before killing with liquid nitrogen/conductivity of leachate after killing with liquid nitrogen X100. In these preliminary experiments nonhardened Target leaves were used. An example of the type of results found is given in Table 4.

These results were interpreted as follows. The leaves cooled to the test temperature -12.0°C were completely killed. Ice formation in each leaf was indicated by the five exotherms. The leaves cooled to the test temperature -8.0°C were either not injured or completely killed. Two of

the leaves cooled to -8.0°C were completely killed and the two exotherms indicated that two leaves had frozen. This suggested that when freezing occurred death resulted. The differential response at -8.0°C may have been due to the randomness of nucleation. It is believed that these results provide evidence that nonhardened rape leaves die when freezing occurs.

Table 4. Percent conductivity of individual leaves cooled slowly to a test temperature.

	Treatment (test temperature)		
	control (not frozen)	-8.0°C	-12.0°C
Leaf of treatment			
1	7	86	93
2	5	7	88
3	3	5	85
4	6	8	93
5	4	90	97
Number of exotherms per thermos	0	2	5

Next, an experiment to determine if hardened leaves of rape die as a result of freezing was performed. The exotherm curve of a single detached leaf was measured with a copper constantan thermocouple and recorded with a Honeywell continuous recorder. A slow freezing rate of 6.0°C per hour was achieved by putting the leaf in a stopperd thermos bottle in a deep freezer at -20°C. The temperature decreased to the supercooling point then rose due to the heat of crystallization. When the temperature again decreased to the value equivalent to the supercooling point, the thermos was removed from the freezer and allowed to rewarm to room temperature. Injury to the leaf was measured by the conductivity method. Eight leaves were frozen and none were injured (Table 5). This

is evidence that hardened leaves can withstand freezing.

Table 5. Effect of freezing on the survival of hardened Target leaves.

Plant	Injury (Percent conductivity) Control Leaf	Frozen Leaf
1	7.3	6.8
2	6.2	6.4
3	23.2	3.6
4	4.8	3.6
5	6.4	7.5
7	4.8	3.1
8	4.9	9.2
Average	8.0	5.8

If fast freezing rates are used during exotherm analysis, the tissue is killed. However when fast freezing was compared to slow freezing (1200°C/hr to 6°C/hr) it was found that the exotherm measurements were not significantly different (Table 6). This justified using a fast freeze so that more samples could be analyzed in a shorter time.

Table 6. Comparison of fast and slow freezing rates on the exotherm measurements.

Tissue	Exotherm Measurement	Slow Freezing (6.0°C/hr) $\bar{x}$	sd	n	Fast Freezing (1200°C/hr) $\bar{x}$	sd	n
Hardened Target Leaves	Supercooling Point	-5.4	1.2	7	-4.2	1.8	10
	Freezing Point	-1.8	0.6	7	-2.6	0.7	10
Hardened Nugget Leaves	Supercooling Point	-9.1	1.8	3	-7.2	1.7	6
	Freezing Point	-4.6	1.2	3	-4.6	0.9	6

Because nonhardened leaves are killed if freezing occurs, the exotherm measurements should be related to frost hardiness. The supercooling point would be related to frost hardiness during moderate freezes of short duration and the freezing point during freezes of long duration.

Because the hardened tissue was not killed by extracellular freezing (indicated by the exotherm), the exotherm values would not be a direct indicator of frost hardiness. However the facts that during hardening the leaves changed so that they could withstand extracellular freezing and that their freezing point decreased indicated that there may be a relationship, probably indirect, between hardiness and freezing point. For example, Young and Peynado (1965) found that the freezing point decreased at a slower rate than hardiness increased during hardening of leaves of grapefruit seedlings. They found no correlation between freezing point of leaves and hardiness of leaves.

Lipid Studies

Effect of a three day hardening treatment on the lipid composition of seedlings of two spring rape cultivars.

Preliminary studies indicated that the hardiness of Target and Nugget seedlings increased after only one day at a hardening temperature. In these studies hardiness was determined by exposing seedlings growing in flats to -4.0°C for eight hours, then visually rating plant injury. Additional studies using exotherm measurements as a measure of hardiness showed that the freezing point of Target cotyledons increased with four days of hardening (Table 3).

Lipid changes, accompanying frost hardiness changes, have been re-

ported in the literature. Theories of frost hardiness based on these lipid changes have also been reported. Thus a study was undertaken to determine whether lipid changes were associated with the apparent increase in hardiness and decrease in freezing point of rape seedlings, with a short hardening treatment. A three day hardening treatment was used and tissue from two cultivars, Nugget and Target, was analyzed.

Comparison of the lipid data for Nugget indicated no significant differences (Tables 7 and 8). Each estimate contained a large variation and so this may have masked any differences that existed. The data for the Target cultivar were less varied (Tables 9 and 10). The large variation of the estimates of the Nugget cultivar may have been due to the experimentor, since during these analyses he was just becoming familiar with the methodology. Another possibility is that the Nugget seed used may have had more genetic variability than the Target seed or the genetic composition of the Nugget may have made it more responsive to micro-environmental changes. It could also have been related to seed ageing, since the Nugget seed was older than the Target seed.

Only a few estimates of lipid quantities are available for the Target cultivar. This was due to a series of silicic acid columns on which the separation of complex lipid from neutral lipid failed to occur. The total lipid was separated into neutral and complex lipid since complex lipid is considered to be one of the better indicators of extent of cell membranes (Tremolieres and Lepage, 1971). The estimates available indicated that some changes in lipid composition occurred (Tables 9 and 10). The quantity of chlorophyll expressed on both the basis of plant tissue and total lipid decreased during hardening. Total lipid also decreased but only at a significance level of $P=0.10$. These results with Target rape indi-

TABLE 7. The lipid composition of seedlings of the spring rape cultivar Nugget, before and after a hardening treatment.

Component	Hardening time (days)		Significance of the Student's t Test
	0	3	
Concentration (g per 100g tissue dry weight)			
TL	10.53±3.24	8.25±0.97	NS
NL	4.36±1.22	3.12±0.39	NS
CL	6.17±2.25	5.13±0.79	NS
Chl	1.12±0.09	1.04±0.14	NS
TL minus chl	9.41±3.20	7.21±0.84	NS
NL minus chl	3.24±1.23	2.08±0.38	NS
NL fatty acids	1.14±0.20	0.70±0.16	NS
CL fatty acids	2.73±0.83	2.28±0.76	NS
Total fatty acids	3.87±1.03	2.98±0.88	NS
Concentration (μ Moles per g tissue dry weight)			
Lipid phosphorus	28.9+7.7	25.1+3.8	NS

Values represent mean and standard deviation of three samples.

*, ** and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

The abbreviations are TL, total lipid; NL, neutral lipid; CL, complex lipid; Chl , chlorophyll.

In this and subsequent tables, differences (for example TL minus Chl) were calculated for each sample. Means and standard deviations were then determined from the sample values.

TABLE 8. Composition of lipid and lipid fractions of seedlings of the spring rape cultivar Nugget, before and after a hardening treatment.

Component	Hardening time (days)		Significance of the Student's t Test
	0	3	
Percent of TL			
NL	41.8±5.5	38.0±4.1	NS
CL	59.2±5.5	62.0±4.1	NS
Chl	11.4±2.2	12.6±0.6	NS
NL minus chl	30.4±6.6	25.4±4.8	NS
Total fatty acid	11.2±1.9	8.6±2.2	NS
Percent of TL minus chl			
Total fatty acid	42.2±6.3	41.0±9.1	NS
μ Moles/g TL			
Lipid phosphorus	277.0±15.8	303.4±15.4	NS
Percent of NL			
Chl	11.4±3.7	12.6±0.6	NS
NL fatty acids	27.1±5.4	22.3±3.5	NS
Percent of NL minus chl			
NL fatty acids	38.5±12.9	33.5±2.4	NS
Percent of CL			
CL fatty acids	44.9±2.9	44.5±12.4	NS
μ Moles/g CL			
Lipid phosphorus	478.6±47.5	489.8±11.3	NS

Values represent mean and standard deviation of three samples.

*, **, and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

TABLE 9. The lipid composition of seedling tissue of the spring rape cultivar Target, before and after a treatment of three days in the coldroom or one day in the greenhouse.

Component	Treatment[A]			Comparison[B]		
	1 Greenhouse (Before treatment)	2 Coldroom (3 days)	3 Greenhouse (1 day)	1 vs 2	1 vs 3	2 vs 3
Concentration (g per 100g tissue dry weight)						
TL	9.50±0.28(3)	8.93±0.25(3)	8.43±0.14(3)	*	***	*
NL	3.47(1)		3.30±0.07(3)	-	-	-
CL	6.34(1)		5.13±0.05(3)	-	-	-
Chl	1.16±0.09(3)	0.96±0.03(3)	1.11±0.09(3)	**	NS	*
TL minus chl	8.34±0.20(3)	7.97±0.24(3)	7.32±0.04(3)	NS	***	**
NL minus chl	2.22(1)		2.19±0.00(3)	-	-	-
NL fatty acids	0.87(1)		0.91±0.15(3)	-	-	-
CL fatty acids	2.23(1)		1.69±0.58(3)	-	-	-
Total fatty acids	3.10(1)		2.60±0.73(3)	-	-	-
Concentration (μ Moles per g tissue dry weight)						
Lipid phosphorus	26.1 ±2.5(3)	24.1(2)	23.3 ±1.2(3)	NS	NS	NS

A Seedlings grown in the greenhouse were analyzed before and after a temperature treatment. One treatment was three days in the coldroom and the other one day in the greenhouse.

B Where possible, the difference between means was tested by the Student's t test.

Values represent mean, standard deviation and sample size, respectively.

*, **, and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

TABLE 10. Composition of lipid and lipid fractions of seedlings of the spring rape cultivar Target, before and after a treatment of three days in the coldroom or one day in the greenhouse.

Component	Treatment[A]		
	Greenhouse (before treatment)	Coldroom (3 days)	Greenhouse (1 day)
Percent of TL			
NL	35.4(1)	-	39.2±0.26(3)
CL	64.6(1)	-	60.8±0.26(3)
Chl	12.2±0.6(3)[A]	10.7±0.3(3)[B]	13.2±0.9 (3)[A]
NL minus chl	22.6(1)	-	26.0±0.7 (3)
Total fatty acid	8.9(1)	-	10.8±1.9 (3)
Percent of TL minus chl			
Total fatty acid	36.2(1)	-	35.6+10.0(3)
μ Moles/g TL			
Lipid phosphorus	274.5±18.3(3)[A]	270.8	276.4±12.8(3)[A]
Percent of NL			
Chl	33.4(1)	-	33.6±2.1 (3)
NL fatty acids	25.1(1)	-	27.7±4.9 (3)
Percent of NL minus chl			
NL fatty acids	39.2(1)	-	41.5±6.7 (3)
Percent of CL			
CL fatty acids	35.2(1)	-	33.0±11.4(3)
μ Moles/g CL			
Lipid phosphorus	457.4(1)	-	454.3±22.5(3)

A Seedlings grown in the greenhouse were analyzed before and after a temperature treatment. One treatment was three days in the coldroom and the other one day in the greenhouse.

Values represent mean, standard deviation and sample size, respectively.

Means with different superscripts are significantly different at the level P = 0.05.

cated that lipid changes can occur within a short hardening period sufficient to increase the freezing point.

Experiments involving analyses of plants grown for a constant time period at different temperatures have been reported in the literature. The lipid differences found may represent adaptive changes which caused an increase in hardiness; or the lipid difference may be due to the different physiological age of the plants caused by the different rates of growth at different temperatures. Thus the corresponding changes in hardiness and lipids may not indicate a casual relationship. To try and determine whether a causal relationship exists, Target seedlings grown in the greenhouse for one extra day were analyzed. This gave a comparison between lipid change during one day in the greenhouse and lipid change during three days in the cold room. If the Q10 was two (my assumption) and with a 20°C temperature differential, then changes occurring during three days in the cold room should be equivalent to changes during one day in the greenhouse. If the application of QI0 does not hold then the change in the lipid measurements should represent an adaptive change in response to the low temperature. To allow for misinterpretation due to choosing the wrong QI0 the trends must be considered and not the absolute changes. The weight of total lipid and total lipid minus chlorophyll per gram dry weight of tissue decreased significantly during the one day treatment in the greenhouse (Table 9). Neither decreased significantly during the three day cold treatment at P=0.05. In contrast the quantity of chlorophyll per gram of tissue decreased significantly during the cold treatment, but not during the greenhouse treatment. It appeared that the

plants under the two conditions had different synthetic or degradation rates not due simply to the thermodynamic influence of temperature. The fact that the response for chlorophyll is opposite to that for total lipid eliminated negation of this conclusion due to the choice of a wrong QI0. Thus the change in lipid metabolism at lower temperatures may be related to the decrease in freezing point. The change in lipid after only one day in the greenhouse apparently does not affect the freezing point since the freezing point did not change with time for plants at constant temperature (Table 3).

The weight of total lipid and total lipid minus chlorophyll per gram of tissue (dry weight) decreased significantly within one day at greenhouse temperatures, showing that the lipid composition is in a very dynamic state. Thus, a single analysis, say of the seedling, may not be representative of the seedling stage of growth.

The lipid composition of seedlings of two spring rape cultivars with different exotherm values.

Nugget cotyledons have a lower supercooling point, freezing point and degree of supercooling than Target cotyledons (Table 3). Thus, if lipids are involved in controlling any of these, lipid differences should exist between the two cultivars. Data from Tables 7, 8, 9 and 10 were used for the comparison. Since there were no significant difference between the two sets of data for Nugget (Tables 7 and 8) they were combined to increase the sample size. This combined average was compared to the Target which had received a one day treatment in the greenhouse (Tables 9 and 10). This was considered a valid comparison because Nugget germinates slower than Target thus invalidating a chronological comparison.

No significant differences between cultivars were found.

Changes in fatty acid composition of alfalfa roots have been observed during hardening (Gerloff, et al. 1966). Therefore the fatty acid composition of each lipid fraction was determined by gas liquid chromatography of fatty acid methyl esters. Peak area was determined by the triangulation method. The relative quantity of each component (based on peak area) was expressed as a percent of total fatty acids. The degree of unsaturation was expressed as mole percent unsaturated fatty acid or as the double bond index. The double bond index is, "the sumation of weight percent of each acid in a mixture multiplied by the number of double bonds it contains per molecule, divided by 100," (Lyons and Asmundson, 1965).

Comparison of the fatty acid data did show differences between the cultivars. The complex lipid fraction of the Nugget seedlings had more 16:0 and less 16:3 fatty acids than Target (Table 11). No differences in the mole percent unsaturated fatty acid or double bond index of the complex lipid fractions were found (Table 12).

There also were differences in the neutral lipid fraction. The fatty acids of the neutral lipid fraction of Nugget had less 16:3, 18:0 and 18:3 and more 20:0 than Target (Table 13). The Nugget sample had an unidentified peak with a retention time very similar to the 18:3 component (Z, Table 13). This component may be present in the 18:3 peak of Target and if it is, then the amount of 18:3 in the two cultivars would not be different. There was no significant difference in the mole percent unsaturated fatty acid of the neutral lipid fraction between the two cultivars. Target

TABLE 11. Fatty acid composition of the complex lipid fraction of seedlings of two cultivars of spring rape.

Fatty acid	Cultivar		Significance of the Student's t Test
	Target	Nugget	
16:0	15.7±1.2	20.0±2.3	**
16:1	2.8±0.2	2.8±0.9	NS
16:2	4.0±0.4	4.3±0.7	NS
16:3	2.9±0.3	1.4±0.6	**
x	2.4±0.9	2.3±0.8	NS
18:0	9.1±2.2	7.2±1.8	NS
18:1	5.9±1.8	6.0±2.3	NS
18:2	14.5±1.6	14.5±1.8	NS
18:3	42.6±3.5	41.5±6.0	NS

Values indicate the mean percent fatty acid composition and standard deviation.

The sample size for Target was three and for Nugget four.

*, ** and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

TABLE 12. The unsaturation status of the complex lipid fatty acids of two cultivars of spring rape.

Measurement	Cultivar		Significance of the Student's t test
	Target	Nugget	
Mole percent unsaturation	74.5±1.6	72.0±0.8	NS
Double bond index	1.8±0.0	1.8±0.1	NS
Ratio of fatty acids			
mono-unsaturated/ saturated	0.4±0.1	0.3±0.1	
di-unsaturated/ saturated	0.8±0.1	0.7±0.1	
tri-unsaturated/ saturated	1.8±0.1	1.6±0.2	

Values are the mean and standard deviation. The sample size for Target was three and for Nugget four.

*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

TABLE 13. Fatty acid composition of the neutral lipid fraction of seedlings of two cultivars of spring rape.

Fatty acid	Cultivar		Significance of the Student's t Test
	Target	Nugget	
14:0	1.2±0.3	1.1±0.4	NS
16:0	8.1±1.0	7.0±0.9	NS
16:1	2.7±0.0	2.6±0.4	NS
16:3	1.4±0.3	0.0	***
x	2.4±0.5	3.1±0.8	NS
18:0	12.7±2.6	6.7±1.0	***
18:1	13.7±2.5	13.2±1.7	NS
18:2	10.2±0.9	11.8±1.4	NS
18:3	42.1±4.0	31.5±4.1	**
z	0.0	8.5±0.6	***
20:0	4.7±2.0	14.5±2.6	***

Values indicate the mean percent fatty acid composition and standard deviation.

The sample size for Target was three and for Nugget five.

*, ** and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

however, had a significantly higher double bond index than Nugget (Table 14). This also may be due to the unidentified peak Z.

The effect of a long hardening period on the lipid composition of leaves of target spring rape.

Membranes are considered by many to be the site of frost injury. Siminovitch, et al.(1968) used lipid phosphorus as a quantitative measure of membranes. They compared the lipid composition of bark of black locust trees harvested in winter and summer. They found that total lipid expressed on a dry weight basis changed very little from summer to winter. However, the polar lipids and lipid phosphorus expressed on a wet weight basis were significantly greater in the winter. The increase in polar lipids was accompanied by a decrease in nonpolar lipids. Lipid phosphorus also increased when expressed on a dry weight basis.

Kuiper (1969) found that leaves of frost resistant citrus cultivars had more phospholipid and less neutral lipid expressed as a percent of total lipid than susceptible cultivars. He found no consistent relationship of percent glycolipids to frost resistance. However, studying alfalfa leaves, Kuiper (1970) found that leaves grown at lower temperatures had more mono- and di-galactosyl diglyceride.

An experiment was performed to see if hardening had the same effect on the lipid composition of leaves of Target spring rape. Plants were grown in the greenhouse for four weeks. At this time they were in the rosette stage of growth. Samples were taken for lipid analysis and the rest of the plants were transferred to the cold room. Sampling for lipid analysis was carried out after 35 days of hardening.

TABLE 14. The unsaturation status of the neutral lipid fatty acids of two cultivars of spring rape.

Measurement	Cultivar		Significance of the Student's t test
	Target	Nugget	
Mole percent unsaturation	72.2±0.0	69.5±2.8	NS
Double bond index	1.6±0.2	1.2±0.2	**
Ratio of fatty acids			
mono-unsaturated/ saturated	0.6±0.1	0.5±0.1	
di-unsaturated/ saturated	0.4±0.0	0.4±0.1	
tri-unsaturated/ saturated	1.6±0.2	1.4±0.2	

Values are the mean and standard deviation. The sample size for Target was three and for Nugget five.

*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

Complex lipids and lipid phosphorus expressed on a dry weight basis were greater in the hardened leaves (Table 15). Neutral lipid minus chlorophyll of the hardened plants was less at a significance level of P=0.10. If this is a true decrease it agrees with the decrease in neutral lipid found by Siminovitch, et al. (1968). During hardening there was a significant decrease of lipid hexose (a measure of glycolipids) expressed on a dry weight basis (Table 15). This is opposite to results found by Kuiper (1970) for alfalfa leaves.

According to Tremolieres and Lepage (1971) chloroplast membranes are characterized by large amounts of glycolipid whereas extrachlorophlastic membranes are characterized by large amounts of phospholipid. Thus the decrease in lipid hexose and the increase in lipid phosphorus (Table 15) may indicate that the membranes associated with the photosynthetic apparatus decreased while those associated with the nonphotosynthetic apparatus increased on hardening. In this experiment the decrease in photosynthetic membranes could be a result of the low light intensity in the cold room. Kuiper (1970) who found an increase of glycolipid in alfalfa leaves during hardening, maintained a constant light intensity at all growing temperatures.

There were no differences in chlorophyll content of hardened and nonhardened leaves (Table 15). The change in lipid hexose and lipid phosphorus in the absence of chlorophyll changes, suggested that the type of photosynthetic membranes may have changed and not the quantity. Godnev, et al. (1969, cited by Alden and Hermann, 1971) found that the chlorophyll content was retained during the winter, in two varieties of Picea.

Neutral lipid, expressed as a percent of total lipid, decreased and

TABLE 15. The lipid composition of hardened and nonhardened leaves of the spring rape cultivar Target in the rosette stage of growth.

Component	Hardening time (days)		Comparison (Student's t test)		
	0	35	Nonhardened to hardened	Rosette hardened to seedling[A]	Rosette hardened to bolted[A]
Concentration (g per 100g tissue dry weight)					
TL	11.03±0.22(3)	11.56±0.31(3)	NS	***	**
NL	4.10±0.08(3)	4.28±0.46(4)	NS	**	**
CL	6.93±0.14(3)	7.51±0.16(3)	**	***	NS
Chl	1.66±0.04(4)	1.67±0.03(4)	NS	***	NS
TL minus chl	9.35±0.23(3)	9.87±0.33(3)	NS	***	**
NL minus chl	2.42±0.10(3)	2.24±0.07(3)	*	NS	***
NL fatty acids	0.62±0.10(3)	0.73±0.10(4)	NS	NS	NS
CL fatty acids	2.77±0.13(3)	2.74±0.82(4)	NS	NS	**
Total fatty acids	3.39±0.06(3)	3.46±0.91(4)	NS	NS	**
Concentration (μ Moles per g tissue dry weight)					
Lipid phosphorus	27.9±14.(3)	31.9±0.3(3)	**	*	NS
Lipid hexose	110.0±5.5(3)	91.5±3.5(3)	**	**	**
Ratio $\frac{\text{lipid Hexose}}{\text{lipid phosphorus}}$	3.9±0.1(3)	2.8±0.1(4)	***	NS	***

A See Table 19 for data used in these comparisons.

Values indicate mean, standard deviation and sample size, respectively.

*, **, and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

the complex lipid increased during hardening (Table 16). The molar phosphorus content of the total lipid also increased (Table 16). These changes agree with Kuiper's (1969) results on citrus leaves. A decrease in the molar hexose content of total lipid was found (Table 16) contrary to Kuiper's (1970) findings for alfalfa leaves.

Changes in the composition of the complex lipid fraction, as a result of the hardening treatment, were observed (Table 16). The molar concentration of phosphorus remained the same in this fraction, but the molar concentration of hexose decreased. An increase in some other component may have compensated for the decrease of glycolipid. The decrease of hexose could indicate a change from digalactosyl to monogolastosyl diglyceride, a change contrary to the results reported by Kuiper (1970).

The quantity of unsaturated fatty acids generally increases at lower growing temperatures (Gerloff, et al. 1966; Harris and James, 1969; Kuiper, 1970). Lyons and Asmundson (1965), suggest how a greater mole percent unsaturation may cause chilling resistance. The metabolism of chilling sensitive plants, at temperatures between 0°C and 12.5°C, usually results in lethal physiological changes (Greencia and Bramlage, 1971). Chilling resistance involves being able to metabolize without lethal consequences at temperatures between 0°C and 12.5°C. Plants that can withstand frost, harden within this temperature range. Therefore the increase in unsaturation of fatty acids of plants capable of hardening may function similarly to chilling resistance. That is the increase in unsaturated fatty acids may allow the cell to function between 0°C and 12.5°C and thus undergo the chemical changes required to withstand extracellular

TABLE 16. The composition of lipid and lipid fractions of hardened and nonhardened leaves of the spring rape cultivar Target in the rosette stage of growth.

Component	Hardening time (days)		Comparison (Student's t test)		
	0	35	Rosette hardened to nonhardened	Rosette hardened to seedling[A]	Rosette hardened to bolted[A]
Percent of TL					
NL	37.2±0.3(3)	34.7±1.0(4)	**	***	***
CL	62.8±0.3(3)	65.3±1.0(4)	**	***	***
Chl	15.3±0.4(3)	14.6±0.6(3)	NS	NS	*
NL minus chl	21.9±0.5(3)	21.0±1.3	NS	***	***
Total fatty acid	30.7±0.5(3)	27.7±3.7(4)	NS	NS	***
Percent of TL minus chl					
Total fatty acid	36.3±0.8(3)	32.0±3.7(4)	NS	NS	***
μ Moles/g TL					
Lipid phosphorus	253.1±7.9(3)	275.4±7.2(4)	**	*	***
Lipid hexose	996.6±29.9(3)	791.4±9.6(3)	***	NS	**
Percent of NL					
Chl	41.1±1.1(3)	41.6±2.2(3)	NS	**	***
NL fatty acids	15.2±2.4(3)	17.0±1.4(4)	NS	NS	**
Percent of NL minus chl					
NL fatty acid	25.6±3.6(3)	29.5±4.8(4)	NS	*	**
Percent of CL					
CL fatty acids	39.9±2.0(3)	33.4±5.2(4)	NS	NS	***
μ Moles/g CL					
Lipid phosphorus	402.8±13.6(3)	421.5±11.0(4)	NS	**	**
Lipid hexose	1585.9±44.9(3)	1193.6±55.2(4)	***	*	***

A See Table 20 for data used in these comparisons.

Values indicate mean, standard deviation and sample size, respectively.

*, **, and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

freezing. On the other hand, the unsaturation of the fatty acids may aid the cell directly in withstanding frost, possibly by affecting the physical properties of the membranes, enabling them to withstand the forces developed during extracellular freezing.

Analyses of the fatty acid composition of the neutral and complex lipid fraction of spring rape were performed in an attempt to relate these to exotherm measurements. The lipid fractions were analyzed separately because the complex lipid fraction is considered more indicative of the membranes (Tremolieres and Lepage, 1971) and thus should be a more sensitive measurement of membrane changes.

The complex lipid fraction of the hardened leaves had more 16:0, 18:1 and 18:2 and less 16:2, 16:3 and 18:3 fatty acid than the nonhardened leaves (Table 17). The neutral lipid fraction of the hardened leaves had more 16:0, 18:0 and 18:1 and less 16:2, x and y fatty acid than the nonhardened leaves (Table 18). X and y were unidentified peaks on the chromatogram.

Contrary to the observations reported, a reduction in the mole percent unsaturated fatty acid occurred in both the neutral and complex lipid fractions during hardening (Tables 19 and 20). The double bond index was the same for both fractions. In the neutral lipid fraction the presence of unidentified peaks may make the comparison meaningless. However in the complex lipid fraction the unidentified fatty acids accounted for less than one percent of the total composition. Therefore a meaningful comparison should be possible for the complex lipid fraction. The mole percent unsaturated fatty acid content of both lipid fractions of the hardened plants remained within the critical range (60-80%) found for chilling

TABLE 17. Fatty acid composition of the complex lipid fraction of hardened and nonhardened leaves of Target rape.

Fatty acid	Hardening time (days)		Comparison (Student's t test)		
	0	35	Nonhardened to hardened	Hardened to seedling[A]	Hardened to bolted[A]
16:0	7.3±0.3	10.4±0.6	***	**	NS
16:1	0.0	0.0	NS	***	NS
16:2	2.0±0.1	1.4±0.1	***	***	***
16:3	0.5±0.0	0.0	***	***	NS
x	0.3±0.1	0.6±0.0	NS	***	NS
18:0	19.9±1.6	19.0±0.3	NS	***	*
18:1	1.2±0.2	1.5±0.1	**	***	*
18:2	6.7±0.4	7.8±0.3	**	***	NS
18:3	62.2±1.1	59.2±1.0	**	***	NS

A See Table 21 for data used in these comparisons.

Values indicate mean percent fatty acid composition and standard deviation. The sample size for the nonhardened plants was three and for the hardened plants four.

*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

TABLE 18. Fatty acid composition of the neutral lipid fraction of hardened and nonhardened leaves of Target rape.

Fatty acid	Hardening time (days)		Comparison (Student's t test)		
	0	35	Nonhardened to hardened	Hardened to seedling[A]	Hardened to rosette[A]
14:0	1.6±0.2	1.6±0.3	NS	NS	NS
16:0	10.7±0.9	12.3±0.8	*	***	***
16:1	1.5±0.2	1.6±0.5	NS	**	**
16:2	5.7±0.6	3.6±0.7	**	***	**
16:3	0.0	0.0	NS	***	NS
x	3.6±0.4	0.0	***	***	NS
18:0	14.8±1.8	19.6±0.2	***	***	NS
18:1	4.5±0.2	6.0±1.1	*	***	***
y	1.6±0.3	0.0	***	NS	NS
18:2	9.3±0.5	9.2±0.6	NS	NS	***
18:3	46.4±1.2	46.2±2.5	NS	NS	***
20.0	0.0	0.0	NS	***	NS

A See Table 22 for data used in these comparisons.

Values indicate mean percent fatty acid composition and standard deviation. The sample size for the nonhardened plants was three and for the hardened plants four.

*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

TABLE 19. Effect of stage of growth and hardening treatment on the degree of unsaturation of the fatty acids of the neutral lipid fraction.

Growth Stage	Mole percent unsaturation	Double bond index	Ratio of fatty acids mono-unsaturated/saturated di-unsaturated/saturated tri-unsaturated/saturated
1. Seedling	72.2±0.0	1.6±0.2	0.6±0.1 0.4±0.0 1.6±0.2
2. Rosette	71.1±1.3	1.8±0.1	0.2±0.0 0.5±0.0 1.7±0.1
3. Bolted	68.3±2.6	1.9±0.1	0.1±0.0 0.3±0.0 1.8±0.2
4. Rosette hardened	66.6±1.6	1.7±0.1	0.3±0.0 0.4±0.0 1.4±0.1
Comparison (Student's t test)			
1 vs 2	NS	NS	
1 vs 3	*	NS	
1 vs 4	***	NS	
2 vs 3	NS	NS	
2 vs 4	**	NS	
3 vs 4	NS	*	

Values indicate mean and standard deviation. Sample size was three for all stages except the hardened rosette stage which was four.

*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

TABLE 20. Effect of stage of growth and hardening treatment on the degree of unsaturation of the fatty acids of the complex lipid fraction.

Growth Stage	Mole percent unsaturation	Double bond index	Ratio of fatty acids mono-unsaturated/saturated di-unsaturated/saturated tri-unsaturated/saturated
1. Seedling	74.5±1.6	1.8±0.0	0.3±0.0 0.7±0.1 1.9±0.1
2. Rosette	72.6±1.0	2.0±0.0	0.0 0.3±0.0 2.3±0.1
3. Bolted	70.5±2.0	2.0±0.0	0.0 0.4±0.0 2.0±0.2
4. Rosette hardened	70.5±0.7	2.0±0.0	0.0 0.3±0.0 2.0±0.1
Comparison (Student's t test)			
1 vs 2	NS	***	
1 vs 3	*	***	
1 vs 4	***	***	
2 vs 3	NS	NS	
2 vs 4	**	NS	
3 vs 4	NS	NS	

Values indicate mean and standard deviation. Sample size of the seedling and nonhardened rosette was three and of the bolted and hardened rosette four.

*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

resistant species, Lyons and Asmundson (1965). Thus the decrease of mole percent unsaturated fatty acid may not adversely affect the membrane functions.

In conclusion, these results agree with the theory involving an increase in cell membranes during hardening.

Leaf lipid composition of three morphological growth stages of spring rape as related to exotherm measurements.

After the seedling stage, spring rape grows in the rosette form for several weeks. The leaves appear to mature during this stage and flower bud initiation occurs. This is followed by bolting which involves stem elongation. Indeterminate flowering then occurs in the bolted stage. Young strap-shaped leaves grow after bolting.

The leaves of these three growth stages have different functions, which may be reflected in different leaf chemical composition. These chemical differences may be related to the different exotherm values of the growth stages. Table 1, indicated that the freezing point decreased from the seedling to the rosette to the bolted stage. The supercooling point was equal for all growth stages and the amount of supercooling was greatest for the cotyledon and equal in the other two stages. The lipid results are organized into Tables 21 and 22. Data for seedlings may be of limited value for purposes of comparison because the total seedling was analyzed for lipids. However, I made the assumption that the composition of the seedlings represents the composition of the cotyledons.

Total lipid, complex lipid and neutral lipid per gram dry weight of tissue increased from the seedling to the rosette to the bolted stage (Table 21). The leaves of the rosette and bolted stage contained equal

TABLE 21. The lipid composition of leaf tissue of the spring rape cultivar Target during different morphological stages of growth.

Component	Growth stage			Comparison (Student's t test)		
	1 Seedling[A]	2 Rosette	3 Bolted	1 vs 2	1 vs 3	2 vs 3
Concentration (g per 100g tissue dry weight)						
TL	8.43±0.14(3)	11.03±0.22(3)	12.77±0.36(3)	***	***	***
NL	3.30±0.07(3)	4.10±0.08(3)	5.31±0.39(4)	***	***	**
CL	5.13±0.05(3)	6.93±0.14(3)	7.64±0.19(3)	***	***	**
Chl	1.11±0.09(3)	1.66±0.04(4)	1.73±0.09(4)	***	***	NS
TL minus chl	7.32±0.04(3)	9.35±0.23(3)	11.05±0.25(3)	***	***	***
NL minus chl	2.19±0.00(3)	2.42±0.10(3)	3.41±0.07(3)	**	***	***
NL fatty acids	0.91±0.15(3)	0.62±0.10(3)	0.56±0.22	*	NS	NS
CL fatty acids	1.69±0.58(3)	2.77±0.13(3)	0.72±0.07(4)	*	**	***
Total fatty acids	2.60±0.73(3)	3.39±0.06(3)	1.25±0.17(3)	NS	**	***
Concentration (μ Moles per g tissue dry weight)						
Lipid phosphorus	26.5±2.8(3)	27.9±1.4(3)	30.2±2.4(4)	NS	NS	NS
Lipid hexose	71.2±6.9(3)	110.0±5.5(3)	108.3±4.8(3)	***	***	NS
Ratio $\frac{\text{hexose}}{\text{phosphorus}}$	2.7±0.5(3)	3.9±0.1(3)	3.8±0.3(4)	**	**	NS

A The total seedling was analyzed, however, for this comparison I assume that it represents the cotyledons, only.

The values indicate mean, standard deviation and sample size, respectively.

*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

TABLE 22. The composition of lipid and lipid fractions of the leaves of three morphological stages of growth of the spring rape cultivar Target.

Component	Growth stage			Comparison (Student's t test)		
	1 Seedling[A]	2 Rosette	3 Bolted	1 vs 2	1 vs 3	2 vs 3
Percent of TL						
NL	39.2±0.3(3)	37.2±0.3(3)	39.7±0.9(4)	***	NS	**
CL	60.8±0.3(3)	62.8±0.3(3)	60.3±0.9(4)	***	NS	**
Chl	13.2±0.9(3)	15.3±0.4(3)	13.0±1.0(4)	**	NS	**
NL minus chl	26.0±0.7(3)	21.9±0.5(3)	26.7±0.4(4)	***	NS	***
Total fatty acid	30.9±8.8(3)	30.7±0.5(3)	4.0±1.2(3)	NS	***	***
Percent of TL minus chl						
Total fatty acid	35.6±10.0(3)	36.3±0.8(3)	10.6±1.0(3)	NS	**	***
μ Moles/g TL						
Lipid phosphorus	314.2±29.8(3)	253.1±8.9(3)	226.1±12.4(4)	**	***	**
Lipid hexose	845.0±91.4(3)	996.6±29.9(3)	853.6±25.4(4)	**	NS	***
Percent of NL						
Chl	33.6±2.1(3)	41.1±1.1(3)	32.7±1.9(4)	**	NS	***
NL fatty acids	27.7±4.9(3)	15.2±2.4(3)	10.3±3.2(3)	***	***	NS
Percent of NL minus chl						
NL fatty acids	41.5±6.7(3)	25.6±3.6(3)	15.4±4.2(3)	**	***	*
Percent of CL						
CL fatty acids	33.0±11.4(3)	39.9±2.0(3)	9.9±0.7(3)	NS	NS	**
μ Moles/g CL						
Lipid phosphorus	516.7±51.2(3)	402.8±13.6(3)	375.2±23.1(4)	**	***	NS
Lipid hexose	1388.9±144.4(3)	1585.9±44.9(3)	1415.9±37.3(4)	NS	NS	***

A The total seedling was analyzed, however, for this comparison I assume that it represents the cotyledons, only.

The values indicate mean, standard deviation and sample size, respectively.
*, **, and *** indicate significance at P equal to 0.10, 0.05, and 0.01, respectively.

amounts of lipid hexose and chlorophyll per gram dry weight of tissue and significantly more of these than the seedlings. The lipid molar phosphorus content of the tissue was the same at all growth stages. The total fatty acid content and complex lipid fatty acid content (calculated on a dry weight basis) were lowest in the bolted stage and equal in the other stages.

Complex lipid expressed as a percent of total lipid was highest in the rosette stage and equal in the seedling and bolted stages (Table 22). The neutral lipid expressed as a percent of total lipid was equal in the seedling and bolted stage and was lowest in the rosette stage. Molar phosphorus composition of total lipid decreased from the seedling to the rosette to the bolted stage (Table 22).

The molar phosphorus composition of the complex lipid was equal in the leaves of the bolted and rosette stage and this was less than in the seedlings (Table 22). The hexose content of complex lipid (on a molar basis) was higher in the rosette stage than the bolted stage. The complex lipid hexose component in leaves (cotyledons) of the seedlings did not differ from that of the other two stages.

In conclusion, the lipid measurements that appeared to be related to the progressive decrease in the freezing point of each growth stage during maturation, were the increase in total lipid, neutral lipid and complex lipid per gram dry weight of tissue. In addition the decrease in molar phosphorus content of total lipid with each more mature growth stage indicated a direct relationship to the freezing point. The decrease in the hexose content of complex lipid (on a molar basis) from rosette to

the bolted stage may have also indicated a trend since the seedling value was too variable to give it an accurate ranking.

The fatty acid composition of the complex lipid fraction of the seedlings was significantly different from that of the other two growth stages (Table 23). The bolted stage differed from the rosette stage by containing more 16:0 and 16:2. No consistent trend existed relative to the freezing point.

The fatty acid composition of the neutral lipid fraction of each growth stage was different (Table 24). Changes in this fraction indicate a consistent trend in that 16:1 and 18:1 change in the same direction as the freezing point.

The mole percent unsaturated fatty acid content of both lipid fractions from the bolted stage was lower than that of the seedlings at a significance level of P=0.10 (Tables 19 and 20). The double bond indices of the neutral lipid fraction were not significantly different for any growth stage (Table 19). The double bond indices of the complex lipid fraction of the rosette and bolted stages were equal and significantly higher than the value of the seedlings (Table 20).

General conclusions.

Tables 25 and 26 summarize the lipid results of the experiment studying hardening of true leaves and the experiment studying growth stages. They show the direction of change of the quantity of lipid and exotherm measurements between the different possible combinations. The only lipid quantity that changed consistently as the freezing point changed was the weight of complex lipid per gram dry weight of tissue (Table 25). By

TABLE 23. Fatty acid composition of the complex lipid fraction of Target rape at different stages of growth.

Fatty acid	Growth stage			Comparison (Student's t test)		
	Seedling	Rosette	Bolted	1 vs 2	1 vs 3	2 vs 3
16:0	15.3±1.8	7.3±0.3	11.8±1.1	***	**	***
16:1	2.1±0.2	0.0	0.0	***	***	NS
16:2	2.8±0.2	2.0±0.1	2.7±0.2	***	NS	***
16:3	1.6±0.4	0.5±0.0	0.0	**	***	NS
x	1.7±0.2	0.3±0.1	0.0	***	***	NS
18:0	9.5±3.2	19.9±1.6	17.8±0.9	***	***	NS
18:1	6.1±0.7	1.2±0.2	1.6±0.3	***	***	NS
18:2	14.6+1.9	6.7+0.4	7.4+0.4	***	***	NS
18:3	46.0+1.7	62.2+1.1	58.6+2.4	***	***	*

Values indicate mean percent fatty acid composition and standard deviation. The seedling and rosette stage had a sample size of three and the bolted stage a sample size of four.

*, ** and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

TABLE 24. Fatty acid composition of the neutral lipid fraction of Target rape at different stages of growth.

Fatty acid	Growth stage			Comparison (Student's t test)		
	1 Seedling	2 Rosette	3 Bolted	1 vs 2	1 vs 3	2 vs 3
14:0	1.2±0.3	1.6±0.2	1.3±0.2	NS	NS	NS
16:0	8.1±1.0	10.7±0.9	7.3±1.3	**	NS	**
16:1	2.7±0.0	1.5±0.2	0.7±0.1	***	***	***
16:2	0.0	5.7±0.6	1.9±0.4	***	***	***
16:3	1.4±0.3	0.0	0.0	***	***	NS
x	2.4±0.5	3.6±0.4	0.0	**	***	***
18:0	12.7±2.6	14.8±1.8	23.2±3.7	NS	**	**
18:1	13.7±2.5	4.5±0.2	2.6±0.3	***	***	***
y	0.0	1.6±0.3	0.0	***	NS	***
18:2	10.2±0.9	9.3±0.5	6.5±1.0	NS	**	**
18:3	42.1±4.0	46.4±1.2	56.2±1.6	NS	***	***
20:0	4.7±2.0	0.0	0.0	***	***	***

Values indicate mean percent fatty acid composition and standard deviation. The sample size for each growth stage was three.

*, ** and *** indicate significance at P equal to 0.10, 0.05 and 0.01, respectively.

TABLE 25. Relationship between the relative quantity of lipid and the relative exotherm values.

Component	rosette rel. to seedling	bolted rel. to seedling	bolted rel. to rosette	rosette hardened rel. to nonhardened	hardened rel. to bolted	hardened rel. to seedling
Concentration (g per 100g tissue dry weight)						
TL	+	+	+	x	-	+
NL	+	+	+	x	-	+
CL	+	+	+	+	x	+
Chl	+	+	x	x	x	+
TL minus chl	+	+	+	x	-	+
NL minus chl	+	+	+	-z	-	x
NL fatty acids	-z	x	x	x	x	x
CL fatty acid	+z	-	-	x	+	x
Total fatty acid	x	x	-	x	+	x
Concentration (μ Moles per g tissue dry weight)						
Lipid phosphorus	x	x	x	+	x	+z
Lipid hexose	+	+	x	-	-	+
Ratio $\frac{\text{lipid hexose}}{\text{lipid phosphorus}}$	+	+	x	-	-	x
Supercooling point	x	-	x	-	x	-
Freezing point	-	-	-	-	x	-
Amount of supercooling	-	-	x	x	x	x

\+ indicates that the first plant mentioned has a higher value than the second.

\- indicates that the first plant mentioned has a lower value than the second.

x no significant difference.

z significant difference at P=0.10.

making a few assumptions, it can be concluded that this represents an increased amount of membrane per cell, thus agreeing with results reported in the literature.

In three out of five cases the stage with the lower freezing point had a higher percent complex lipid of total lipid (Table 26). In one of the other cases there was no significant difference. This suggested a possible trend.

If lipid molar phosphorus and hexose content of complex lipid are considered together, in all cases where the freezing point decreased one of these lipid components increased and the other remained constant (Table 26). This could indicate that a third component of the complex lipid always increases with the decreasing freezing point.

Because the total seedling was analyzed for lipid, it may not have been valid to compare it to the leaves of the other two stages. Therefore a comparison of the lipid measurements of the leaves may be of more value. The freezing point of the leaves of the hardened rosette stage and the bolted stage were equal and lower than the freezing point of the leaves of the nonhardened rosette stage (Table 1). Both the hardened leaves and leaves of the bolted stage had more complex lipid per gram of tissue than the nonhardened leaves (Table 25). They also both had a lower molar hexose content in the total and complex lipid (Table 26).

It was concluded in the hardening experiment of the true leaves that the hardened plants likely had more membranes per cell than the nonhardened plants. The bolted stage, which had a lower freezing point than the rosette stage, also had more complex lipid (based on dry weight of tissue)

TABLE 26. Relationship between the relative quantity of lipid constituents to the relative exotherm values.

Component	rosette rel. to seedling	bolted rel. to seedling	bolted rel. to rosette	rosette hardened rel. to nonhardened	hardened rel. to bolted	hardened rel. to seedling
Percent of TL	-	x	+	-	-	-
NL	+	x	-	+	+	+
CL	+	x	-	x	x	x
Chl	+	x	-	x	x	x
NL minus chl	-	x	+	x	-	-
Total fatty acid	x	-	-	x	+	x
Percent of TL minus chl						
Total fatty acid	x	-	-	x	+	x
μ Moles/g TL						
Lipid Pi	-	-	-	+	+	-z
Lipid hexose	+	x	-	-	-	x
Percent of NL						
Chl	+	x	-	x	+	+
NL fatty acids	-	-	x	x	+	x
Percent of NL minus chl						
NL fatty acids	-	-	+z	x	+	-z
μ Moles/g CL						
Lipid Pi	-	-	x	x	+	-
Lipid hexose	x	x	-	-	-	-z
Percent of CL						
CL fatty acid	x	x	-	x	+	x
Supercooling point	x	-	x	-	x	-
Freezing point	-	-	-	-	x	-
Amount of supercooling	-	-	x	x	x	x

\+ indicates that the first plant mentioned has a higher value than the second.

\- indicates that the first plant mentioned has a lower value than the second.

x no significant difference

z significant difference at P=0.10

which suggested more membranes per cell. However the lipid phosphorus content, which is a better indicator of membranes than complex lipid was not significantly different. However it is likely that the dry weight of a cell in the bolted stage is greater than in the rosette stage due to additional cell wall growth and lignification. It has been mentioned that it is believed that cell division and elongation in leaves is completed by the end of the rosette stage. Thus the bolted stage may actually contain more membranes per cell than the rosette stage, thus accounting for the apparent increase in hardiness.

If one compares Nugget to Target; hardened to nonhardened leaves; and leaves of the bolted stage to the rosette stage; the leaves of the first plant listed in each comparison has a significantly lower freezing point (Tables 1 and 3). They also all have more 16:0 in their complex lipid fraction (Tables 11, 17 and 23).

Comparison of the fatty acid double bond status of lipid fractions indicated a significant inverse relationship between hardiness and double bonds (Tables 14, 19 and 20). This was opposite to what was expected from reading the literature. However all the values are within the critical range found by Lyons and Asmundson (1965) for chilling resistant plants. The decrease in unsaturated fatty acids of the complex lipid fraction was probably due to the decrease in glycolipids (lipid hexose) which are especially rich in unsaturated fatty acids (Kuiper, 1970).

In the first section of the lipid experiments, it was concluded that low temperatures induce adaptive changes in metabolism. When lipid change from the rosette stage to the hardened and bolted stage was compared, some

lipid components change in opposite directions (Tables 25 and 26). For example, percent complex lipid of total lipid and lipid phosphorus content changed in opposite directions. Thus the effect of low temperature was on the rate of specific reactions and not a general slowing down of all reactions. This supports the earlier conclusion that plants growing at a low temperature have different synthetic or degradation rates, not due simply to the thermodynamic influence of temperature.

SUMMARY

1. During a five week hardening treatment, the freezing point of cotyledons decreased to a minimum value within one week and remained lower than the freezing point of the nonhardened cotyledons during the rest of the treatment. The freezing point of true leaves responded to hardening in a similar manner except they required two weeks to reach their minimum freezing point.

2. After equal hardening periods, cotyledons had a higher freezing point than true leaves.

3. The supercooling point and amount of supercooling of cotyledons changed significantly during hardening, whereas the supercooling point and amount of supercooling of true leaves did not.

4. The supercooling point and freezing point of leaves from plants germinated and grown in the greenhouse decreased with maturity.

5. The cotyledons and incompletely expanded leaves of plants germinated in the cold room had lower freezing points than the cotyledons and incompletely expanded leaves of plants germinated in the greenhouse. The freezing points were equal to those produced by directly hardening the cotyledons and leaves.

6. The supercooling point of fully expanded leaves of plants germinated in the cold room remained constant during maturation, whereas for plants germinated in the greenhouse it decreased. This suggested that the cold treatment during germination had an effect throughout the total growth of the plant.

7. During hardening the supercooling point and freezing point of the cotyledons were independent of the percent moisture. The supercooling point and freezing point of the true leaves were positively correlated to the percent moisture.

8. For both plants germinated in the coldroom and plants germinated in the greenhouse, the amount of supercooling was negatively correlated to the weight of water per leaf disc.

9. Nugget cotyledons had a lower freezing point, supercooling point and amount of supercooling than Target cotyledons.

10. Lipid analysis before and after a 35 day hardening treatment, showed that the quantity of complex lipid and lipid phosphorus increased during hardening. This suggested that an increase in quantity of membranes occurred, which agreed with theories reported in the literature.

11. The mole percent unsaturation of both the neutral and complex lipid fractions decreased during the 35 day hardening treatment. However it remained within the critical range found by Lyons and Asmundson (1965) for chilling resistant species.

12. Plants with a lower freezing point tended to have more 16:0 fatty acid in their complex lipid fraction.

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APPENDIX

Sample Calculations

Correction factors employed in calculating the weight of the various lipid components.

Treatment: nonhardened true leaves of the rosette stage, sample two.

Raw data:

- weight of plant material analyzed 2.3612g.
- weight of various lipid components per sample (value for lipid in the weighing bottle)

TL 0.2516g
NL 0.0880g
CL 0.1499g
NL fatty acids 0.0043g
CL fatty acids 0.0181g

- other components

Chlorophyll 0.0002g/ml
Lipid phosphorus 2.32 μ moles/ml
Lipid hexose 0.93 μ moles/ml

Calculations:

i Correction factors due to experimental procedure.

a Chlorophyll 0.0002x200 = 0.0401g/sample

b Multiply by $\frac{200}{197}$ to allow for sample lost for chlorophyll determination.

TL $\frac{200}{197}$ x 0.2516 = 0.2554g.
NL $\frac{200}{197}$ x 0.0880 = 0.0893g.
CL $\frac{200}{197}$ x 0.1499 = 0.1521g.

c Fatty acids also correct for using only $\frac{2}{5}$ of sample for analysis.

NL fatty acids $\frac{200}{197} \times \frac{5}{2} \times .0043 = 0.0110$g.
CL fatty acids $\frac{200}{197} \times \frac{5}{2} \times .0181 = 0.0459$g.

d Lipid phosphorus analyzed $\frac{1}{25}$ of sample for each determination.

$\frac{200}{197} \times 25 \times 2.32 = 59.0$ μ moles.

e Lipid hexose - hydrolyzed $\frac{1}{25}$ of the complex lipid and did a color reaction on $\frac{1}{10}$ of this, thus multiply by 250.

$\frac{200}{197} \times 250 \times 0.93 = 236.0$ μ moles.

ii The silicic acid column yielded only 94.5% of the applied sample. When you calculate percent NL and CL of TL using the recovered material you get

$$\% \text{ NL of TL } \frac{.0893}{.0893+.1521} \times 100 = 37.0$$

$$\% \text{ CL of TL } \frac{.1521}{.0893+.1521} \times 100 = 63.0$$

All values can then be corrected for this.

TL remains the same 0.2516g.

NL $0.37 \times 0.2516 = 0.0945$g.

CL $0.63 \times 0.2516 = 0.01609$g.

NL fatty acids $\frac{.0945}{.0893} \times .0110 = 0.0117$g.

CL fatty acids $\frac{.1609}{.1521} \times .0459 = 0.0679$g.

Lipid phosphorus $\frac{.1609}{.1521} \times 59.0 = 62.4$ μ moles

Lipid hexose $\frac{.1609}{.1521} \times 236.0 = 249.7$ μ moles

iii All values from ii were then expressed on the basis of 100g of plant material by dividing by 2.3612g and multiplying by 100. Lipid phosphorus and hexose were expressed on the basis of one g of plant material.

TL 10.82g
NL 4.00g
CL 6.82g
NL fatty acid 0.50g
CL fatty acid 0.67g
Chlorophyll 1.70g

Lipid phosphorus 26.4 μ moles
Lipid hexose 105.8 μ moles

All other methods of expressing the results were calculated from these values.

TABLE 27. Advantage of correcting values to the silicic acid column yields.

Sample	Data[A] Not corrected	Corrected
	g/100g plant material	
1	6.75	6.88
2	6.54	6.82
3	7.09	7.10
	Statistical results	
$\bar{x}$	6.79	6.93
S.d.	0.28	0.14
Coefficient of variation	4.0	2.0

[A] Complex lipid of the nonhardened true leaves from the rosette stage. The coefficient of variation shows that the correction factor decreases the variation of the estimate.

Calculation of the mole percent unsaturation.

TABLE 28. Calculation of the mole percent unsaturation.

TABLE 28 a. Raw data.

Fatty acid	Sample[A] 1	2	3
16:0	6.9	7.4	7.5
16:2	1.9	2.1	2.0
16:3	0.5	0.5	0.5
18:0	21.7	19.4	18.6
18:1	1.1	1.4	1.1
18:2	6.3	7.0	6.8
18:3	61.3	61.8	63.4

A For the complex lipid fraction of the nonhardened true leaves of the rosette stage.

TABLE 28b. Organize data in Table 28a as follows:

Fatty acid	Sample 1	2	3
		saturated	
16	6.9	7.4	7.5
18	21.7	19.4	18.6
		unsaturated	
16	2.4	2.6	2.5
18	68.7	70.2	71.3

Flame ionization detectors give a weight percent response. Correction factors may have to be applied to the different components (McNair and Bonelli, 1969). However because the values for the mixture of fatty acid standards were within that of experimental error, I assumed that the detector response was the same for each fatty acid.

If it is assumed that the detector response is to moles of material and not weight the following is calculated from Table 28b.

TABLE 28c.

	Sample 1	2	3
Saturated (16 + 18)	28.6	26.8	26.1
Unsaturated (16 + 18)	71.1	72.8	73.8
Total	99.7	99.6	99.9
"Mole percent unsaturation"	71.3	73.1	73.9

However one can correct for the fact that the response is to weight. This is done by dividing the values in Table 28b by the proper molecular weight to give a relative molecular content.

Assumptions:

	Molecular Weight
16:0, 16:1, 16:2, 16:3	270
18:0, 18:1, 18:2, 18:3	298

TABLE 28d. Relative molecular content.

Fatty acid	Sample 1	2	3
	saturated		
16	.0256	.0274	.0278
18	.0728	.0651	.0624
	unsaturated		
16	.0089	.0096	.0093
18	.2305	.2356	.2393

From Table 28d one calculates the following (Table 28e).

TABLE 28e

	Sample 1	2	3
Saturated (16 + 18)	.0984	.0925	.0902
Unsaturated (16 + 18)	.2394	.2452	.2486
Total	.3378	.3377	.3388
Mole percent unsaturation	70.9	72.6	73.4

The statistical results for Tables 28c and 28e are now calculated.

TABLE 28d.

	Table 28c	Table 28e
$\bar{x}$	72.7	72.3
s.d	1.3	1.3
n	3	3
Coefficient of variation	1.8	1.7

It is seen that the mole percent unsaturation is not statistically different when calculated by two different methods. Thus I used the former method because it was the simpliest.

TABLE 29. Percent yields from silicic acid columns.

Treatment	Sample	Percent yield
Nugget cotyledons nonhardened	1	100.6
	2	99.0
	3	97.6
Nugget cotyledons hardened	1	96.8
	2	99.2
	3	94.3
Target cotyledons nonhardened	1	94.2
	2	91.3
	3	99.3
Target true leaves rosette stage nonhardened	1	96.7
	2	94.5
	3	98.4
	4	101.7
Target true leaves rosette stage hardened	1	84.1
	2	76.2
	3	85.9
	4	85.4
Target true leaves bolted stage nonhardened	1	71.4
	2	91.9
	3	98.4
	4	96.2

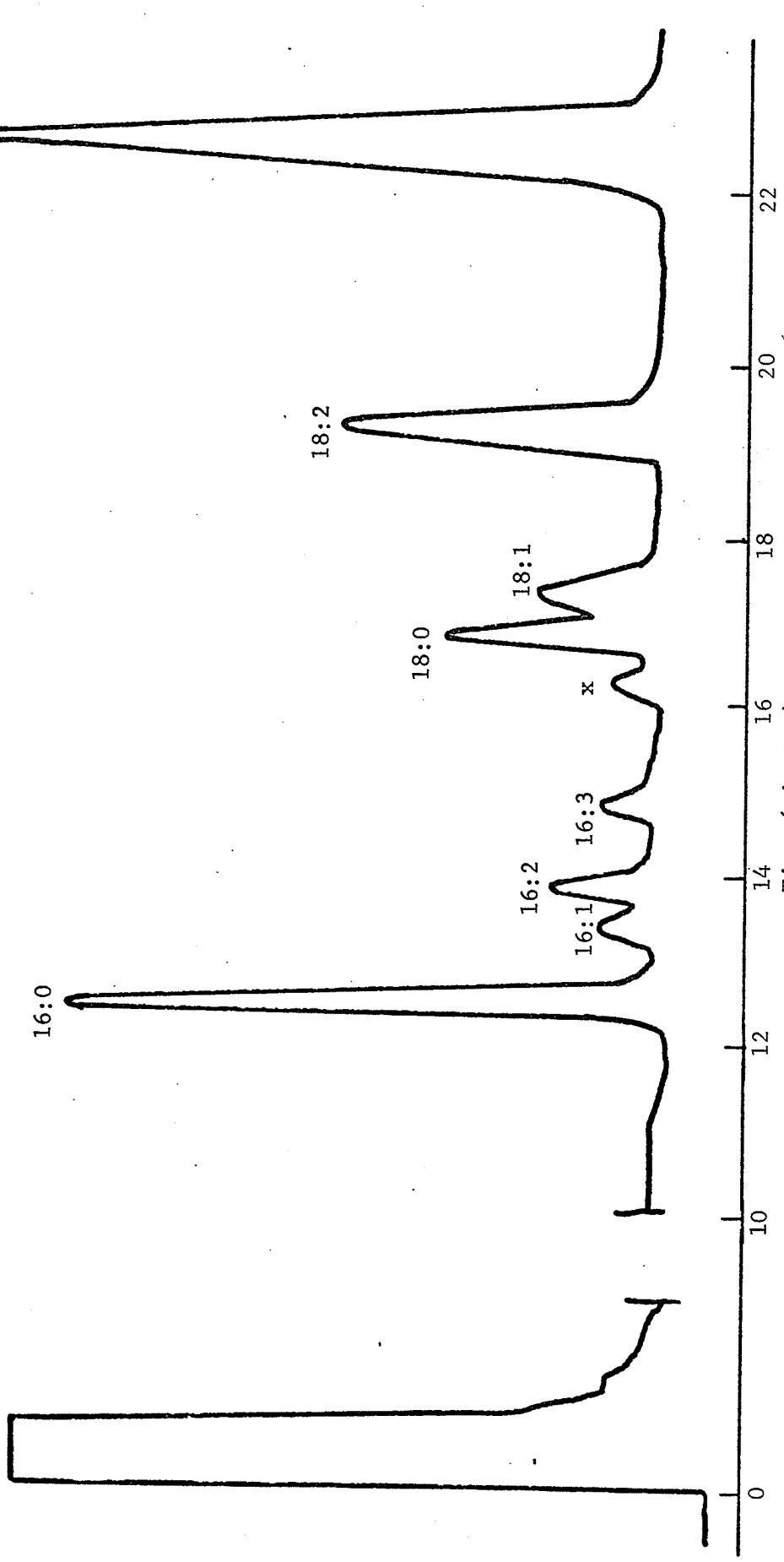

FIGURE 13. Cromatogram of the fatty acids of the complex lipid fraction of the nonhardened cotyledons of Target rape. Date analyzed 22/2/71. Same size as original chromatogram. Sensitivity used 2 x 10.

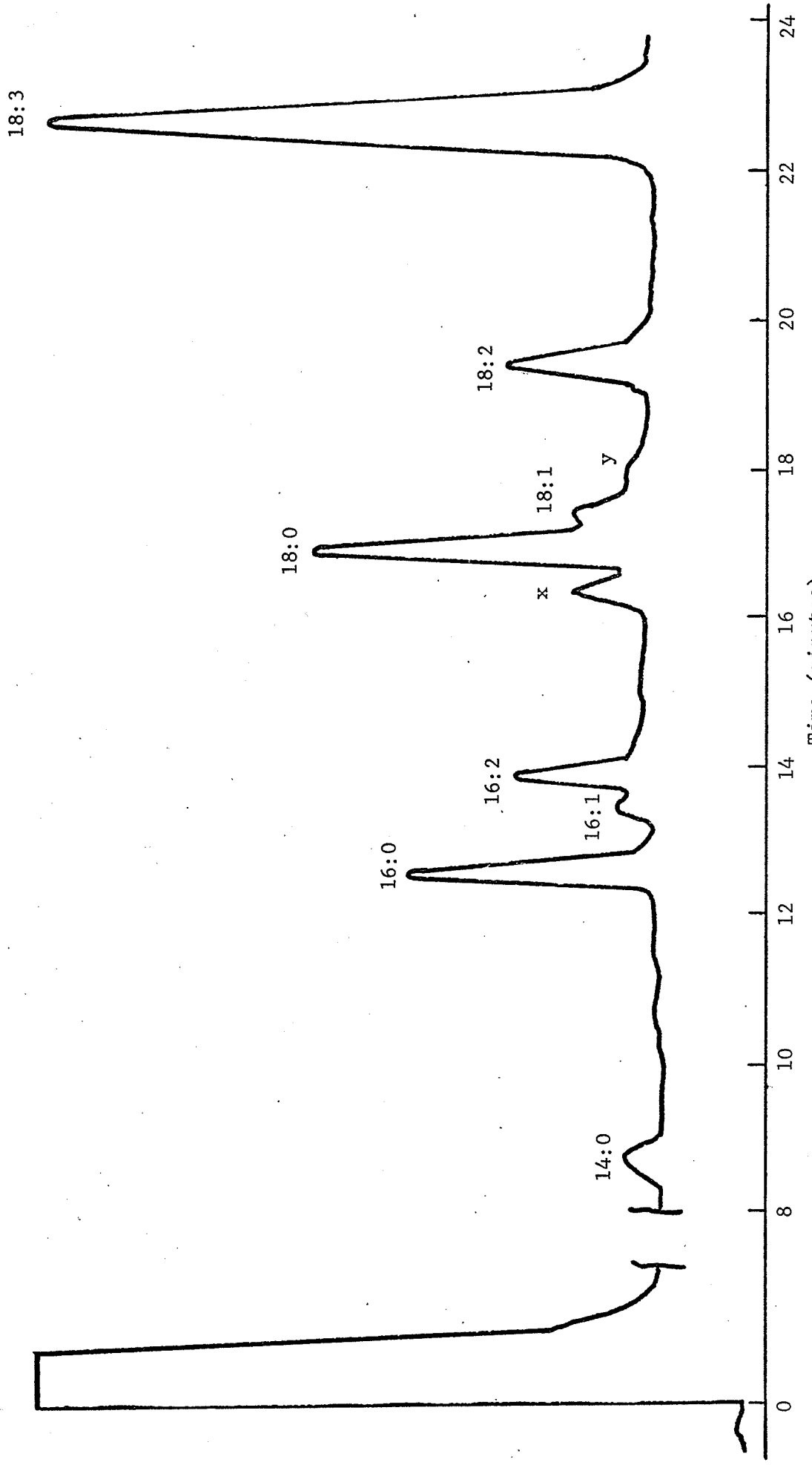

FIGURE 14. Chromatogram of the fatty acids of the neutral lipid fraction of the nonhardened leaves of the rosette stage of growth of Target rape. Date analyzed 25/2/71. Same size as original chromatogram. Sensitivity used 1 x 10.

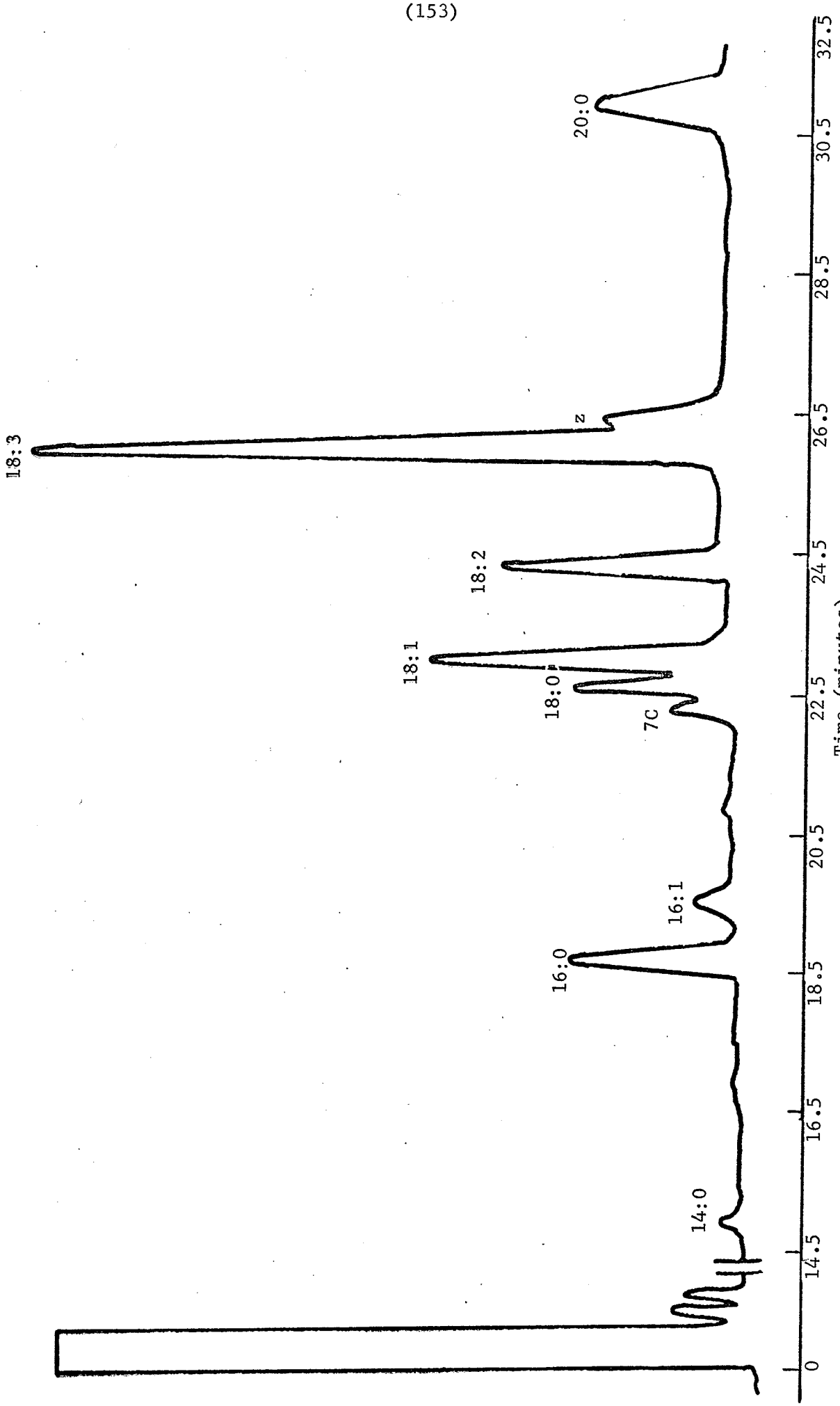

FIGURE 15. Chromatogram of the fatty acids of the neutral lipid fraction of the nonhardened cotyledons of Nugget rape. Date analyzed 15/11/70. Same size as original chromatogram. Sensitivity used 1 x 10.

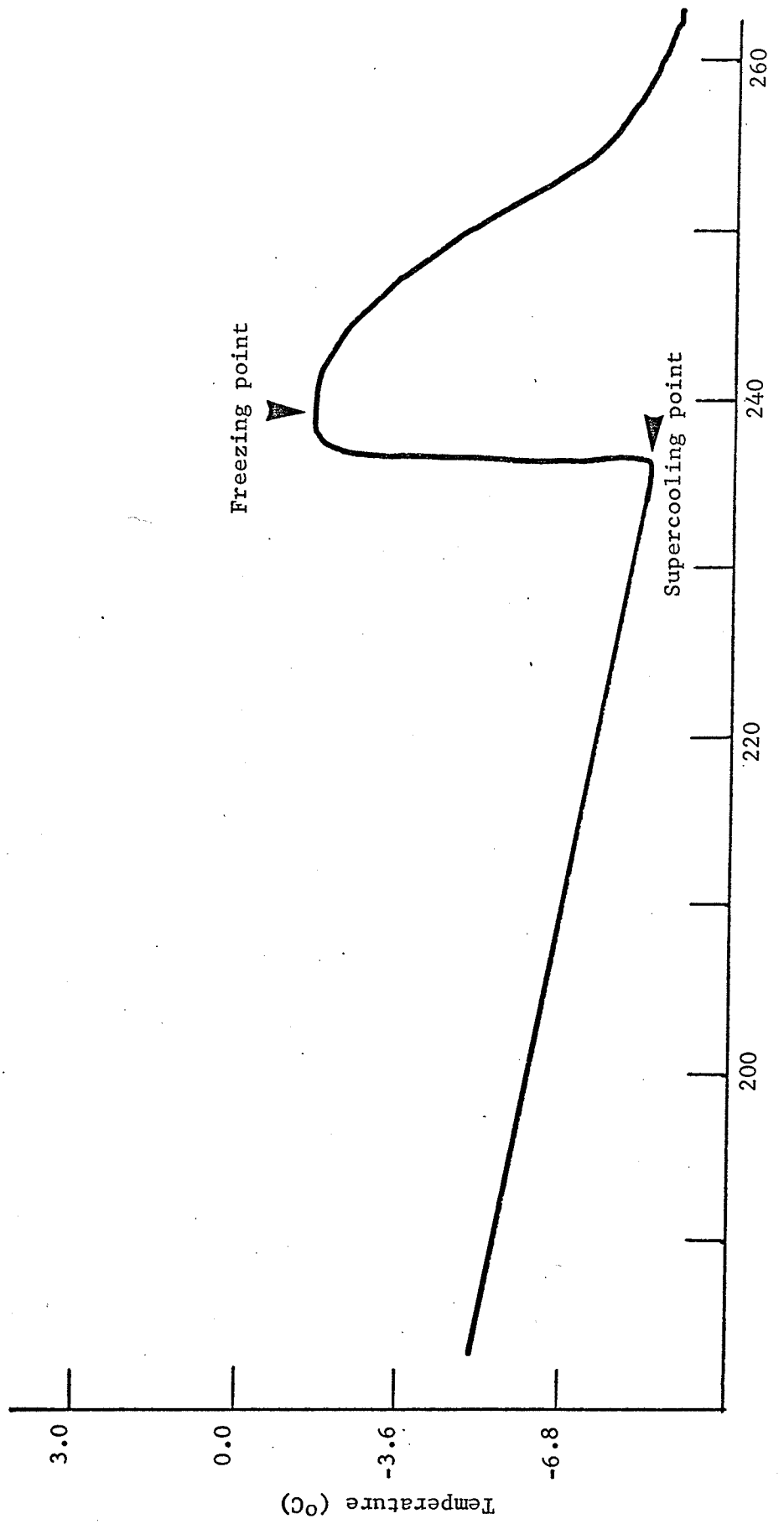

FIGURE 16. Tissue temperature during a controlled slow freeze of a nonhardened leaf of Target rape. The amount of supercooling was equal to the freezing point minus the supercooling point.

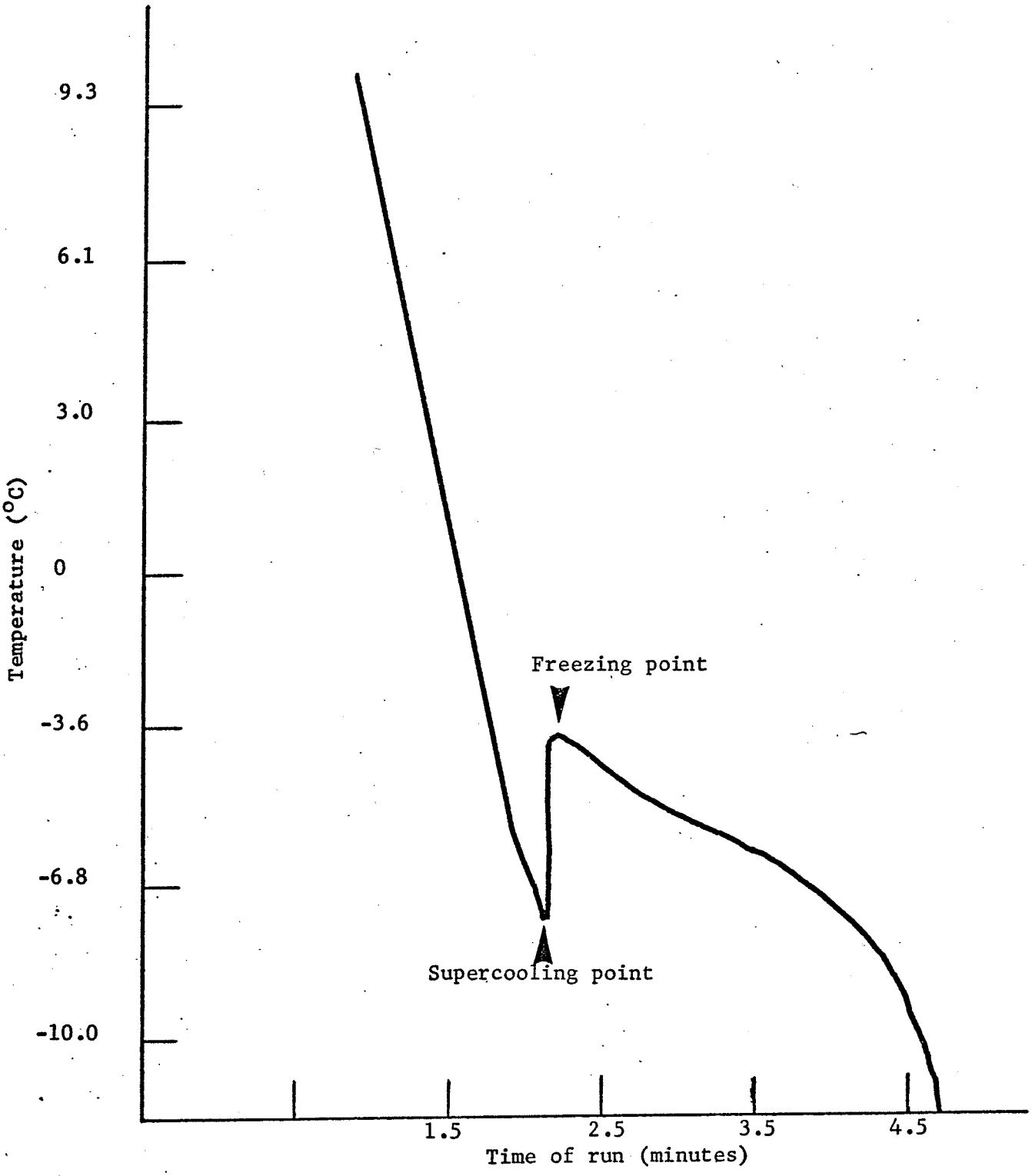

FIGURE 17. Tissue temperature during a controlled fast freeze of a hardened cotyledon of Target rape. The amount of supercooling was equal to the freezing point minus the supercooling point.